VIRAL INFECTION IN A MURINE MODEL OF ALLERGIC AIRWAYS INFLAMMATION: ACTIONS OF CORTICOSTEROIDS

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SUMMARY

Viral respiratory infection exacerbates asthma symptoms in almost all patients with allergic asthma. Asthma symptoms in viral associated asthma exacerbation are often severe and require urgent care as well as hospitalisation. Corticosteroids are the mainstay treatment for asthma. However, they are less effective in treating virus associated asthma exacerbation. The main aim of the thesis is to determine the role of virus infection in airway allergic inflammation and then define the effects of corticosteroids in virus associated exacerbations of airway allergic inflammation.

Mice sensitised and challenged with ovalbumin demonstrated most of the main features of asthma including lung cellular inflammation with eosinophilia, early phase asthmatic responses (EAR), late phase asthmatic responses (LAR), and airway hyperresponsiveness (AHR) to methacholine provocations. Treatment with either systemic (dexamethasone: DEX) or inhaled (fluticasone propionate: FP) corticosteroids in the murine ovalbumin allergic airways inflammation model attenuated inflammatory cells influx and eosinophilia, LAR, and the AHR.

Influenza A (H1N1/PR8) is the most infective to mice compared to human parainfluenza virus type 3 (HPIV3), and a synthetic dsRNA, poly (I:C). Influenza infection in mice caused a significant increase of inflammatory cell influx in the airways with marked neutrophilia, and AHR. Ovalbumin challenge in the acute course of influenza infection on a murine model of allergic airways inflammation exacerbated the inflammatory cells influx, LAR, and AHR. Treatment with either DEX or FP attenuated the airway cellular inflammation, LAR, but not the AHR. Mice only infected with influenza were resistant to the corticosteroids (DEX and FP) treatment. DEX but not FP showed antiviral activity against HPIV3 and influenza A *in vitro*.

These data suggest that influenza infection in a murine model of allergic airways inflammation exacerbates the inflammation and alters the sensitivity toward corticosteroids. It is also suggested that some elements in the influenza associated exacerbation of murine model of allergic airways inflammation are refractory or not regulated by corticosteroid treatment.

CONTENTS

CHAPTER I

GENERAL INTRODUCTION

I. RESPIRATORY VIRAL INFECTIONS

Respiratory tract infections which are predominantly viral, are responsible for the majority of acute illnesses worldwide and have been established as leading causes of morbidity and mortality particularly at the extremes of age and in high-risk individuals. It is estimated, about 5-6% of all deaths and about 60% of respiratory disease related deaths are caused by respiratory viruses (Benson and Marano, 1998).

Various respiratory viruses are known to cause acute respiratory infections in humans, including the common cold, bronchiolitis, and pneumonia (Tregoning and Schwarze, 2010). The major known pathogens are respiratory syncytial virus (RSV), human rhinovirus (RV), human metapneumovirus (HMPV), human parainfluenza virus, enterovirus, influenza viruses, adenoviruses, and human bocavirus (Fabbiani *et al.*, 2009). Some of the mentioned viruses produce local infection which is primarily restricted to the respiratory tract. However, adenovirus, coxcackie, and echoviruses have been identified to cause more serious respiratory and systemic disease (Welliver and Ogra, 1988).

Respiratory syncytial virus, human parainfluenza virus, adenovirus, and influenza virus are known as the most common causes of serious lower respiratory tract disease in infants and children. Epidemiological studies suggest that most children are infected at least once with human parainfluenza virus early in life, and re-infections occur throughout life (Henrickson, 2003). This thesis will evaluate the nature of infection of human parainfluenza virus type 3 (HPIV3) and influenza A virus (H1N1/PR8) *in vitro* and *in vivo* (in mouse). The nature of those virus infections on their susceptible cells and the possible antiviral effects of corticosteroids are presented in chapter 3. Chapter 4 will mainly evaluate the nature of infection of some infective agent candidates in mice which will then be utilised to generate exacerbation of allergic inflammation, in subsequent studies.

I.1. ANTIVIRAL IMMUNE RESPONSES

A typical antiviral immune response of the host involves a combination of nonspecific (innate) and specific (adaptive) immunity.

Innate Immunity or the nonspecific type of immunity include: phagocytes, natural killer (NK) cells and components of body fluids such as enzymes and complements which are capable of neutralizing viral infections independently, or in combination with antibodies (Message and Johnston, 2001). The phagocytosis action of macrophages which engulf and destroy virus are triggered by pattern recognition receptors (PRRs) on the surface of the phagocyte and are activated by pathogen-associated molecular patterns (PAMPs) on the surface of the virus. Macrophages might also play a significant role in phagocytosis of virions or debris from virus-infected cells (Crowe and Williams, 2003).

Nonspecific immune mechanisms are important for early defence against the virus during the early stages of infection. When viruses infect the respiratory tract, multiplication will peak on the second day of infection when type I interferons which are released by the infected cells, are first detected. The interferons reach maximum levels at day 3 and are undetectable by day 8. Interferons then activate NK cells, which destroy viral infected cells and release cytokines, including Type II interferon (Interferon-γ) which activates additional inflammatory cells in the airway. NK cells are first detected at day 3 and then peak at day 4 (Message and Johnston, 2001).

The second type of immunity is adaptive immunity. The cells of specific immunity are lymphocytes, especially B and T lymphocytes. Antigens are processed and presented by dendritic cells to naive T-cells in the lymph node. Virus will be presented by both MHC class I and class II. MHC class I activates CD8+ T-cells to produce IFN-γ, which skews the differentiation of CD4+ T-cells into Th1 phenotype. The activated T-cells

may then stimulate B-cells to produce specific antibodies which will aid virus eradication (Message and Johnston, 2001; Openshaw and Tregoning, 2005).

During the first few days of infection, dendritic cells process viral antigens locally in regional lymph nodes and then present them to T-cells. T-cell recruitment depends on the expression of adhesion molecules on the vascular endothelium at the site of inflammation and on the production of chemokines. CD4+ and CD8+ T-cells are detected on day 4 and day 6. CD8+cytotoxic T-cell responses peak on day 7 and are then undetectable by day 14, although memory CD4+ and CD8+ responses may remain a lifetime (Message and Johnston, 2001).

B Cells are responsible for the production of antibodies. Mucosal Ig (Immunoglobulin) A – IgA is detected at day 3, becoming undetectable after 3 to 6 months, serum IgM is detected at day 5 to 6 and IgG is detected at day 7 to 8; these antibodies then increase in quantity and activity for 2 to 3 weeks. Serum IgG may remain detectable for life. Within 7 days after infection, the infectious virus is eradicated because the activity of specific immune system namely CD8+ T-cells and Immunoglobulin (Ig) (Message and Johnston, 2001).

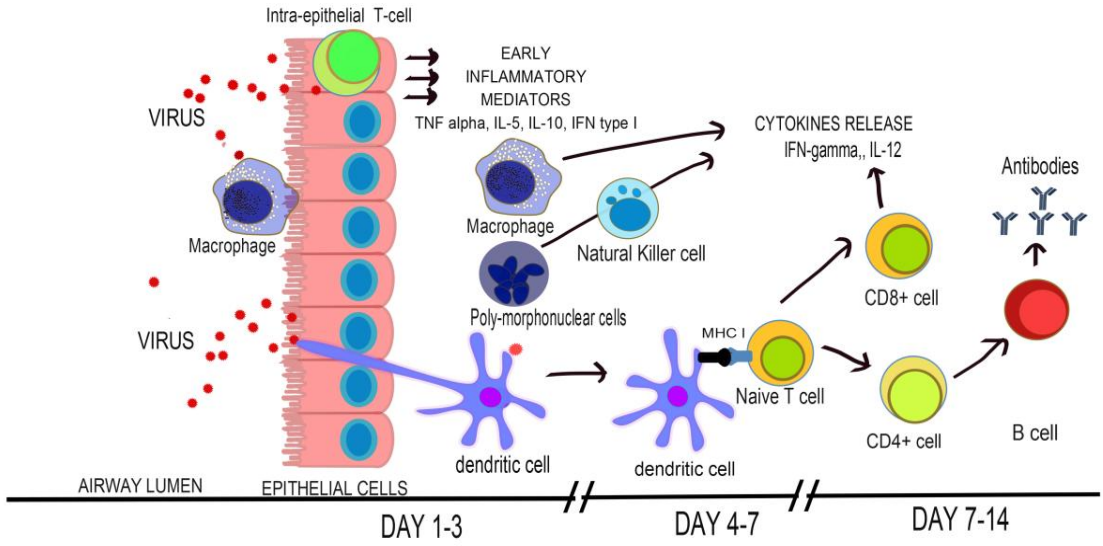


Figure 1.1. Inflammatory cells and mediators involve in viral respiratory infection immune response. Infection of the epithelial cells triggers the release of early inflammatory mediators which leads to the recruitment of inflammatory cells at the site of infection (Openshaw and Tregoning, 2005).

In the case of re-infection, adaptive immunity is important. When the same serotype of virus re-infects, B- and T-cell- will be mobilised even faster. The level of T-cells and NK cells peak earlier on day 3–4. Antibodies, which still exist in the circulation, neutralize the virus. Viral replication will be slowed down or stopped by this mechanism. As a consequence, in secondary infection, fewer cells are infected, allowing nonspecific immunity to be less active (Crowe and Williams, 2003).

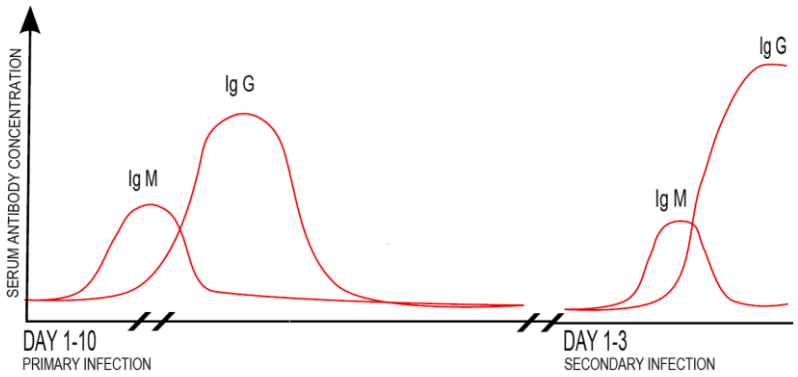


Figure 1.2. Acquired immune response by production of IgM and IgG is directed against respiratory viral infection. The concentration of serum immunoglobulin varies depending on whether it is primary or secondary viral infection (Openshaw and Tregoning, 2005).

II. ALLERGIC INFLAMMATION OF THE AIRWAYS

Allergy is an inappropriate susceptibility of some individuals to certain substances, usually environmental proteins (antigens), which lead to the development of signs and symptoms of reactivity or hyperreactivity. The cornerstone of an allergic response is the production of allergen specific immunoglobulin E (IgE) and the response of the developed IgE to the future exposure of naturally harmless substances. The production and exaggerated response of IgE toward allergen involve activation and interactions of a number of leukocytes particularly T- cells populations (Ishmael, 2011).

In allergic individuals, high levels of allergen activated T-cells are present in the peripheral blood circulation. These CD4+ T cells produce abnormal levels of Th2

specific cytokines; IL-4, IL-5, IL-9, and IL-13, which contributes to the clinical features of allergic inflammation pathophisiology. Manifestation of this cascade is allergic disorders; one of them is allergic (atopic) asthma (Galli *et al.*, 2008).

II.1. ASTHMA: DEFINITION AND PREVALENCE

Asthma, as defined by The Global Strategy for Asthma Management and Prevention Report (Report, 1995) is "*a chronic inflammatory disease of the airways in which many cell types play a role, in particular mast cells, eosinophils and T lymphocytes. In susceptible individuals the inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness and cough particularly at night and/or early morning. These symptoms are usually associated with widespread but variable airflow obstruction that is at least partly reversible either spontaneously or with treatment. The inflammation also causes an associated increase in airway responsiveness to a variety of stimuli. Reversibility of airflow limitation may be incomplete in some patients with asthma*"

Asthma is a serious worldwide problem affecting approximately 300 million people of all ages (GINA, 2012). There is an increasing trend of asthma prevalence, especially in children; in the UK alone around 20–33% of children have asthma. The financial impact of this condition is significant in terms of un-productivity at work or school, general practice consultations, hospital admissions and mortality.

As a multi-factorial disorder, asthma expresses different phenotypes but shares similar symptoms: difficulties in breathing and airways hyperresponsiveness with underlying cascade of inflammation. At the moment, asthma has been classified based on the objective phenotypes (Wenzel, 2006) according to different observable components of asthmatics such as clinical features, immunological mechanism, and response to treatment (Lotvall *et al.*, 2011).

One categorisation which receives a particular interest is atopic and non-atopic asthma. This classification, in convention, is based on the response to common allergen and supported by the presence of allergen specific IgE. Atopic asthma usually begins before the age of 6 and is associated with atopy with a higher level of serum total IgE concentration compared to normal individuals which persists into adulthood. While the non-atopic or instrinsic variant of asthma shows normal serum total IgE concentration with no evidence of specific IgE antibody towards a certain allergen. Non atopic asthma usually begins later in life with more severe clinical consequence compared to the atopic asthma. Non atopic asthma comprised only a third of total asthmatics (Humbert *et al.*, 1999).

Atopic asthmatics express significantly increased number and activity of eosinophils compared to non-atopic (Amin *et al.*, 2000). While in the non atopic airways, neutrophil infiltration seems to be more elevated, even though neutrophils tend to be elevated in all type of more severe asthmatics. The level of IL-4 and IL-5 are also significantly higher in atopic asthmatics, whereas in non-atopic asthmatics, IL-8 expression is higher (van Aalderen, 2012).

II.2. PATHOGENESIS OF ASTHMA

II.2.1. ALLERGEN SENSITISATION

The underlying features of asthma are closely associated with interaction between genes and the environment, between genetically susceptible individuals and an exaggerated immune response to environmental allergens. Allergen sensitisation is a central element in allergic asthma. In allergic sensitisation, the airway is capable of recognising usual allergens to develop Th2 cytokines responses, which also involves a cascade of inflammation caused by the interaction of respiratory epithelium, innate and adaptive immune system, which drives a chronic inflammatory response (Ishmael, 2011).

Inhaled allergens, which are typically common environmentally proteins such as pollen, house-dust mites, and mould are processed by one of the antigen presenting cells (APCs) commonly, dendritic cells. Processed allergens are then loaded into to the major histocompatibility complex II (MHC II), a cell surface molecule which presents the allergens on the surface of cells and enables APC to interact with CD4+ T-cells. The MHC II – allergen combination interacts with naive T-cells, which initiates sensitisation and triggers the subsequent immune response to the allergen. Dendritic cells also contribute to the differentiation of the nature of the immune response (Th1/Th2) by producing inflammatory mediators such as IL-12 and type I interferons which stimulate naive T cells differentiation into Th1 cells. The production of IL-4 by eosinophils and mast cells inhibits the expression of IL-12 and subsequently Th1 development, therefore directing the immune response to the production of Th2 effector cells. Expression of IL-4, OX40L and the CD86 (cluster of differentiation (CD) marker) polarises T -cells differentiation in favour of Th2 responses which is the hallmark of allergic response (Lambrecht and Hammad, 2010).

Activated and sensitised T-cells then produce a range of cytokines fundamental to allergy; IL-3, IL-4, IL-5, IL-6, IL-9, IL-13 and granulocyte–macrophage colony stimulating factor (GM-CSF). IL-4 and IL-13 produced by T-cells, drive B-cells to proliferate and produce allergen-specific antibodies (IgE) which is the main pathway of allergen sensitisation. The sensitisation results in the formation of IgE memory B-cells and allergen specific memory T-cells which are responsible for future allergic responses (Levine and Wenzel, 2010).

II.2.2. ANTIGEN CHALLENGE

Allergen challenge in sensitised asthmatics can provoke two phases of temporal airway responses, namely early-phase reaction and late-phase reaction.

Early-phase reaction occurs immediately (within minutes) after allergen encounter. The early phase reaction is often also named as immediate hypersensitivity reactions. In allergic patients, inhaled allergen challenge initiates an early allergic inflammatory reaction and this may be followed sometime later with a late-phase reaction (Bousquet *et al.*, 2000). The early phase reaction mainly portrays the cascade of responses as a result of mediator secretion by mast cells. Mast cells in allergic individuals bear high affinity IgE receptors (FcεRI) on their surfaces. Allergen specific IgE produced by B cells binds to high affinity allergen-specific IgE receptors (FcεRI) on the surface of mast cells. When the sensitised individual is exposed to inhaled allergen, the antigen will bind to the allergen specific IgE on the surface of mast cells, which leads to the activation of mast cells. The activated mast cells subsequently release their granules, which contain inflammatory mediators such as histamine, leukotrienes, reactive oxygen species, and lipid mediators (Jarjour *et al.*, 1997). Degranulation, the process when the mast cells cytoplasmic granules membrane fuses with the plasma membrane, is responsible for the release of the mediators. The outcome of the mediators release includes bronchoconstriction, vasodilatation, increased vascular permeability and increased mucus production, and enhanced airway responsiveness. Mast cells activation also induces slower expression of other cytokines and chemokines (TNF (tumour necrosis factor) -α, IL-4 and IL-5) up to 72 hours after allergen challenge (Okayama *et al.*, 2003), which contributes to the inflammatory cascade of allergen-induced late phase reaction (Holgate, 2008).

Late-phase reaction peaks at six to nine hours after allergen provocation. The orchestration of the late phase reaction is thought to be the result of long term

outcomes of mast cells activation during the early phase reaction and allergen activated T-cells. The release of Th2 cytokines, which is initiated by the activation of T cells after allergen challenge, is an important mechanism of the late-phase response (Kay, 1991). However, the release of cytokines by mast cells has been thought to initiate the early recruitment of inflammatory cells (Bradding *et al.*, 1994). The nature of the late phase reaction represents the increasing number and activity of resident cells and recruited leucocytes to the airways such as eosinophils (De Monchy *et al.*, 1985), neutrophils (Montefort *et al.*, 1994), basophils (Guo *et al.*, 1994), macrophages (Calhoun *et al.*, 1992) and CD4+ T cells (Robinson et al., 1993). Airway hyperresponsiveness is usually observed after the late phase reaction but not after the early phase reaction (Bousquet et al., 2000).

II.2.3. RECRUITMENT OF INFLAMMATORY CELLS INTO THE AIRWAYS

One of the hallmarks of asthma is infiltration of the airways by inflammatory cells including T cells, mast cells, basophils, macrophages, and eosinophils. Upregulation of the production of proinflammatory cytokines, chemokines, mediators, and cell surface adhesion molecules are responsible for the recruitment of the inflammatory cells into the site (Bousquet *et al.*, 2000). The inflammatory response, tissue damage and remodelling in asthma is orchestrated by the interactions of all these effector cells and their pro inflammatory products (Hamid *et al.*, 2003).

A. EOSINOPHILS

Although not specific to asthma, airway eosinophilia is a characteristic of asthma. In fact, activated eosinophils and T cells are the hallmarks of asthma. There is a significant correlation between eosinophil activation and asthma severity and bronchial hyperresponsiveness (Bradley *et al.*, 1991). Eosinophil priming, activation, and survival are known to be regulated by IL-3, IL-5, and GMCSF cytokines, which are released from Th2 cells. Eosinophils are recruited into airways through the release of

TNF-α by degranulated mast cells and the release of IL-5 by activated T cells (Barnes, 2002). Eosinophils are also involved in airways remodelling because they release growth factors, elastase, and metalloproteases, which contribute in the process of tissue remodeling and fibrosis (Bousquet *et al.*, 2000).

B. T-LYMPHOCYTES

T lymphocytes also play an important effector role in asthma. T cells are activated and produce high levels of cytokines during acute asthma exacerbations in response to antigen stimulation. According to their cell surface markers and distinct functions, T lymphocytes are grouped into two types: the CD4+ (T helper) and the CD8+ (T cytotoxic) cells. Based on the type of cytokines they produce, CD4+ cells are subdivided into Th1, Th2, Th17, Th9, T follicular helper, and regulatory T cells. Th2 cells, which are more prominent in asthmatics release IL-4, IL-5, and IL-13. IL-4 is important in allergic sensitisation, IL-5 is essential in esoinophil survival, while IL-13 leads the induction of bronchial hyperresponsiveness, airway smooth muscle proliferation, subepithelial fibrosis, and goblet cell hyperplasia (Lloyd and Hessel, 2010). IL-13 also increases eosinophil numbers and activation via up regulation of multiple chemokines including CCL11 (Robinson *et al.*, 1993). Th-9 cells express IL-9 which plays a role in mast cell proliferation, activation, recruitment, and survival (Kearley *et al.*, 2011). Th17 cells produce IL-17 which is thought to contribute to the severity of the disease in cortisteroid-resistant asthma. T regulatory (Treg) cells inhibit the development of allergic airway inflammation. Treg control inflammation by preventing the activation of dendritic cells by allergen (Lloyd and Hessel, 2010). T follicular helper cells are thought to have a role in a B cells isotype switching (Kemeny, 2012).

C. MAST CELLS

Within the airway tissue, mast cells make up approximately 20% of inflammatory cells, although they only make up a small portion (0.02% to 0.48%) of total cells recovered in broncholaveolar lavage (BAL) fluids. In atopic or non-atopic asthma, there is an increase of normal mast cell numbers by 2- to 6-fold. Mast cells seem to be an important contributor during episodes of acute asthma, which leads to acute bronchoconstriction, oedema, and mucus secretion (Hamid *et al.*, 2003). On allergen challenge of the airways, mast cells respond immediately (within minutes) by degranulation, which then release histamine and other vasoactive and bronchoactive mediators such as Prostaglandin D2 ($PGD_{2)}$ and cysteinyl leukotrienes (Broide *et al.*, 1991). Other stimuli such as exercise, aspirin, and chemicals are also known to trigger mast cell degranulation, causing bronchoconstriction and vascular changes. Mast cells are also involved in the production of cytokines including IL-3, IL-4, IL-5, IL-13, IL-6, and TNF-α (Hamid, 2003). Mast cell products may also be involved in airways remodeling by stimulating the migration and proliferation of fibroblasts (Bousquet et al., 2000).

D. MACROPHAGES

In both non-asthmatics and asthmatics, the most prominent cells found in BAL fluid are macrophages. Increased activation of macrophages has been linked to the severity of asthma. Airway macrophages may participate in airway inflammation through several mechanisms. Macrophage is one of professional antigen presenting cells (Sprent, 1995). Macrophages may also be involved in the development of airways inflammation and the airways obstruction via release of enzymes, eicosanoids, platelet-activating factor (PAF), oxygen free radicals and cytokines, and mucus secretagogues. They also produce pro-inflammatory cytokines such as IL-1, TNF-α, IL-6 and GM-CSF,

which may induce endothelial cell activation, cellular recruitment, and prolonged eosinophil survival (Poulter *et al.*, 1990).

Macrophages may be involved in mast cell activation during the late-phase reaction in asthma by releasing histamine-releasing factors which act on basophils and mast cells by binding to surface IgE (Liu *et al.*, 1986). Macrophages may also play a role in the regulation of the airway remodeling via the secretion of growth-promoting factors for fibroblasts, cytokines, and growth factors such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), or TGF-β (Kovacs and DiPietro, 1994).

E. NEUTROPHILS

Neutrophil number is higher in some patients with severe asthma, which has been linked with the high dose corticosteroid treatment on severe asthmatics (Lacoste *et al.*, 1993). Elevated numbers of neutrophils in lavage fluids have also been found in fatal asthma. Some studies showed that there is an association between the number of neutrophil found in sputum and the degree of airway obstruction (Woodruff and Fahy, 2002). Neutrophils also seem to have a role in corticosteroid insensitivity (Green *et al.*, 2002).

F. DENDRITIC CELLS

There are two main subsets of dendritic cells: plasmacytoid dendritic cells and conventional dendritic cells. Plasmacytoid dendritic cells are the main producers of type I interferons while conventional dendritic cells act mainly as antigen presenting cells (Manh *et al.*, 2013). As an antigen presenting cell (APC), the role of the dendritic cell network within the human airways is to express major histocompatibility complex (MHC). Dendritic cells are specialized in antigen processing and presentation, so they play an important role in the induction of immune responses in the airways (Hance, 1993).

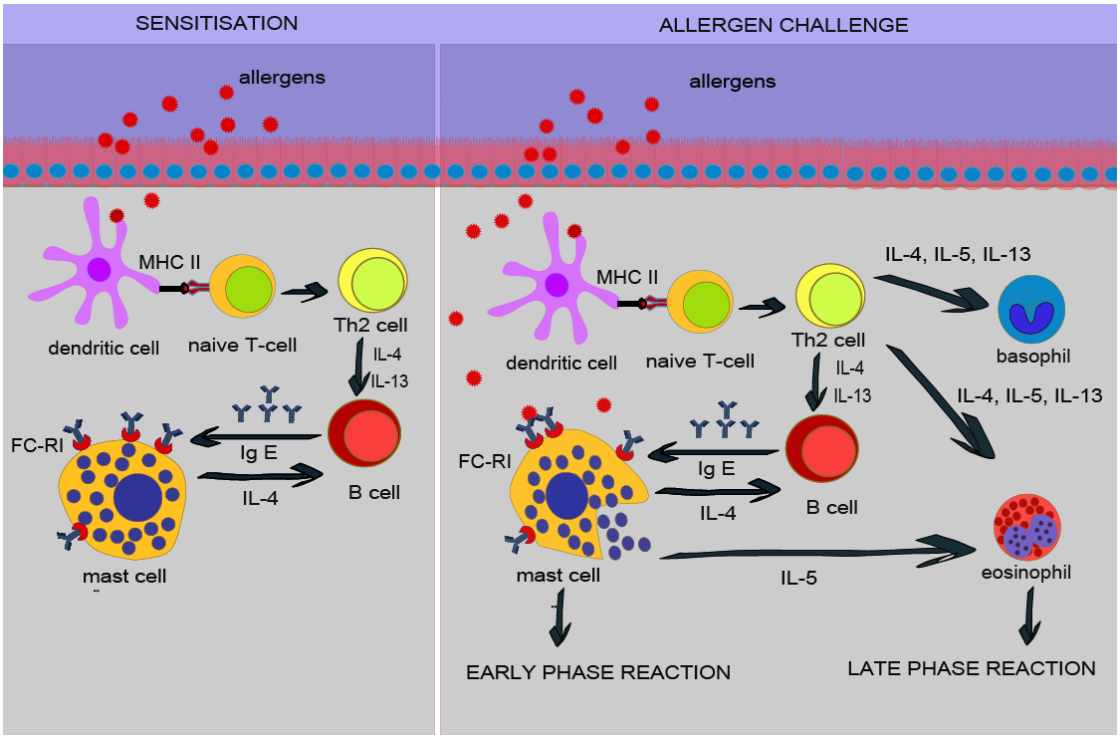


Figure 1.3. Schematic figures of pathophysiology of asthma. In atopic individual, exposure to allergen will activate Th2 lymphocytes and stimulate IgE production. Future exposure to the same allergen will cause immediate release of pro-inflammatory mediators via mast cells degranulation as well as eosinophil activation and airway hyperresponsiveness (Taher *et al.*, 2010).

II.2.4. AIRWAY HYPERRESPONSIVENESS

Measurements of airway responsiveness to inhaled bronchoconstrictor mediators (most often histamine or methacholine) are important in asthma studies. In most asthma studies, exposure to a single allergen, repetition of low dose allergen, and exacerbations of asthma may increase methacholine or histamine responsiveness in the range of 1–2 doubling doses from a stable baseline in average (Zosky *et al.*, 2004). This is airways hyperresponsiveness (AHR).

The term AHR takes both airway hyperreactivity and airway hypersensitivity into account. Airway hyperreactivity is a greater degree of closure in the airways to a variety of stimuli and the maximum bronchoconstriction to methacholine is elevated (Fig. 1.4). While airway sensitivity is a decrease in the threshold of the airways to react to stimuli, therefore sensitive airways respond to a dose of stimuli that would have no effect on

normal airways (O'Connor *et al.*, 1999). The term of stimuli can be classified into 2 types; direct acting stimuli which include histamine and methacholine, and indirect acting stimuli such as exercise, hyperventilation, cold and dry air which induce AHR by releasing bronchococstrictor mediators from cells (O'Byrne and Inman, 2003). Non asthmatics need more than 16 mg/ml of methacholine to reduce the FEV1 (*the volume of air exhaled in 1 second of the forced expiratory manoeuvre*) by 20%, whereas asthmatics would only need less than 3 mg/ml to achieve the same PC_{20} (*provocative concentration of a substance causing a 20% fall in FEV1*) (Cockcroft and Davis, 2006) (Fig. 1.4).

There is an established relationship between the level of AHR and the severity of airway inflammation. In asthmatic subjects, exposure to inhaled allergen induces a cascade that increase airway inflammation and enhances reactivity (Meurs *et al.*, 2008). Many different factors have been suggested to be involved in the AHR seen in asthma. Sterk and Bel suggested that change in airway smooth muscle contractility, thickening of the airway wall, and decreased elastic loads may increase the airway reactivity, while epithelial damage, alteration in neural control, inflammatory cells, and their mediators contribute to the hyper sensitivity of the airways (Sterk and Bel, 1989).

Eosinophilia is one airway inflammation process thought to play a role in AHR (O'Byrne and Inman, 2003). Eosinophila in asthmatic airways is suggested to contribute to epithelial damage, thickening of the basement membrane, and the release of mediators which cause bronchial smooth muscle contraction and exudation of plasma. All of these processes result in thickening of the airway wall which causes change in airway geometry. The airway will be narrower as a result of the swelling and smooth muscle shortening which are associated with AHR (Cockcroft and Davis, 2006).

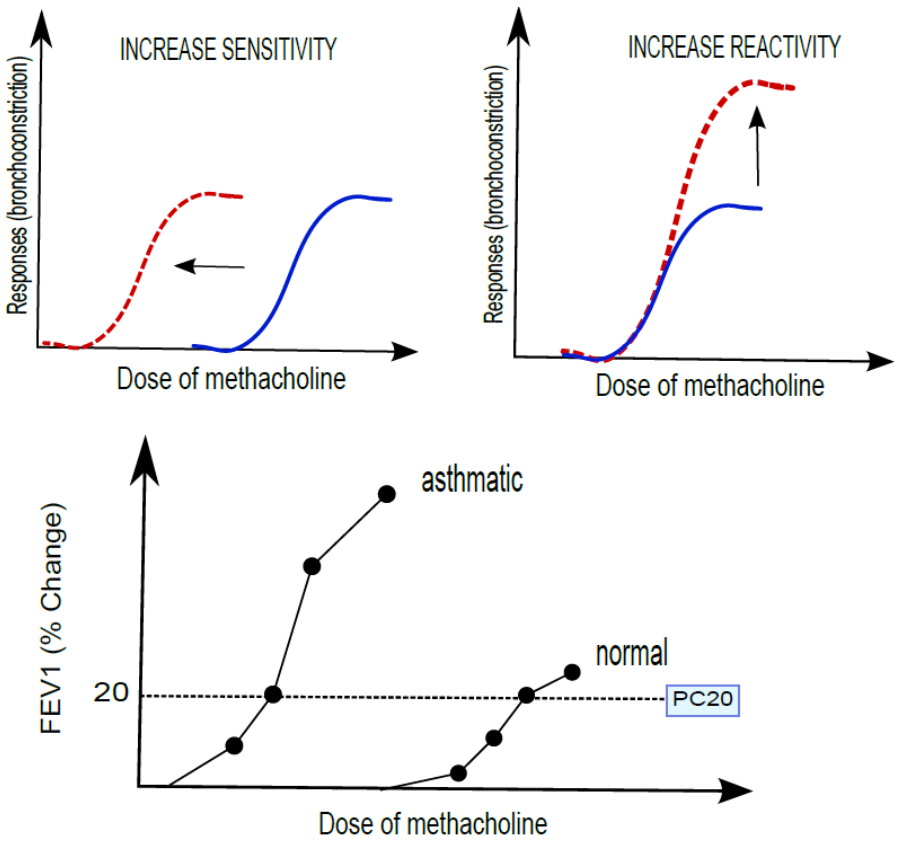


Figure 1.4. The change of FEV1 compared to baseline provoked by the exposure of methacholine (bronchocostrictor) in normal and asthmatic individuals. An increase in slope indicating increased sensitivity while increasing maximal response indicating increased reactivity of the airways (O'Byrne and Inman, 2003).

II.3. ASTHMA EXACERBATIONS

For most asthmatics, the disease would be stable with some episodes of disease exacerbations. Asthma exacerbations are “acute or sub-acute episodes of progressively worsening shortness of breath, cough, wheezing, and chest tightness, or some combination of these symptoms, characterized by decreases in expiratory airflow and objective measures of lung function (spirometry and peak flow) (Busse and Lemanske Jr., 2007). Asthma exacerbations are classified into moderate and severe exacerbations. Moderate asthma exacerbation features include deterioration in asthma symptoms and lung functions, increase use of bronchodilator, but not severe enough to require hospital care. Severe asthma exacerbations warrant increasing dose of corticosteroids for 3 days and usually need hospitalization (Reddel *et al*., 2009).

Asthma exacerbation triggers are multifactorial, including viral infections, allergens (pollen, animal danders, dust mite), exercise, airborne irritants (cigarette smoke, strong odors, dust), occupational irritants, hormones, and drugs (aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs)). These diverse triggers can produce symptoms that range from mild brief to life-threatening episodes of respiratory distress (Maddox and Schwartz, 2002).

There is a common pattern of asthma exacerbations. Phase 0 is the baseline of stable maintenance asthma. Phase 1 is where the stable baseline of lung functions abruptly and progressively decline with variation in onset, the rate of decline and the extent of decline, and phase 2 when the exacerbations progress to death or recovery (Tattersfield *et al.*, 1999).

III. VIRUS INFECTION AND ASTHMA

Respiratory viruses are closely associated with acute exacerbations of asthma. Evidence shows that they are responsible for 80–85% of acute asthma exacerbations (Johnston, 1995). In almost all studies of asthmatics, the predominant viruses are RVs (rhinoviruses), RSVs (respiratory syncytial viruses), influenza and parainfluenza viruses. Of the common respiratory viruses, RV is the most frequent causative agent. Adenoviruses, enteroviruses, coronaviruses, and metapneumoviruses, may also be associated with exacerbations of asthma but less frequently. A study in the UK estimated the cost of asthma exacerbations to be 3.5-times higher per patient compared to asthma patients who did not experience exacerbation (Hoskins *et al.*, 2000). Although asthmatics have the same probability of encountering viral infection as normal people do, they have more severe, as well as longer duration of, lower airway symptoms and also increased reduction in lung function compared to normal healthy people. Hence, respiratory tract viral infections

cause a higher level of morbidity in patients with asthma than in the healthy population (Corne, 2002). The more severe consequences of viral infection in asthmatics is a result of both pre-existing asthmatic pathology and the toxic effect of viral pathology. The underlying asthmatic Th2 type immune response may interfere with effective antiviral responses, equally, virus infection may amplify the hypersensitivity of asthmatic airways to allergen exposure (Message and Johnston, 2001).

Respiratory viruses infect the bronchial epithelium and upregulate a range of pro-inflammatory cytokines, chemokines, adhesion molecules, mucins and growth factors, all of which contribute to loss of lung function and causing airway inflammation. In virus-induced asthma exacerbations neutrophils seem to play a more significant role, while in allergen-induced asthma exacerbations eosinophils seem to be more prominent. T lymphocytes appear to be important to both (Pizzichini *et al.*, 1998).

Most respiratory viruses enter and replicate within airway epithelial cells and damage both ciliated and non-ciliated respiratory epithelial cells, leading to necrosis of the epithelium, ciliostasis, loss of cilia, and impairment of mucociliary clearance (van der Schans, 2007; Thornton *et al.*, 2008). Damaged epithelial cells increase epithelial permeability, which in turn increases penetration of allergen, including penetration into respiratory tract smooth muscle cells which causing an increase in airway hyperresponsiveness. The clinical manifestations of viral infection on top of pre-existing asthma are also likely to be a result of pro-inflammatory mediators released by damaged epithelial cells, as well as a direct cytotoxic effect of the virus (Message and Johnston, 2001).

Asthma is characterized by type 2 T-cell (Th-2) infiltration with production of their specific cytokines (Jackson *et al.*, 2008). On the other hand, viral infection is characterized by type 1 T-cell (Th-1) responses leading to production of interferons (Message and Johnston, 2001). Therefore, the balance between Th-1 and Th-2

cytokine production can be important to viral clearance. Studies have shown that within a pre-existing type 2 cytokine allergic asthmatic environments, the normally effective type 1 antiviral immune response might be inhibited, the immune response would be skewed toward type 2 responses, or both (Gern *et al.*, 2000).

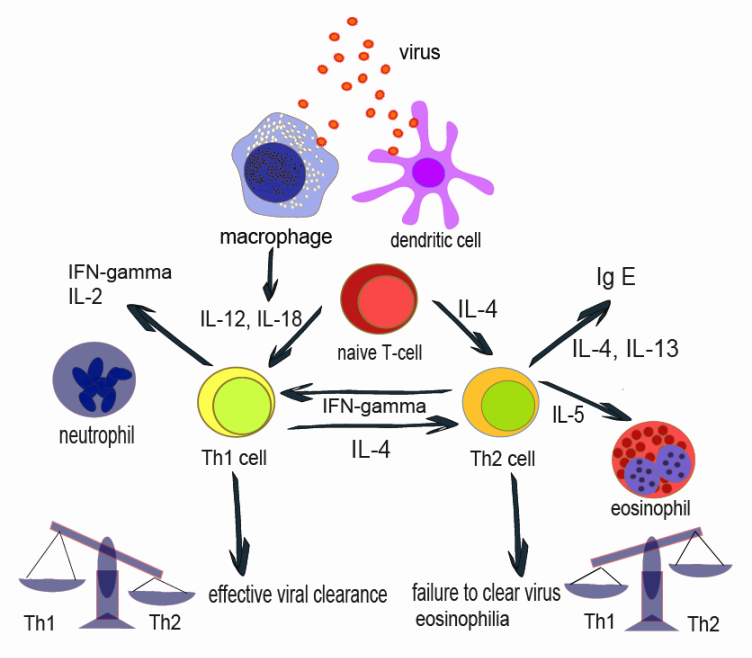


Figure 1.5. The immune response of respiratory viral infection with underlying asthma. The Th2 immune response in pre-existing asthma might alter the Th1 antiviral immune response skewing towards Th2 or mixed response which leads to increased immunopathology and tissue damage due to delay in viral clearance and prolonged viral induced inflammation (Message and Johnston, 2001).

IV. CURRENT THERAPIES FOR ASTHMA

Asthma is a chronic respiratory disease which is characterised by variable episodes of impaired breathing attacks. The hallmarks of asthma include bronchoconstriction, airway hyperresponsiveness with underlying airway inflammation, and some symptoms such as shorthness of breath, wheezing, chest tightness and cough due to narrowing of small airways (Taylor *et al.*, 2008). Based on the pathophisiology of asthma, the two most important classes of medications for asthma are controllers and relievers.

1. Controllers basically keep asthma under control to achieve target management of asthma which is defined as: asthma without any daytime or night symptoms, no needs of relievers, no exacerbations, and preserve a normal lung function (BTS/SIGN Asthma Guidelines (British Thoracic Society/Scottish Intercollegiate Guidelines Network, 05/08), 2012). Controllers available in the market and clinical practice include corticosteroid, cromones (i.e. sodium cromoglycates and necrodomil sodium), leukotrienes modifiers (i.e. montelukast, pranlukast, and zafilukast), and sustained release theophylline (GINA, 2012). Although these controllers have anti-inflammatory properties, at the moment, inhaled corticosteroids remain the cornerstone of asthma therapy because they are proven to be the most effective agents in this category of asthmatic medications (GINA, 2012). In patients with mild to moderately persistent asthma, inhaled corticosteroids significantly reduce exacerbations and asthma death (Suissa *et al.*, 2000) Another study shows corticosteroids improved pathological signs of airway inflammation, pulmonary function, and reduced airway hyperresponsiveness (Djukanovic *et al.*, 1992). Asthma exacerbations can also be treated to a lesser extent by cromones (Holgate, 1996) and theophylline (Bousquet *et al.*, 2000).
2. Reliever function is mainly to quickly reverse bronchocostriction and relieve its symptoms. Relievers are mainly bronchodilators such as short-acting inhaled or oral β2-agonists, inhaled anticholinergics, and short acting theophylline (GINA Report, 2012). The relievers are the first line therapy of acute asthmatic exacerbations to reverse airway obstruction because this class of medicines relax airway smooth muscle and also increase mucociliary clearance. In most asthma treatment guidelines, short acting β2-adrenergic agonists are regarded as the most effective and widely used bronchodilator (GINA Report, 2012).

IV.1. ASTHMA TREATMENT LADDER

Most asthma guidelines classify asthmatics based on the frequency of attack and severity of symptoms i.e: mild intermittent, mild persistent, moderate persistent and severe persistent asthma. Evidence based management of asthma also recommends gradual steps of treatments based on the disease classification with aims to improve or achieve asthma control (GINA Report, 2012).

In mild intermittent asthmatics whose symptoms occur less than once a week and night symptoms less than twice a month, with small (<20%) variability of their PEF (peak expiratory flow rate), inhaled short acting $ß_2$ –agonists are the main therapy. The short acting $ß_2$ –agonists should only be used when needed because their regular use could be harmful and have no additional benefit (Walters and Walters, 2000). When the patient uses more than 1 canister of Inhaled short acting $ß_2$ –agonists per month, the disease is regarded as poorly controlled therefore there is a need to step up on the treatment ladder with the addition of regular controller or anti inflammatory treatment. Inhaled corticosteroids are also given to this group of patients who experience severe exacerbation

Controller treatment is started at any level of non-well controlled (persistent) asthma. The mild persistent asthmatic has one or more symptom a week but less than once a day and more than twice per month night symptoms. They also have 20 – 30% variability of PEF value. This group of patients will benefit from additional inhaled corticosteroid therapy (GINA Report, 2012). It has been proven that administration of regular low dose of inhaled corticosteroids significantly reduced asthma exacerbation in patients with mild persistent asthma compared to control (Suissa et al., 2000). Leukotriene antagonist could also be used as additional anti inflammatory agents in mild persistent asthma albeit they appear to be less effective than inhaled corticosteroids (Bleecker *et al.*, 2000).

Moderate persistent asthmatics are those who are using inhaled short acting $ß_2$ agonist on a daily basis and whose symptoms occur daily with nocturnal symptoms more than once a week. They have more than 30% variability of their PEF values. Whereas severe persistent asthmatics refer to patients who have symptoms continuously therefore limiting their physical activity. They have high variability (more than 30%) of their PEF (GINA Report, 2012).

Although moderate and severe persistent asthmatics seem to benefit from increase of their current corticosteroid dose, studies on this matter remain controversial. Some studies showed no significant clinical benefit with higher dose of inhaled corticosteroid (Adams and Jones, 2006) and some studies demonstrated improvement in lung functions and reduction of symptoms optima (O'Byrne *et al.*, 2001). Based on modest benefit in increasing the dose of inhaled corticosteroid which might largely outweighed by the greater risk of side effects, this approach is not a first choice in managing moderate persistent asthma.

The first choice for moderate and severe persistent asthmatic patients is long acting $ß_2$ –agonist add-on therapy. Many studies demonstrated those patients may benefit from additional long acting $ß_2$ –agonists (e.g. salmeterol and formoterol) on top of regular inhaled corticosteroids. The FACET and OPTIMA randomised controlled trial study results showed that the addition of formoterol to inhaled budesonide improved lung function, reduced symptoms and reduced acute exacerbation optima (Tattersfield *et al.*, 1999; O'Byrne *et al.*, 2001). Long acting $ß_2$ -agonists do not have significant anti-inflammatory properties, therefore they are recommended to be given only alongside regular inhaled corticosteroids (BTS/SIGN Asthma Guidelines, 2012; GINA Report, 2012).

Other therapeutic choices to be coupled up with regular inhaled corticosteroids to manage persistent asthma are leukotriene receptor

antagonists and theophylline. Leukotriene modifiers may reduce the dose of inhaled corticosteroid in this group of patients (Löfdahl et al., 1999). However, some studies showed that this group of medicines are inferior to long acting ß2-agonists as additional therapy (Ringdal et al., 2003; Deykin et al., 2007). The last choice of add-on therapy for moderate and persistent asthma is theophylline. It is reserved as the last choice due to adverse side effects. Theophylline is a bronchodilator and has modest anti-inflammation activity in the lower dose (Barnes and Adcock, 2003).

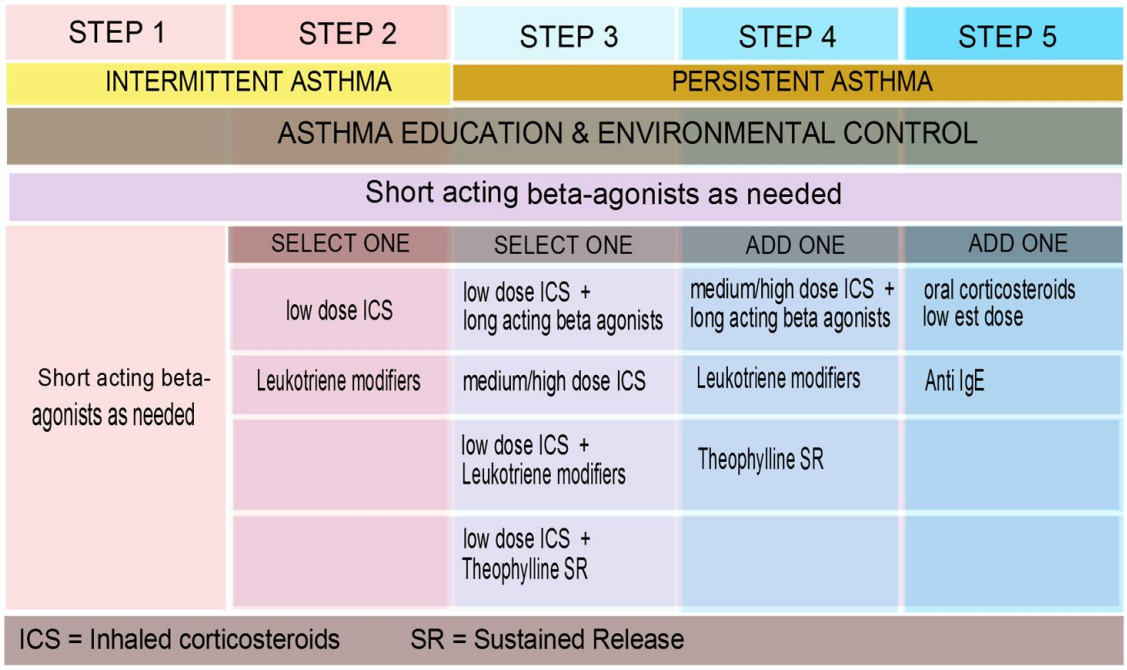


Figure 1.6. Asthma treatment ladder with stepwise approach as recommended by Global Strategy for Asthma Management and Prevention-GINA (2012).

There are some patients whose severe asthma persists and is difficult to control albeit being given the highest dose of corticosteroids and coupled with the listed add-on therapeutics. This group of patients might benefit from the regular (once daily) oral corticosteroid, prednisolone, to control the symptoms and prevent exacerbation (GINA Report, 2012).

IV.2. TREATMENT OPTIONS FOR VIRUS-INDUCED EXACERBATIONS OF ASTHMA

Viral induced exacerbation of asthma has not been yet treated effectively. The range of treatment options including antiviral therapy, interferons therapy, and corticosteroid therapy, have been studied and applied with mixed results. According to clinical guidelines, current treatment for virus-induced exacerbations of asthma is still limited to high-dose corticosteroids combined with bronchodilators for symptomatic treatment. Although antiviral therapy exists for influenza; it is not available for the most common respiratory viruses (Message and Johnston, 2001).

The use of corticosteroids in virus-induced exacerbations of asthma remains controversial. There is growing evidence showing that the use of corticosteroids alone is only partially protective against virus-induced exacerbations of asthma (Farr *et al.*, 1990; Grunberg *et al.*, 2001). There are studies on the use of prednisone or intranasal beclomethasone given for 3–5 days before antigen challenge as a prophylactic treatment in a model of experimental RV infection. The corticosteroids treatments were effective in reducing nasal obstruction, nasal kinin and mucus concentration but had non-significant effects on other symptoms (Gustafson *et al.*, 1996). However ,there is evidence that giving systemic corticosteroid in early onset of respiratory viral infection in asthmatics prevented the symptoms from progressing to severe acute exacerbation of asthma which require hospitalisation (Storr *et al.*, 1987).

In viral induced asthma exacerbations, corticosteroids may be given in combination with other reliever agents, mostly long-acting β_2 agonists (LABA). Several studies have demonstrated that the combination of corticosteroids and LABA has advantages in terms of alleviating inflammation, controlling smooth muscle remodelling and improving lung function compared to the use of corticosteroids alone, in severe or persistent asthma (Greening *et al.*, 1994; Matsunaga *et al.*, 2013).

The use of corticosteroids in respiratory viral infection alone is also still in debate. There are some findings which showed treatment of corticosteroids in acute respiratory distress syndrome (ARDS) induced by H1N1 infection was associated with significant increase in morbidity and mortality (Estenssoro *et al.*, 2010). Even though those findings were not concluded from randomized clinical trial, the reasons of not using corticosteroids in this setting are understandable. Corticosteroids are immunosuppressant which might interfere with the effectivity of eradication of the invading viruses by the host immune system. There is also a chance that the virus might take hold effectively in the immunosuppressive host.

For those who support the use of corticosteroids in viral infection, however, there is strong biological rationale underlining the decisions. In acute respiratory distress syndrome due to H1N1 infection, acute lung injury was more a consequence of uncontrolled lung and systemic inflammation rather than uncontrolled viral infection (Mauad *et al.*, 2010). There is a storm of pro-inflammatory cytokines, reactive nitrogen species, and reactive oxygen species released in the lung of the patient with ARDS (Vlahos *et al.*, 2011). Therefore, the use of corticosteroids in ARDS is justified.

v. CORTICOSTEROIDS

Corticosteroids are highly effective in the control of many inflammatory and immune diseases, including asthma. Inhaled corticosteroids are by far the most effective anti-inflammatory treatment for asthma and have now become the first-line therapy in all patients with persistent asthma (WHO/NHLBI Workshop Report, 1995).

Corticosteroids have been proven to have many benefits in any asthmatic patients needing to use β2-adrenoreceptor agonist inhaler more than once a day. The benefits include decreased mortality (Barnes *et al.*, 1998), reducing exacerbations and improving health status (Barnes, 2006). The majority of the benefits from inhaled corticosteroids are achieved in adults at relatively low doses (equivalent to 100 µg of fluticasone a day). In addition, increasing the dose might only give a little additional benefits in asthma control and increases the risk of side-effects (Kankaanranta *et al.*, 2004).

The benefit of using corticosteroids must be weighed against their side effects. Corticosteroid therapy exerts various side effects including osteoporosis, fluid retention, hypertension, cataract formation, fat deposition, cushing syndrome, and myopathy. Giving corticosteroids to children also put them in the risk of growth and sexual maturation retardation. Unlike oral corticosteroid, inhaled steroid therapy has been shown to decrease the risk of side effects. However, some molecules of inhaled corticosteroids deposited in the lung or swallowed into gastrointestinal tract are still able to undergo systemic absorption. Therefore, increasing the dose of inhaled corticosteroids to more than 5mg of cortisol per day may still increase the risk of osteoporosis (Ledford et al., 1998).

V.1. EFFECT CORTICOSTEROID ON CELL FUNCTIONS

Corticosteroids are a group of chemicals which include endogenous cortisol produced by the adrenal cortex of the vertebrae. Corticosteroids are potent anti-inflammatory agents consisting of a 21-carbon that have inhibitory actions on several inflammatory cells implicated in asthma. Corticosteroids are also very effective in blocking the release of proinflammatory cytokines, which play an important role in the recruitment and survival of several inflammatory cells involved in asthmatic inflammation (Schleimer, 1990).

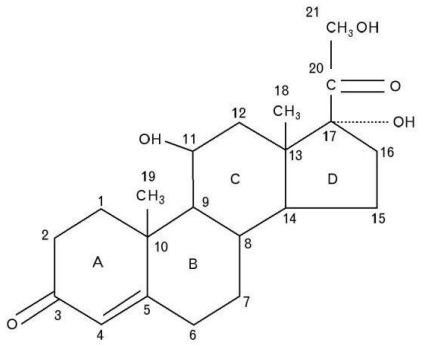


Figure 1.7. Basic chemical structure of corticosteroid (Torres and Canto, 2010).

Corticosteroids exert their biological effects via interaction with glucocorticoid receptors (GR), which are predominantly localized in the cytoplasm of target cells, and only on binding of the corticosteroids does it move into the nuclear compartment (McMaster and Ray, 2007). Different responses of cells and tissues to corticosteroids and its effect on different signaling pathways may be the results of interaction between the glucocorticoid receptor (GR) with different tissue specific co-activators or co-repressors (Yudt and Cidlowski, 2002).

V.2. GLUCOCORTICOID RECEPTOR

The glucocorticoid receptor is a member of the hormone receptor super family of proteins which is part of the basic transcription apparatus to initiate transcription (transcription factor) (Robinson-Rechavi *et al.*, 2001). GR is encoded from a single gene but several isoforms have been identified as splice variants (Adenuga and Rahman, 2007). The most common one encodes GRα. A C-terminal variation results in GRβ. GRβ does not bind the glucocorticoid ligand and has a dominant negative action on GRα. Therefore GRβ is thought to affect corticosteroid responses in some groups of inflammatory diseases (Chikanza, 2002). GRα (subsequently will be mentioned as GR) is a single polypeptide chain consisting of 777 amino acids (Adenuga and Rahman, 2007) localized in the cytoplasm of almost all cell types (Rhen and Cidlowski, 2005). Corticosteroid function is depend on its binding to GRα (Yudt and Cidlowski, 2002).

Corticosteroids exert their action by diffusing trough the plasma membrane by a passive process where they bind to the intracellular GRα which is inactive and bound to Hsp90 (heat shock proteins 90) and immunophilins. Ligand binding causes dissociation of Hsp90 thereby exposing the nuclear localization signal (NLS), which allows active GRα -ligand complex to translocate to the nucleus. In the nucleus, GRα-ligand complex could either stimulate transcriptional responses (transactivation) or inhibit transcriptional responses (transrepression) of inflammatory genes by protein-protein interactions or binding directly to the DNA (Ito *et al.*, 2006).

V.3. MECHANISM OF GLUCOCORTICOID RECEPTOR INTERACTIONS

Gene expression is controlled by how chromatin is packed in the nucleus. Chromatin consists of nucleosomes which contain DNA wound into a core of octomer of histone proteins (2 H2A, 2 H2B, 2 H3, 2 H4). The process of switching on and switching off the genes is associated with alteration of the chromatin structure. In the resting cells, the

gene expression is switched off, chromatin is condensed tightly into the histone core thus non accessible to the transcription factors. The gene is switched on when the chromatin structure is unwound allowing access of the transcriptional apparatus (Adenuga and Rahman, 2007) to start the transcription process of the DNA sequence of the gene into a molecule of mRNA which is subsequently translated into protein (i.e. enzymes, receptors, cytokines)

The winding and unwinding structures of the chromatin depend on acetylation status of the histone complexes. Histone dissociates when acetylated, the DNA is then uncoiled and the sequence is exposed to the transcription factor leading to initiation of transcription. Histone acetylation is regulated by a group of histone- acetyltransferases (HATs). Some nuclear receptor co-activators such as cyclic AMP response element binding (CREB)-binding protein (CBP)/adenoviral protein E1A (p300) protein, steroid receptor co-activator 1 (SRC-1), activator transcription factor- 2 (ATF-2) and CBP/p300 associated factor (P/CAF) have intrinsic HAT activity (Rahman *et al.*, 2004). ATF-2 and CBP/p300 are important for the co-activation of important transcription factors for inflammation such as Nuclear factor κB (NF-κB) and activator protein 1 (AP-1) (Rahman *et al.*, 2004). Transcription is initiated when the co-activator/s-transcription factor complex interacts with RNA Pol II. Histone acetylation is reversed by histone deacetylases (HDACs) which deacetylate the histones and make them rewind back to the DNA thus blocking the gene expression (Smoak and Cidlowski, 2004).

V.3.1. TRANSCRIPTIONAL ACTIVATION OF GR DEPENDENT GENES

In the nucleus, upon nuclear translocation, two corticosteroid-GRα complexes bind together to form a homodimer where it associates to DNA at specific sequences in the promoter region of corticosteroid-responsive genes known as glucocorticoid response elements (GREs) and switches on gene transcription (Ito *et al.*, 2006). The association of ligand-GRα complex to the GRE causes its conformational change which promotes

the recruitments of coactivators such as CBP/p300, P/CAF and SRC-1 to the GRα-GRE site. The mentioned coactivators have intrinsic HAT (histone acetylase) activity which seems to be important in remodeling chromatin and unwinding the DNA which subsequently allows transcription of anti-inflammatory proteins (Smoak and Cidlowski, 2004). This mechanism of actions however, has only been proven at high concentration of corticosteroids (Ito and Adcock, 2000).

Corticosteroids are also known for their action to switch on the genes responsible for the synthesis of several anti inflammatory proteins, including annexin-1, serum leukoprotease inhibitor (SLI), IL-1 receptor antagonist, IL-10, neural endopeptidase, and mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1) (Barnes, 2006). Corticosteroids also induce the production of IκBα which is an inhibitor of NF-κB and thus decreases the cellular inflammatory response which is known to be primarily through the NF-κB pathway (Adenuga and Rahman, 2007).

V.3.2. TRANSCRIPTIONAL REPRESSION OF GENE EXPRESSION BY CORTICOSTEROIDS

The primary anti-inflammatory mechanism of corticosteroids is via repression of proinflammatory genes which encode the production of proinflammatory cytokines, chemokines, and adhesion molecules. It was originally believed that GRα switches off the proinflammatory genes by direct binding to a negative GRE (nGRE). However, most genes that are repressed in the presence of corticosteroids are not known to possess nGREs in their promoter regions (Barnes and Adcock, 2003).

On the other hand, abundance of evidence shows that the repression of proinflammatory genes seems to be the result of direct protein-protein interaction between GRα and other transcription factors, mainly NF-κB and AP-1. The antagonism interaction results in repression of the synthesis of several cytokines such as TNF-α,

GM-CSF, IL-1β, IL-2, IL-3, IL-6, IL-8, and IL-11 and chemokines including eotaxin, MIP, and RANTES, iNOS, COX-2, ICAM-1 and VCAM-1 (Smoak and Cidlowski, 2004).

1. TRANSREPRESSION BY INTERACTION WITH TRANSCRIPTION FACTORS

Nuclear factor κB (NF-κB) is an abundant cytosol to nucleus translocating transcription factor which has an established important role in inflammatory signalling process. NF-κB is a homo or heterodimer formed by an assembly of subunits. The most common assembly is the p65/p50 heterodimer. When inactive, NF-κB forms a complex with inhibitor IκB to maintain its position in the cytoplasm. IκB prevents NF-κB's nuclear translocation and subsequent DNA binding activity by masking its nuclear localization signals. NF-κB is activated by some inducers including proinflammatory cytokines (IL-1 and TNFα), lipopolysaccharides from microbes, and viral infections (dsRNA) (Barnes and Karin, 1997). These inducers transmit signal to the IκB kinase (IKK) then phosphorylates the complex which leads to post-translational modification and proteasome-dependent degradation of IκB which then set the NF-κB free. The released NF-κB then translocates to the nucleus to activate transcription of wide arrays of inflammatory genes including cytokines and some inflammatory mediators (Almawi and Melemedjian, 2002) . GRα is able to generate direct physical interaction with NF-κB subunit p65 (RelA) which results in reciprocal inhibition (Barnes and Karin, 1997). The protein-protein interaction and antagonism between GRα and NF-κB has been thought to repress the expression of many proinflammatory genes.

AP-1 is also an abundant transcription factor in the cytoplasm which is responsible for the expression of many proinflammatory genes and tissue destructive enzymes such as collagenase (Johnson and Lapadat, 2002). AP-1 is formed by dimerisation of a Jun family member (c-Jun, v-Jun, Jun-B, or Jun-D) with another jun proteins or with a Fos protein (c-Fos, Fos-B, Fra-1, or Fra-2). GRα repress AP-1 via similar mechanisms on

which GRα represses NF-κB, such as direct protein–protein interactions between the c-Jun subunit and GRα which results in a mutual antagonism of transcription (Schule *et al.*, 1990). AP-1 is also able to interact with GRα by the same mechanism that it interacts with NF-κB which results in mutual inhibition of activity via the interaction of heterodimeric complexes of AP-1 and GR (Tuckermann *et al.*, 1999).

2. TRANSREPRESSION BY GR RECRUITMENT OF HDAC

GRα may repress pro-inflammatory gene activation via histone modifications on chromatin remodeling. Activated GRα may bind to CBP to inhibit its HAT activity which leads to silencing the gene (Adcock and Caramori, 2001). Other reported mechanism on which GRα switched off proinflammatory genes is by GRα binding to the transcriptional complex which followed by recruitment of co-repressors (HDACs) leading to repressing pro-inflammatory genes. HDACs, especially HDAC2 have been shown to be crucial in corticosteroid mediated anti-inflammatory activity particularly at low concentration which relevant to asthma therapy (Ito *et al.*, 2005).

V.3.3. OTHER MECHANISMS

These particular corticosteroid effects are thought not to be mediated by chromatin remodeling but by reducing stability of mRNA which in turn reduce the production of protein synthesis. In the inflammatory gene that codes GM-CSF, corticosteroids are suggested to inhibit the proteins that stabilize mRNA so they are more prone to break down and switching off the inflammatory protein expression (Bergmann *et al.*, 2000).

V.3.4. NON-GENOMIC EFFECTS

Research has shown that corticosteroids can also have a relative rapid response which can occur within minutes. This effect does not seem to go through genomic mechanisms which requires hours to occur (Stellato C., 2004). Inhaled corticosteroids have shown to cause vasonconstriction and reduce blood flow by inhibiting

extraneuronal monoamine transporter uptake of norepinephrine in smooth muscle cells (Horvath and Wanner, 2006). *In vitro*, corticosteroids interact with their receptors in the cytosol and block the production of arachidonic acid production (Croxtall *et al.*, 2000), although this effect was failed to be shown *in vivo* (Bisgaard, 2001).

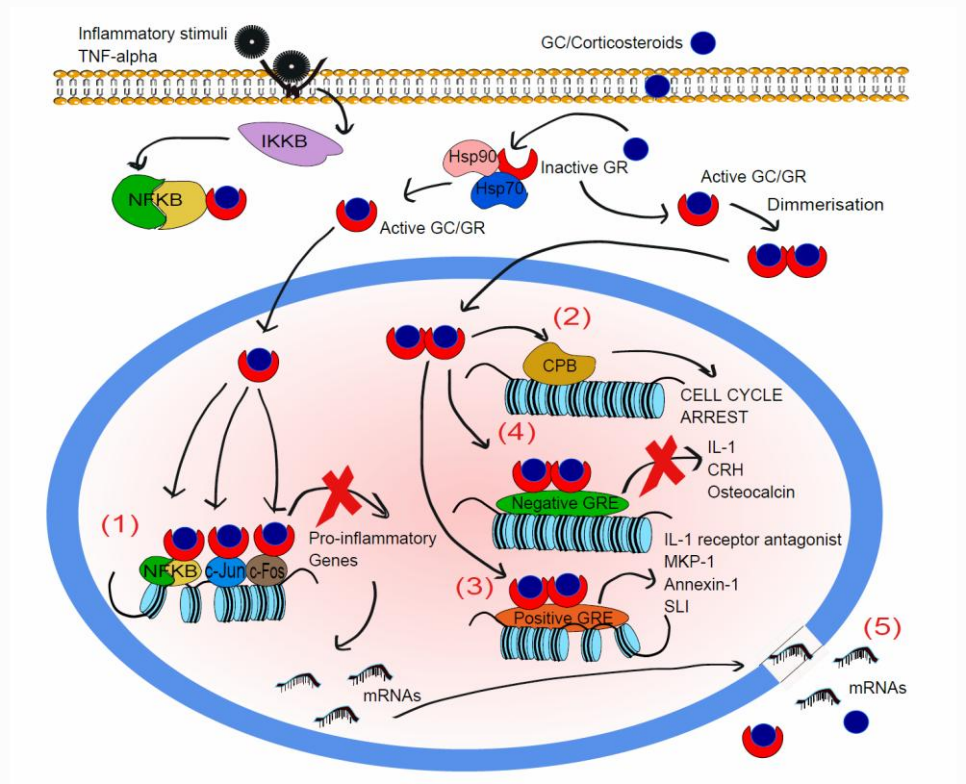


Figure 1.8. The mechanism of action of corticosteroids. After entering cells, glucocorticoids (GC) bind with glucocorticoid receptors (GR) in the cytoplasma forming GC/GR complex which then dimmerise. The complex or the dimmers might exert the anti-inflammatory effects via multiple mechanism (1). Transrepression of pro-inflammatory gene expression by blocking and binding into transcription factors such as NF-κB and AP-1; (2). Transrepression by binding into CPB which inhibits the HAT's activity; (3). Transrepression by binding to negative GRE of pro-inflammatory genes; (4). Transactivation of anti-inflammatory genes; (5). Reduce the stability of pro-inflammatory mRNAs (Fan and Morand, 2012).

VI. MODELLING ASTHMA IN MICE

The best approach to investigate pathophysiology underlying asthma disorder, and to identify crucial pathways and potential novel targets for drug therapy, is to perform studies in human asthmatics. However, the required human studies are not acceptable because of ethical reasons. Experimental animal models provide an alternative for investigating disease mechanisms and progression as well as developing new therapies. Asthma is a complex disease with many factors involved, so, there is no single animal model of asthma which reflects all of the pathophysiological features of human asthma. However, experimental animal model can be used to simulate specific features of the disease, and disease processes in asthma and in particular their responses to treatment. Laboratory animals such as guinea pigs, rats and mice have been used as a model to understand the pathobiology of asthma (Wegmann, 2008).

Mouse is the most widely used species. Many studies have used murine models of asthma for at least three reasons. First, transgenic animals are available. In addition, dense genetic and physical maps of the murine genome have already been constructed. Second is the wide range of specific reagents available for the need to investigate the cellular and mediator response. Third, mouse is a non endangered species and also relatively cheap (Wegmann, 2008).

As for other animal species, murine models also have limitations. Mice are not human and do not spontaneously develop asthma. So, to study and evaluate the fundamental processes in asthma, an asthmatic-like reaction has to be induced. A murine acute allergic reaction mimicking the reaction of acute human asthma triggered by inhaled allergens has been widely used to investigate the immunopathological mechanism underlying asthma (Nials and Uddin, 2008).

Mostly, the murine models of asthma are performed by systemic sensitization to one of a number of allergens, which is then followed by a second exposure of the allergen via inhalation or intranasal (Epstein, 2004). Such models are also capable of portraying the immunological mechanisms of acute allergic inflammation with the involvement of Th2 cytokines such as interleukin 4 (IL-4), IL-5 and IL-13 which play fundamental roles in the immunopathology of asthma (Wegmann, 2008). In this thesis, allergy in mice is generated by ovalbumin (OVA) adsorbed to the adjuvant aluminium hydroxide to skew the immune-response into a T-helper-2 (Th2) type responses with significant production of OVA-specific IgE. Inhalation or intranasal application of OVA in these sensitized animals will result in airway hyperresponsiveness and the migration of eosinophils into the airways. Airway hyperresponsiveness to a bronchoconstricting agent (methacholine) is measured post challenge.

Acute viral respiratory infections trigger wheezing and exacerbations of asthma in children and adults. Viral respiratory tract infections are also responsible for around 80% of wheezing episodes in school children (Johnston, 1995). Several animal models have been used to investigate the immunological mechanisms involved in the respiratory tract infections with respiratory viruses, allergic airway sensitization, and the development of obstructive airway disease and asthma. In these models, a range of respiratory viruses have been utilised, human parainfluenza virus (HPIV) 3 and influenza. These viruses are all proven to have potential in elucidating the mechanisms of interplay between respiratory tract viral infections and underlying allergic airways inflammation (Schwarze and Gelfand, 2002).

The anti-inflammatory properties of corticosteroids might be expected to inhibit the virus-induced airway dysfunction due to the influx of inflammatory mediators and cells in the airways. However, the studies on the efficacy of corticosteroids in virus-induced exacerbations of asthma showed controversial results. Contributing to the controversy are the variables of dose, duration, and timing of corticosteroid therapy, as well as the uncertainties of which exacerbations are caused by viral illness, even in the presence of viral-like symptoms (Message and Johnston, 2001).

VII. THESIS HYPOTHESIS AND AIMS

VII.1. HYPOTHESIS

Acute respiratory viral infection in ovalbumin-induced allergic airway inflammation will alter corticosteroid effectiveness to alleviate the allergic inflammation features in the murine model.

VII.2. AIMS

1. Develop a murine model of allergic airway inflammation exacerbation using respiratory virus infection which displays a worsening of airway inflammation and respiratory functions.

   ***OBJECTIVES**:*

   - Characterisation of respiratory virus infections on their susceptible/permissive cells and evaluation of possible antiviral effects of corticosteroids, compared to reference antiviral drugs
   - Characterisation of infection of some infective agent candidates *in vivo*
   - Reproduce a murine model of ovalbumin-induced allergic inflammation
   - Generation of exacerbation in the murine model of allergic airway inflammation which shows a worsening airway inflammation and respiratory functions.

2. Determine the role of corticosteroids in the exacerbations or worsening of allergic airway inflammation associated with viral respiratory infection.

   ***OBJECTIVE**:*

   - Establish the sensitivity of the ovalbumin airway allergic inflammation model to corticosteroids in the absence and presence of respiratory viral infection.

CHAPTER II

GENERAL METHODS

I. *IN VITRO* METHODS

I.1. HUMAN PARAINFLUENZA VIRUS TYPE-3 (HPIV3)

I.1.1. BSC-1 CELL CULTURE

BSC-1 cells, epithelial cells of African green monkey kidney origin were purchased from ECACC Salisbury UK at passage 57. BSC-1 cells were grown in Dulbecco's modified essential medium (DMEM) supplemented with 1% 2mM L-glutamine, 1% non essential amino acids, and 10% foetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO_2. The cell line was passaged when the cells achieved confluency using trypsin ethylene di-amine tetra acetate (EDTA) to detach the cells. Once cells had detached, the trypsin was neutralized with media. Harvested cells in the medium were then passaged into several dilutions in the new flask as required. Cell lines were fed with the medium 2-3 times a week. Splitting of the cells can be started either from the frozen cells or the new passaged cell line when it reached 100% confluence.

I.1.2. PREPARATION OF HPIV3 SUSPENSION (VIRUS STOCK)

The HPIV3 strain MK9 stock was purchased from the National Collection of Pathogenic Viruses (NCPV). To make batches of the HPIV3 stock, confluent BSC-1 monolayer was used. The medium was removed from the cells in a 75 cm^2 flask and virus solution was added to the cell monolayer to give ~ 2.8 x 10^7 viral particles per flask. After 5 minutes adsorption period at 37°C in a humidified incubator with 5% CO_2, 10 ml BSC-1 medium as virus growth medium was added to the flask. The infected cells were then incubated at 37°C as above for 2-3 days before harvesting. As a control or sham, a flask containing BSC-1 cells without viral infection was also treated similar to that containing HPIV3 infected cells.

At the end of the incubation period, after a clear cytopathic effect (CPE) characterised by a formation of syncytia, the cells were scraped from the bottom of the flask into the

media. The scraped cells were centrifuged at 1000 G for 5 minutes at 20°C to pellet the cells. Virus containing media (supernatant) or sham/control media was aliquoted and stored at -80°C until required.

I.1.3. QUANTIFICATION OF HPIV3 VIRAL TITRE

A wide range of techniques can be used to identify virus isolates. The number of infectious or non-infectious viral particles can be quantified by PCR, by determining the highest dilution of virus stock to produce cytopathic effects in 50% of cells ($TCID_{50}$), or by enumerating the number of plaques which originated from a single viral particle. These viral quantification methods are also essential in testing antiviral activities. Compounds which have antiviral activity will reduce the ability of virus to infect or replicate thus should also reduce the titre of the virus (Cann, 1999).

Several standard virus quantification techniques (RT-PCR, $TCID_{50}$, and plaque assay using crystal violet as well as immunocytochemistry) were conducted and compared in this thesis to determine which was the most appropriate for subsequent experiments.

I.1.3.1. QUANTIFICATION OF HPIV3 RNA BY LIGHT CYCLER PCR

PCR (Polymerase Chain Reaction) has become a new standard in virology. In this method, each of a pair of oligonucleotides called primers hybridises one of a double stranded DNA (dsDNA) target. The primers along with DNA polymerase enable selective and repeated amplifications of the target gene region. The primer used in the experiments was purchased from Primerdesign, but the primer sequence is not available due to commercial reasons. PCR usually uses heat stable DNA polymerase derived from thermophilic bacterium *Thermus aquaticus* and called *Taq.* The DNA template is amplified exponentially as the PCR progresses. There are 3 important steps in PCR; dsDNA separation at >90°C, primer annealing at lower temperature 50–75°C, and optimal extension at 72–78°C. A thermal cycle program control the cycle, the rate of temperature change, and how long the incubation is needed at each

temperature (Holland *et al.*, 1991). The LightCycler is a new technology which was designed to combine the features of rapid PCR and real-time detection of an amplification product by FRET (fluorescent resonance energy transfer) analysis (Uhl *et al.*, 2003). Rapid thermocycling as the feature of the LightCycler instrument has made rapid PCR possible (Uhl *et al.*, 2003). In "real-time PCR", the detection of the amplification product occurs after each PCR cycle.

I.1.3.2. NUCLEIC ACID EXTRACTION

RNA was extracted from HPIV3 stock solution using the High Pure Nucleic Acid Kit, according to the manufacturer instructions. Two hundred µL working solution containing poly (A) and binding buffer as well as 50 µL of 2 mg Proteinase K carrier RNA solution was added to 200 µL HPIV3 stock solution. The mixture was incubated for 10 minutes at 72°C to extract the nucleic acid content. After being washed with washing buffer and filtered with high filter tube with the aid of centrifuge at 8,000 G several times, the extracted viral RNA was then eluted with 50 µL elution buffer in nuclease free sterile distilled water. The extracted RNA was immediately stored at –80°C until use.

I.1.3.3. REVERSE-TRANSCRIPTASE PCR

RT-PCR was performed according to the manufacturer instructions by mixing 8 µl viral RNA, 1 µl of 10 mM deoxynucleoside triphosphates (dNTPs), 1 µl primer (0.5 µg/ µl oligo (dT)12-18, and DEPC-treated water to make a final volume of 10 µl in a sterile 0.5 ml tube). The mixture was incubated at 65°C for 5 minutes in a cycle, and then was placed on ice for 1 minute. A reaction mix consisting of 2 µl of 10x reverse transcription (RT)-PCR buffer, 4 µl 25 mM $MgCl_2$, 2 µl 0.1 M DTT, and 1 µl RNaseOUT (40U/µl) was then added to the tube before incubation at 25°C for 2 minutes. After the second incubation, 1 µl (50 units) of Superscript II was added to the tube, followed by incubation at 25°C for 10 minutes, 42°C for 50 minutes, 70°C for 15 minutes, and the

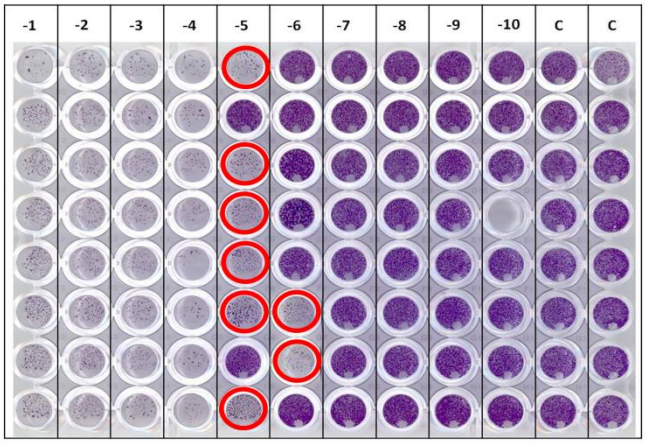
program was terminated at 5°C. The sample was used immediately for the Lightcycler reaction

A Lightcycler® TaqMan Master Kit was used as the basis for the reaction mixture in the LC-PCR assay; a 20-µl volume was used in each reaction capillary. Briefly, 5 µl of DNA template from the RT reaction was added to a capillary previously loaded with a 15 µl mixture of 4 µl mixture of master mix-ready to use hot start PCR reaction mix which contains LightCycler® FastStart Taq DNA polymerase, reaction buffer, $MgCl_2$ stock solution, and deoxynucleoside triphosphates (dNTPs) mix; 1 µl of PIV-3 primer or β-actin (ACTB) primer; and sterile PCR-grade water to make the mixture up to 15 µl. Along with the sample, six positive controls to generate a standard curve and no-target controls consisting of 15 µl of reaction mixture and 5 µl of PCR-grade water were also included. Reaction capillaries were capped, centrifuged at 13,000 g, and then placed into a carousel of the LightCycler® instrument. The LC-PCR protocol included the following parameters: an initial 10 min of pre-incubation at 95°C for FastStart *Taq* DNA polymerase activation followed by 50 cycles of amplification at 95°C for 10 seconds, annealing at 60°C for 60 seconds, and extension for cooling at 40°C for 30 seconds. The data were obtained during the annealing period with the channel set to detect emission from fluorescein which labels the RNA.

I.1.4. QUANTIFICATION OF OF HPIV3 TITRE BY $TCID_{50}$

$TCID_{50}$ is units of virus required to infect 50% of cells. BSC-1 cells were seeded onto 96 well plates at a density of 2.3 x 10^3 cells/ml and then incubated at 37 °C in a humidified atmosphere containing 5% CO_2 until reaching at least 95% confluency. The cell monolayer was then infected with 50 µl/well of serial dilution starting from 1 in 10 dilution of the virus stock in the maintenance medium. After 2 hours of inoculation, 100 µl/well BSC-1 maintenance media was added to each well. After 3 days of incubation at 37 °C in a 5% CO_2, the cell monolayer was washed with PBS three times and then

fixed with 2% formaldehyde in 0.1 M phosphate buffered saline (PBS) for 15 minutes at room temperature. The formaldehyde solution was then removed and subsequently, cells were washed three times for 5 minutes with 0.1 M PBS. After fixing and washing, 0.1% crystal violet in 10% ethanol was added for 15 minutes. The dye was then removed and stained plates were washed with tap water until no colour identified in the washing water, and dried overnight. The viral titres were determined with plaque forming unit/ml or $TCID_{50}$ by using Reed and Muench method (Reed and Muench, 1938) (fig 2.1).



Dilution of Virus	#infected/#total	%wells infected
10^{-1}	8/8	100
10^{-2}	8/8	100
10^{-3}	8/8	100
10^{-4}	8/8	100
10^{-5}	6/8	75
10^{-6}	2/8	25
10^{-7}	0/8	0
10^{-8}	0/8	0
10^{-9}	0/8	0
10^{-10}	0/8	0

Log PD = $\frac{75-50}{75-25}$ x (Log10)

PD = 0.5

Log Dilution above 50 % = 10^{-5}

Infection $10^{-5.5}$ $TCID_{50}$/50 **µl**

Or 6.32 x 10^{6} $TCID_{50}$/ml

Figure 2.1. Illustration on how to calculate quantitative viral biological activity (viral titre) which expressed in units of virus required to infect 50% of cells ($TCID_{50}$)/ml based on Reed and Muench method (1938).

I.1.5. QUANTIFICATIONOF HPIV3 TITER BY PLAQUE ASSAY

I.1.5.1. AVICEL OVERLAY CONCENTRATION OPTIMISATION

Some viruses can be easily quantified using plaque assays in standard culture medium, because these viruses undergo direct cell to cell spread which formed localized plaques. However, HPIV3 does not seem to form a clear plaque (fig.2.2).

In viral quantification using plaque assays, the infected cell monolayer is usually covered with a nutrient medium that causes the formation of a gel type substance before incubation to restrict the original infected cells releasing viral progeny to the neighbouring cells hence produce localised observable plaques (Matrosovich *et al.*, 2006). Matrosovich *et al* (2006) have developed an alternative overlay method which overcomes some of the difficulties of conventional overlay methods such as agar, which is a quite a challenge to be used in 96-well plates. The method utilises Avicel™ (microcrystalline water insoluble cellulose). The avicel overlay also produces larger and more obvious plaques compared to the conventional overlays.

A stock suspension of Avicel™ (which kindly given by FMC Biopolymer, USA) was prepared (Matrosovich *et al* (2006) and a 2.4% Avicel™ suspension in distilled water was then stirred for 1 hour. The suspension was then sterilized by autoclaving and stored at room temperature. To vary the concentration of Avicel™ in the overlays, the original 2.4% stock was diluted in BSC-1 media. Concentrated (2.4%) Avicel™ stocks did not separate during storage. However, 2.4% avicel stock was always mixed before each use to make sure that the suspension was homogenous before being used as an overlay. In the first experiment, the recommended 1.2% Avicel™ solution was used as an overlay. However, no plaques were identified even from the highest concentration of virus stock. Therefore, experiments with lower concentrations of Avicel™ overlay were tested to determine the optimum concentration of Avicel™ overlay to produce visible plaques.

Two overlay concentrations of Avicel (0.3% and 0.6%) were tested for their suitability. BSC-1 cells were seeded in 96 well-plates at the density of 2.3 x 10^3 cells/ml. The cell monolayer was then infected with 50 µl/well of serial dilution of HIPV3 in the maintenance medium starting from 10^5 $TCID_{50}$. After 2 hours of inoculation, 100 µl/well overlay of Avicel™ RC/CL in BSC-1 maintenance media was added to each well. The infected cell monolayers were incubated for 3 days. The overlay medium was then removed and the cell monolayer was fixed and stained as described previously. The dyed 96 well plates were scanned and the plaques produced by HPIV3 were counted.

As shown in Figure 2.2, plaques were visible with 0.6% and 1.2% concentrations of Avicel™ overlays tested, but plaques were clearer with 0.6% Avicel concentration. Therefore, for subsequent experiments, in order to make the plaque visible, HPIV3 infection on BSC-1 cells was incubated for 3 days with 0.6% Avicel™ overlay.

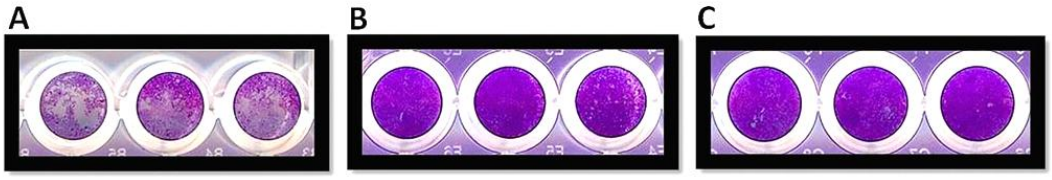


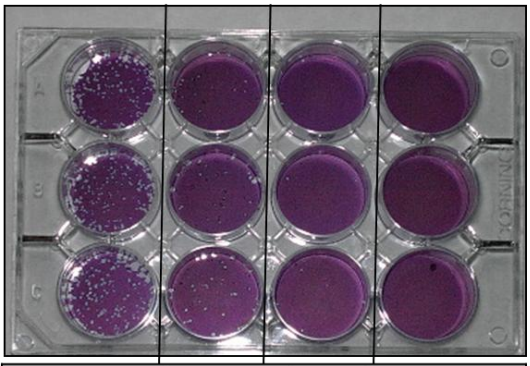
Figure 2.2. HIPV3 infection on BSC-1 cells using different concentration of Avicel overlays. After 2 hours of virus adsorption, different overlays were added: A. 0.3%, B. 0.6%, C. 1.2%. After 3 days cells were fixed with 2% formaldehyde and stained with 0.1% crystal violet solution.

I.1.5.2. QUANTITATIVE DETECTION OF HPIV3 BY PLAQUE ASSAY

Plaque assay of viral infection in cell culture monolayers is the most common and simple method for quantification of infectious viruses and potential antiviral agents (Zhu *et al.*, 2009). The replication of each viral particle produces a circular zone of infected and destructed cells known as plaque. The plaques will eventually become large enough to be visible to the naked eye. The plaque can be observed either as an area of destroyed cells identified by cellular stains such as crystal violet or as an area of infected cells identified by immuno-staining (Cann, 1999).

I.1.5.2.1. CRYSTAL VIOLET PLAQUE ASSAY

BSC-1 cells were seeded onto well plates and then incubated at 37 °C in a humidified atmosphere containing 5% CO_2 until reaching at least 95% confluency. The cell monolayer was then infected with serial dilution of the virus in the maintenance medium. After 3 days of incubation (or clear cytopathic effects were visible), the cell monolayer was washed with PBS three times and then fixed with 2% formaldehyde in 0.1 M Phosphate Buffered Saline (PBS) for 15 minutes at room temperature. The formaldehyde solution was then removed and subsequently, cells were washed three times for 5 minutes with 0.1 M PBS ready to be observed with visualisation methods. The example of how to calculate plaque forming unit (PFU) is displayed on figure 2.3.



Plaques Formed per Well			
43	4	1	0
40	3	0	0
38	6	2	0

$$\text{PFU/ml} = \frac{\text{Average \# Plaques}}{\text{Dilution factor x Volume of diluted virus added to the well}}$$

Figure 2.3. Illustration on how to calculate quantitative viral biological activity (viral titre) which expressed in a pfu (plaque forming unit) per ml

I.1.5.2.2. IMMUNOCYTOCHEMISTRY PLAQUE ASSAY

The confirmative presence of HIPV3 infection can be detected using antibodies against the viral antigen in infected cells. The method is known as immunohistochemical (IHC)/immunocytochemistry (ICC) staining. Monoclonal antibodies (MAbs) have been produced to all four major serotypes of HPIV and are commercially available. Usually these MAbs are very specific, although minor cross-reactivity has been reported (Henrickson *et al.*, 1994)**.** The experiment was performed to determine the HPIV3 antigens using ICC staining of formalin-fixed HPIV3 infected BSC-1 cell lines.

On a well plate, the completed incubation of HPIV3 infected cells were dried and washed as above and stored in PBS at 4°C until use. ICC staining was performed with the avidin–biotin–peroxidase complex (ABC) procedure (Haines *et al.*, 1992), using commercially available immunoperoxidase kits. For immunocytochemistry, infected cell lines were incubated for 30 minutes with 20% methanol/1.5% H_2O_2 to inhibit endogenous peroxidase activity, and then the cells were washed three times for 5 minutes in 0.1 M PBS. Cells were blocked from aldehyde active sites, for 30 minutes with 3% horse serum, 1% bovine serum albumin (BSA). Triton-X-100 (0.1 % in 0.1 M PBS) was also added to the well to permeabilise the cells. Subsequently, cells were the incubated with primary antibody (the monoclonal mouse HPIV3 antibody) diluted to 1 : 500 in blocking solution at 4°C overnight in a sealed plate. On the next day, after three washes in PBS, the cells were incubated for 2 hours with a biotinylated anti-mouse secondary antibody 1:500 in blocking solution. After washing 3 times with PBS, cells were incubated with avidin–biotin–peroxidase complex (ABC) for 45 minutes at room temperature. The ABC mix has to be prepared by mixing the reagent with 0.1% Triton-X-100 in 5 ml of PBS, 30 minutes before its use. Once finished, cells were washed 3 times with PBS, and then incubated with Vector® VIP substrate kit for peroxidase for 15 minutes. Stained plates were washed with tap water to stop the

reaction. As a negative control, non-infected cells were processed in an identical manner to the HPIV3-infected cells. For a negative antibody control, replicate wells of the HPIV3 infected cells were processed by substituting the mouse anti-HPIV3 antibodies with the blocking solution. The ICC staining was also performed with anti β-actin antibodies (ACTB) as a control (1:1000).

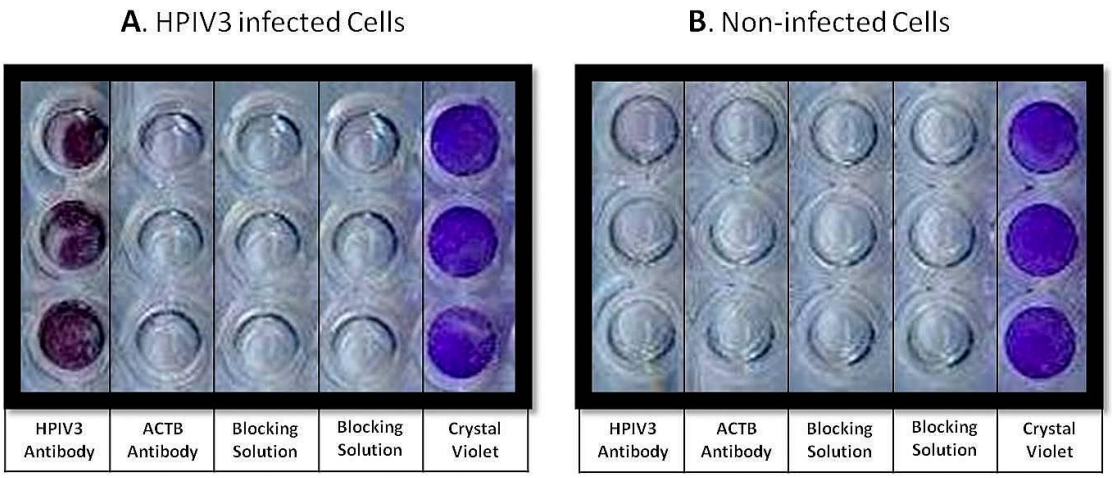


Figure 2.4. Immunocytochemistry (ICC) staining 3 days post HPIV3 infection using avidin–biotin–peroxidase complex (ABC) procedure. A. HPIV3 antigen was raised with monoclonal mouse HIPV3 antibody (1:500) and ACTB, crystal violet was also performed to confirm the infection B. The same procedures of ICC performed on non-infected cells

In ICC staining experiments, the presence of HPIV3 viral antigens was detected in cells infected with virus. The ICC techniques for the detection of HPIV3 in cell monolayer can be more sensitive and reliable (Haines *et al.*, 1992). However in this experiment, the staining did not show the localised and defined plaques which easy to be counted (fig 2.4). Therefore, for the subsequent experiments, crystal violet was used as a staining solution as it offers several advantages over ICC method, including; sharply defined plaques observed, the possibility for counting the plaques accurately and easily; the dry plate can be preserved after being fixed and stained, and thus can be counted at a convenient time. If needed, representative plates can be retained as permanent records of plaque size and morphology for comparison with those of later experiments. In addition, this procedure has been shown to give a good results on monkey kidney cell lines (Holland and McLaren, 1959).

I.1.6. VIRAL GROWTH ASSAY

This study was performed to observe the kinetics of HPIV3 growth on the BSC-1 cell line. Both plaque assay and RT-qPCR were used to quantify the viral titers to allow comparison between the 2 methods of quantification. Therefore the experiments were performed in parallel in 2 different plates. Medium was removed from confluent monolayers of BSC-1 cells on 24 well plates then 200 µl of HPIV3 was added at a concentration of 10^5 $TCID_{50}$ units/ml. After 2 hours incubation at 37°C, 200 µl of 0.6% Avicel™ overlay was added on each well. To determine the time course of viral growth and release of virions into the medium with the RT-PCR method, the culture supernatants were collected at either 1, 2, 3, and 7 days after HIPV3 infection. At the specified day, the bottom of the well plate was scrapped and the whole volume of medium was then taken for measurement of viral content. The same experiment was carried out in a well plate containing only media with no virus. The medium collected on each of the specified days was centrifuged at 300 g for 5 minutes. The supernatants were stored at −80°C for the determination of viral content, by RT-PCR or quantitative cell culture (plaque assay). The other plate which similarly treated (replicate plate) were washed with PBS, 3 times, fixed with formaldehyde and stained with 0.1% crystal violet as mentioned above.

Viral infection and replication were confirmed by RT-qPCR and plaque assay showed that viral titers of lysates from infected cells increased with time, viral content progressively increased between 1 and 7 days after infection (fig.2.5). There was a high correlation of viral load ($r^2 = 0.944$; $n = 15$, $P < 0.0001$) measured by quantitative RT-qPCR and viral titer by plaque assay (fig 2.6.).

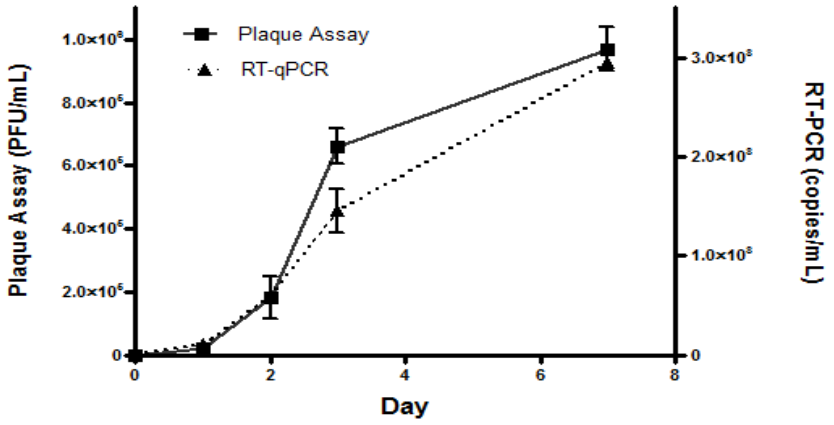


Figure 2.5. Viral titers in supernatants of BSC-1 cells obtained at different times after exposure to 10^5 units of virus required to infect 50% of cells ($TCID_{50}$)/ml of HIPV3. Results are means ± SEM from 3 samples.

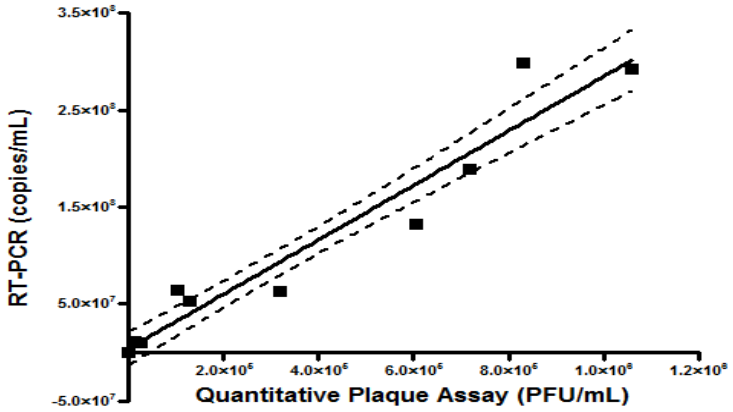


Figure 2.6. Correlation of RT-PCR versus plaque assay (r^2 = 0.944; n = 15, P < 0.0001). All quantifications were performed in triplicate. Dotted lines represent the 95% confidence limits for the slopes.

The new molecular assays like PCR are rapidly replacing the time-consuming plaque assay method in clinical virology. When compared to the specific limitations of quantification by culture, RT-PCR offers several theoretical advantages, including: a lower threshold of detection, the potential stability of the assay after specimen freezing and thawing, and a less subjective assay readout. . However, PCR is often unable to discriminate between infectious (‘alive’) and inactivated (‘dead’) virus. In this study, RT-PCR was used along with tissue culture methods to determine the viral titre. Both methods showed a good reproducibility between experiments and showed a very significant correlation.

I.2. INFLUENZA A (H1N1/PR8) VIRUS

I.2.1. MADIN-DARBY CANINE KIDNEY (MDCK) CELLS

Influenza A virus can be grown in primary or continuous epithelial cells. Epithelial cells, such as MDCK, MEK, Vero, MRC-5, and LLC-MK2 cells have been reported to support the growth of influenza A virus. However, among these influenza A susceptible/permissive cell lines, the highest titres of the virus is isolated from MDCK cells (Schepetiuk and Kok, 1993). Consequently, MDCK epithelial cells are the most widely used cells in influenza A and B studies (Schepetiuk and Kok, 1993; Reina *et al.*, 1997) and therefore being used in the experiments.

MDCK is a kidney epithelial cells of cocker spaniel which were kindly given by Dr. Awen Gallimore and Dr. Sarah Lauder (Cardiff institute of infection and immunity). MDCK cells were cultured in Iscove's Modified Dulbecco's Media (IMDM) supplemented with 0.5% heat inactivated fetal bovine serum (FBS), 10% peptone primatone, penicillin and streptomycin, insulin-transferrin-selenium and β-mercapto-ethanol. The cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C. The cell line was passaged when confluent. Trypsin-EDTA was use to detach the cells. When cells were detached from the flask, IMDM containing 10% FBS was used to neutralise trypsin-EDTA. The detached cells in the media were then centrifuged at 1000 G for 5 minutes at 20°C to pellet the cells. Cell pellet was re-suspended in MDCK culture media in the new flask as required.

I.2.2. INFLUENZA A (H1N1/PR8) VIRUS PROPAGATION

The original influenza virus strain A Influenza A virus (A/Puerto Rico/8/1934(H1N1)) stock was kindly given by Dr. Awen Gallimore and Dr. Sarah Lauder (Cardiff institute of infection and immunity). For propagation, 10-days old embryonated chicken eggs (Dekalb white from Henry and Stewart Co.) were inoculated with 100 µl of virus solution (virus stock 1:1000 in PBS) or PBS, applied to the allantoic cavity of the egg and the

punctured hole was then sealed with molten wax (fig 2.7). The influenza A and PBS inoculated eggs were then kept at 37°C for three days in an egg incubator. After 3 days, egg embryos were killed by storing egg in 5°C for 90 minutes. Chorioallantoic fluid was collected and centrifuged (2000 rpm, 10 min) to remove cell debris. Virus stocks were aliquoted and stored at -80°C until needed to use.

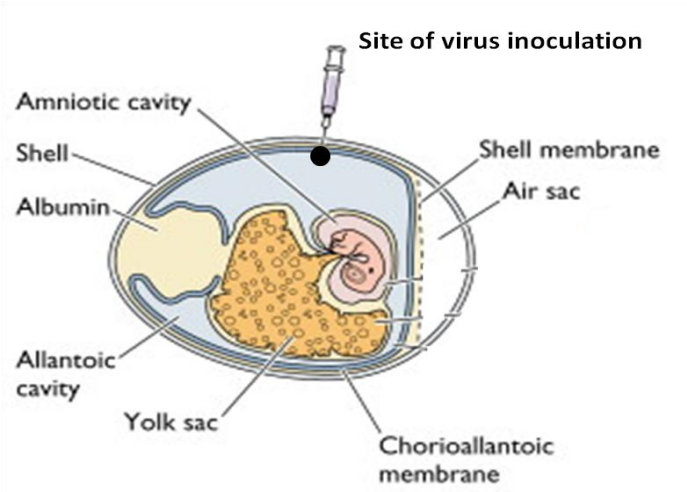


Figure 2.7. Embryonated chicken egg was inoculated with influenza A H1N1/PR8. Virus stock was injected into the allantoic fluid

I.2.3. THE PRODUCTION OF ANTI-INFLUENZA A H1N1/PR8 MONOCLONAL ANTIBODY

The hybridoma cell line producing anti-influenza A (H1N1/PR8) antibody was kindly given by Dr. Awen Gallimore and Dr. Sarah Lauder (Cardiff institute of infection and immunity). The production of antibody was carried out using Iscove's Modified Dulbecco's Media (IMDM) supplemented with 10% heat inactivated fetal bovine serum, 1% penicillin and streptomycin, 10 ppm of IL-6, 0.1% of β -mercapto-ethanol, and 10% of cloning supplement.

Cells were brought up from frozen by adding 20 ml of hybridoma growing media (37°C) into thawed hybridoma cells. The cell suspension was then centrifuged at 15000 rpm for 5 minutes. The cell pellet was suspended in 2.5 ml of media and then transferred

into 25 ml culture flask which was then incubated in a humidified atmosphere containing 5% CO_2 at 37°C until they reached confluence (a week) and were ready to be harvested.

I.2.4. ANTI-INFLUENZA A (H1N1/PR8) MONOCLONAL ANTIBODY PURIFICATION

Once confluent, the media were collected and spun at 1000 rpm for 5 minutes at room temperature. The supernatant was then filtered by transferring into Ultracel® 100K tube which filters protein less than 100 KDa and concentrates the antibody. The tube was then subsequently centrifuged at 3000 G for 30 minutes at 10°C.

The concentrated monoclonal antibody was then purified using MAb Trap Kit™ as per manufacturer instruction. MAb Trap Kit™ employs a chromatography principle to purify monoclonal antibody. The MAb Trap Kit™ column was stored in 20% ethanol so this needs to be washed out with water before being used to elute the monoclonal antibody. The column was then equilibrated with 3 ml of binding buffer (0.2 M sodium phosphate pH 7). The monoclonal antibody was then loaded into the column to bind into the protein G which is highly crossed-linked with agarose beads. The column was subsequently washed with binding buffer to remove contaminant proteins. The bound monoclonal antibody (IgG) was then eluted with 3ml elution buffer (1.0 M glycine-HCl pH 2.7). The concentration of pure IgG produced was detected with Nanovue spectrophotometer™.

I.2.5. QUANTIFICATION OF INFLUENZA A (H1N1/PR8) VIRUS TITRE BY PLAQUE ASSAY

Trypsinised MDCK cells were plated on 24 well-plate at a density of 10 x 10^5/ml (200μl of cells/well) then incubated at 37°C in 5% CO_2 atmosphere for 24 hours. The next day, the media was replaced by 200μl of a serial dilution of virus influenza A (H1N1/PR8) in the MDCK growing media (starting from 1:250 dilution and then diluted 1:3 afterward).

The plate was then incubated at 37°C in 5% CO_2 for 5 hours to allow the virus to infect MDCK cells. After incubation, 300 µl of 1.2% avicel overlay solution (from a mixture of 2.4% avicel stock and MDCK growing media 1:1) was added to the well. The plate was then incubated at 37°C in 5% CO_2 for further 48 hours.

After 48 hours, the media was removed and cells were washed three times with 600 µl of PBS. Cells were then fixed with 400 µl of 4% formaldehyde/well for 45 minutes and washed with PBS. The cells were permeabilised by adding 250µl/well solution containing 0.5% TritonX-100 in PBS for 30 minutes. The residual aldehyde groups were subsequently blocked with 400 µl/well of 10% FCs in PBS for 90 minutes. After being washed with PBS three times, cells were incubated with 200 µl/well of primary antibody (1:500) for 90 minutes, washed with PBS 5 times then followed by 90 minutes incubation with 200 µl/well of antimouse IgG-HRP antibody (1:1000). Finally, a 300 µl/well developing solution of aminoethylcarbazole (AEC) (0.4 mg/ml) in 0.05 M sodium acetate buffer, pH 5.5, containing 0.03% H_2O_2 was added to the well and incubated in the dark for 60 minutes then washed with tap water 3 times. The infected cells will develop pink-reddish colour (fig. 2.8) which can be scanned and analysed.

Figure 2.8. Infection with Influenza A (H1N1/PR8) in MDCK cells. Cultures were immunostained with aminoethylcarbazole (AEC)

II. *IN VIVO* METHODS

II.1. ANIMAL

Male BALB/c mice weighing 20-25 g were obtained from Harlan UK Ltd. All animals were housed at the laboratory animal facilities of Cardiff University. Cages which were equipped with a sawdust base, cardboard tubes, and a removable grid roof were placed in an air-conditioned room at 24°C in a 12-hours light–dark cycle at approximately 50% humidity. Food and water were available *ad libitum*. All animals were acclimated for a period of at least 7 days upon arrival before any experimental work began. The mice were also exposed to the system that measures their airway functions adapt them to a new environment and to make the animal familiar with the equipment when readings were taken. The studies were conformed to the U.K. Animals (scientific procedures) Act 1986, under valid Home Office project and personal licenses.

II.1.1. INDUCTION OF ALLERGIC INFLAMMATION OF THE AIRWAYS

II.1.1.1. SENSITISATION

Mice were sensitized on days 0 and 5 by 0.25 ml intra peritoneal (i.p.) injection of ovalbumin (OVA, 50µg/mouse) and Al $(OH)_3$ (aluminium hydroxide) (10%, 50 mg/mouse) suspension in saline solution which had been mixed for at least 2 hours to ensure ovalbumin being absorbed properly on the particle of aluminium hydroxide. $Al(OH)_3$ is needed as an adjuvant to boost the immune response and promote the production of Th2 cytokines (Kumar *et al.*, 2008).

II.1.1.2. NONINVASIVE MEASUREMENT OF AIRWAY RESPONSIVENESS

Unrestrained whole body plethysmography is commonly used to evaluate bronchial responsiveness in mice because it is easy to use and non-interventional. As no restraint is involved, stress is reduced and normal breathing patterns occur. Despite

this, in this experiment, mice still need to be habituated by introduces them to the chamber at least a week before any experiments began.

In unrestrained plethysmography, mice are placed inside a closed chamber, where they are free to move within. Pressure changes which are caused by respiration are measured within the chamber. Changes in the mouse's breathing pattern can be predicted and measured during bronchoconstriction and the changes in breathing pattern are correlated with the change in airway physiology. Therefore the calculation of enhanced pause (Penh) can be made. Changes in Penh have been commonly used as a measure of airway responsiveness to monitor the respiratory dynamics of unrestrained mice in a quantitative manner before and after bronchococonstrictor (methacholine) challenge. Penh is increased when airway obstruction is observed (Lundblad *et al*., 2002). Figure 2.9 shows the plethysmography system used in the experiments.

On day 11 and 18, body weight of each mouse was recorded and mice were allowed to acclimate to the chamber before recordings were made. Then, respiratory activity was recorded for 5 min, to establish baseline value for Penh. Mice were subsequently exposed to increasing concentration of aerosolized methacholine (acetyl-β-methylcholine chloride) dissolved in saline. The procedures were performed with preselected flow rate; length of intermittent methacholine exposures, and length of dryer time according to buxco equipment guideline. Plethysmographic data are recorded for another 5 minutes after aerosol administration. The Penh values measured during each 5 minutes sequence were averaged and were expressed for each methacholine concentration.

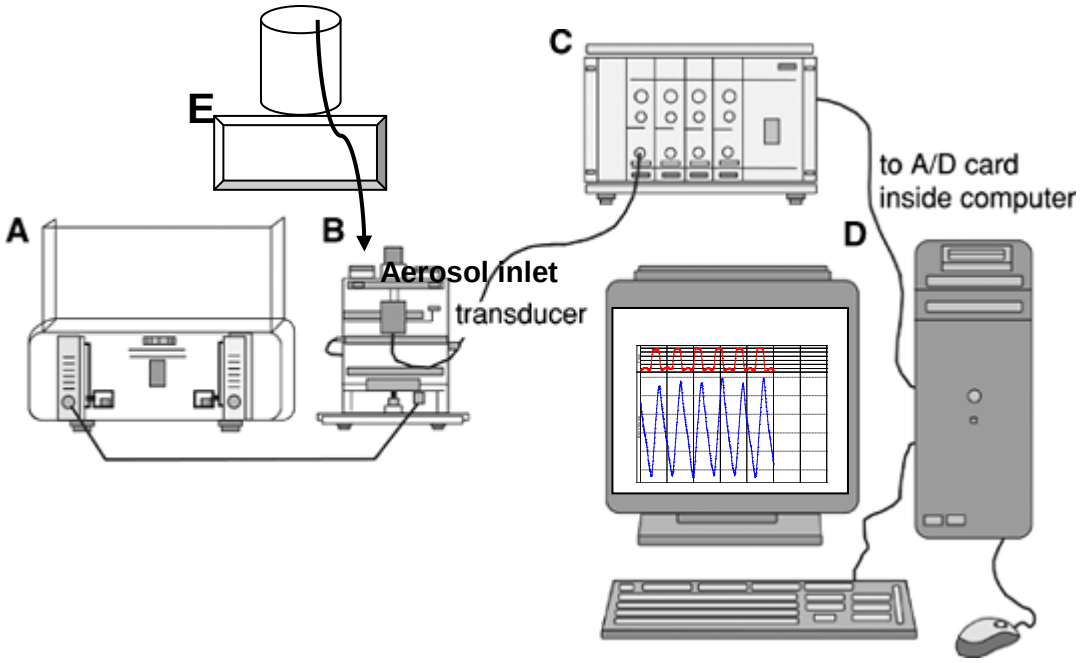


Figure 2.9. A schematic of the equipment for the measurement of respiratory function expressed as enhanced pause (Penh) in unrestrained, conscious mice (Buxco®). The 5 main parts of the equipment are indicated: *A. A*irflow regulator, which maintains a constant flow of clean air inside the plethysmographic chambers; B. Whole body plethysmograph with mouse plexiglas chambers, where it is free to move. A port at the top of the chamber is connected to a nebulizer to allow administration of aerosolized chemicals i.e. methacholine chloride. Each chamber has a coupled transducer that transforms the respiratory signal into an electrical signal and sends it to the amplifier; C. Amplifier, which receives the signals from the transducer in each chamber, amplifies and translates them into data which can visualized and analysed on the computer (D) using FinePointe software; E. Nebulizer, which delivers methacholine chloride as an aerosol into each chamber at a preselected, constant flow rate.

II.1.1.3. OVALBUMIN CHALLENGE

On day 17, to trigger the effector of asthma, mice were challenged by inhalation of a 0.5% (w/v) OVA aerosol in 0.9% sodium chloride solution (saline) for 1 hour, twice on the same day, 4 hours apart. The exposure was carried out in a perspex chamber (38 cm length; 20 cm width; 20 cm height) with a DeVilbiss nebuliser attached. Values of Penh following allergen challenge were recorded for 3 minutes at 0, 20, 40, 60, 90 and 120 minutes, then hourly until 10 hours followed by a final reading at 19 hours (or 24 hours after the first ovalbumin challenge). The Penh values which were measured during each 3 minutes sequence were averaged and were expressed for each time point as the percentage of baseline Penh values.

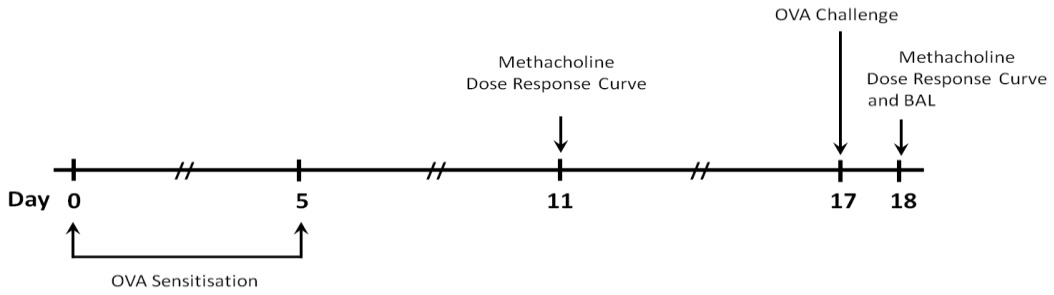


Figure 2.10. Diagram of murine model of ovalbumin induced allergic airway inflammation

II.1.2. ASSESSMENT OF PULMONARY INFLAMMATION

II.1.2.1. CELLULAR INFILTRATION

After the second methacholine chloride provocation, mice were sacrificed. Terminal anaesthesia was induced with pentobarbitone sodium (Euthatal 400 mg/kg) overdose (intraperitoneally). An incision of the skin above the thymus was performed; the tissue was retracted until the trachea was visible. An incision was made in the trachea and a cannula was inserted and held in place. One ml of PBS solution was introduced via the trachea, 3 minutes later, bronchoalveolar lavage (BAL) fluid was withdrawn from the lungs of the mice. This process was repeated three times. The fluid withdrawn was stored on ice. Two measurements of cellular influx i.e. total cell count (per ml of lavage fluid) and differential cell count were performed on BAL fluid (BALF).

II.1.2.1.1. Total Cells Counts

Total cells were manually counted using a Neubauer hemocytometer under a light microscope at 40 x magnification. The tip of pipette contained BAL fluid (100 µl) was held at an angle of approximately 45^{o} (higher or lower to control flow rate) at the leading edge of the coverslip on the haemocytometer. Capillary action allows the fluid to flow quickly and distributed evenly into the chamber. The cells were allowed to set for 1 min. The haemocytometer has two grids of 25 squares which can be observed under the microscope. The number cells in 5 of the squares was always counted in the

same manners starting from the top left, top right, middle, bottom left and then bottom right. The total number of cells obtained was multiplied by 5 to give an estimated amount of cells in 25 squares. The process was repeated for another grid on the haemocytometer and then the results were averaged. 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 was 0.1 mm, therefore giving a number of cells per 0.1 mm^3. As the number of cells per 1 ml (1 cm^3) was required, the cell count figure was multiplied by 10^4.

II.1.2.1.2. Differential Cells Counts

The differential cell count was performed to determine the different number of leucocyte types. Smears of BAL fluid cells on microscope slides were prepared for differential cell count by cytocentrifugation of 100 µl samples at 1000 rpm for 7 min using a Cytospin at room temperature. After they had been air dried for approximately 1 hour, all slides were differentially stained with 0.15% Leishman's stain (eosin-polychrome methylene blue) in 100% methanol, pH 6.4-6.6 for 7 minutes. The stained slides were then air dried overnight. A minimum of 200 cells were counted and differentiated under a light microscope at 100 × magnification. Standard haemocytological procedures were used to identify four different types of leucocytes in the BAL fluid samples: lymphocytes, macrophages, eosinophils and neutrophils. Results are expressed as a percentage of the total number of cells counted. The remaining BAL fluid was centrifuged at 3800 rpm for 6 minutes; the supernatant was then aliquoted and stored at -80^0 C.

II.1.2.2. LUNG OEDEMA/PROTEIN EXTRAVASATION

The albumin concentration in BAL fluid has been used as a marker of the pulmonary tissue permeability (Minne *et al.*, 2008). Increase in tissue permeability leads to the movement of protein-rich fluid into the tissue space. Inflammatory leukocytes

accumulation also contributes to the permeability oedema by releasing their mediators which increase the microvessel permeability and attract more leukocytes into the site of inflammation (Scallan *et al.*, 2010).

Total protein content in the BAL fluid was determined using bicinchoninic acid (BCA) method. Briefly, 10 µl of BALF samples were added to the 96 well-plate. In the same plate, PBS was also added as a negative control (blank) and serial dilution of albumin with a range of 0.025-2 mg/ml was used to determine a standard curve. Samples were mixed with 200 µl working reagent (a mixture of 50 part of reagent A and 1 part of reagent B) and then incubated at 37°C for 30 minutes. After 10 minutes period to cool the plate, it was read at 540 nm on a plate reader. The absorbance of the samples were plotted against the albumin standard curve to determine the BALF protein concentrations.

II.1.2.3. LUNG HYSTOLOGY

Once BALF withdrawal was completed, the ribcage was opened then the trachea and lung were removed from the animal. The right lobes of the lung were excised and frozen at -80^0 C for viral titre quantification or cytokines measurement if required. The large left lobe of the lung was immersed in phosphate buffered formaldehyde for histological analysis.

II.1.2.3.1. Fixation and Tissue Processing

To be able to be viewed and analysed under microscope, lung tissue must be prepared as a very thin translucent section before they were stained. This process involves fixing the tissue in 4% buffered formaldehyde to preserve it and then embed the tissue in a paraffin block to harden and support it in the slicing process.

The fixed tissues were placed in histology cassettes then processed by dehydrating the tissues with ethanol, clearing ethanol with chloroform and infiltrating/embedding them with paraffin/wax as follows:

- 50% ethanol for 1 hour
- 70% ethanol for 1 hour
- 90% ethanol for 1 hour
- 100% ethanol for 1 hour 30 minutes
- 100% ethanol for 1 hour 30 minutes
- 100% ethanol for 1 hour 30 minutes
- 50% ethanol:50% chloroform overnight
- Chloroform for 1 hour 30 minutes
- Chloroform for 1 hour 30 minutes
- Paraffin wax for 2 hours
- Paraffin wax for 2 hours
- Paraffin wax for 2 hours

At the end of the procedure, the tissues were removed from cassettes then placed with the desired orientation on a stainless steel base mould. The mould allowed the wax to be decanted into the cassettes during the embedding and secure the tissue in the wax when the moulds were cooled on a cooling plate at -30^{0}C. The tissue now secured and set in the paraffin wax was ready to be sliced with microtome as a thin section (5 µm). The sliced thin section was placed on a microscope slide by floating the section out on a water bath (30^{0}C) and then picked it up with the slide. The slide sections were then dried at room temperature overnight ready to be stained on the next day.

II.1.2.3.2. Tissue staining

The first procedure in the tissue staining is de-waxing by using Histoclear to remove the wax from the slides and rehydrated it with a series of graded ethanol for 5 minutes before being stained. At the end of staining, the reverse process, dehydration, is

applied to the tissue, and completed with the use of Histoclear to rinse ethanol out of tissue.

II.2.1.3.2. Haematoxylin and Eosin (H and E)

H and E staining is a routine staining procedure because it shows very detailed view of the morphology tissue by clearly stain cell structure including differentiation between the nucleus and cytoplasma. The step by step procedures of H & E staining described as follow:

- Histoclear for 5 minutes
- Histoclear for 5 minutes
- 100% ethanol for 5 minutes
- 100% ethanol for 5 minutes
- 90% ethanol for 5 minutes
- 70% ethanol for 5 minutes
- Distilled water for 5 minutes
- Mayer's Haematoxylin for 3 minutes
- Running tap water for 5 minutes
- Rinse in distilled water
- Scott's tap water for 5 seconds
- Running tap water for 5 minutes
- Rinse in distilled water
- Eosin Y for 1 minute 30 seconds
- Running tap water for 5 minutes
- Rinse in distilled water
- 70% ethanol for 5 minutes
- 90% ethanol for 5 minutes
- 100% ethanol for 5 minutes
- 100% ethanol for 5 minutes
- Histoclear for 5 minutes
- Histoclear for 5 minutes

II.1.2.3.3. Histopathology

When staining was completed, the slide was mounted with Histomount and covered with a coverslip to make the tissue permanent on slide. Analysis of the dried prepared tissue was performed under a Leica DMRAZ microscope equipped with Leica DC500 camera and Leica QWin software to capture the picture.

II.2.1.3.3. Haematoxylin & Eosin for general morphology

The H & E staining is mainly aimed to assess histological change in the lung particularly peribronchial inflammation and inflammatory cells infiltration. The sections were graded subjectively by blinded investigators using semiquantitative scoring method on three (upper, middle, and lower parts) 100 times fields of lung section images. The parameter used in scoring system was peribronchial inflammation (0-3), perivascular inflammation (0-3), epithelial desquamation (0-3), and the evidence of haemorrhage (0-3) to give a score ranging from 0 – 12 (Table 2.1) (Longhi *et al.*, 2007; Nandedkar *et al.*, 2008).

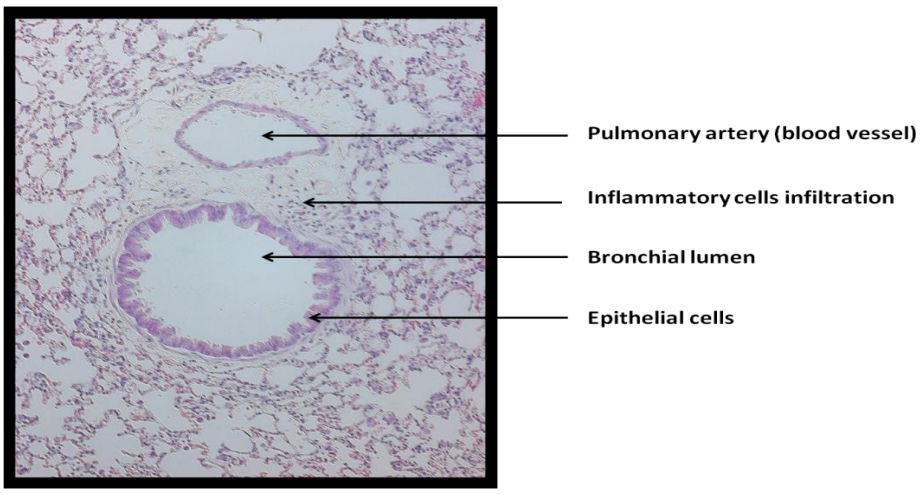


Figure 2.11. Photomicrograph showing lung histology of OVA sensitised and challenged mice. Tissue was stained with H & E, 100X magnifications.

Table 2.1.Lung histology scoring system

Feature Scored	Score	Descriptions
Peribronchial inflammation	0 = None	0 - 3 layers of inflammatory cells
	1 = Mild	3 -6 layers of inflammatory cells
	2 = Moderate	6 - 10 layers of inflammatory cells
	3 = Severe	More than 10 layers of inflammatory cells
Perivascular inflammation	0 = None	0 - 3 layers of inflammatory cells
	1 = Mild	3 -6 layers of inflammatory cells
	2 = Moderate	6 - 10 layers of inflammatory cells
	3 = Severe	More than 10 layers of inflammatory cells
Epithelial desquamation	0 = None	Normal intact airway epithelial lining
	1 = Mild	Elongation and distortion of the cuboidal/columnar epithelial cells lining the airways
	2 = Moderate	Elongation associated with in folding of the epithelium and narrowing of the airway lumen
	3 = Severe	Epithelial cells shedding or loss of epithelial cells with cell debris inside the bronchial lumen
Haemorrhage	0 = None	No haemorrhage
	1 = Mild	Vascular or alveolar haemorrhage
	2 = Moderate	Vascular and alveolar haemorrhage
	3 = Severe	Severe vascular and alveolar haemorrhage, affecting more than 50% of the captured area

III. STATISTICAL ANALYSIS

Statistical analysis was performed using the software package PRISM, version 5.0. ANOVA (One way analysis of variance) followed by Bonferroni's post hoc test were used to compare 2 or more groups. Student's t-test was used to compare the difference between groups. P values <0.05 were considered significant and were denoted with an asterisk.

CHAPTER III

EFFECTS OF CORTICOSTEROIDS ON RESPIRATORY VIRAL INFECTION IN VITRO

I. INTRODUCTION

Viruses are an *infectious agents of small size and simple composition that can multiply only in living cells of animals, plants, or bacteria* (Wagner, 2013). According to its definition, viruses which are composed of either DNA or RNA, are basically parasites because outside the host cell, these organisms do not have any metabolic activity hence are totally dependent on the host cells for life (Harper, 1998).

Respiratory viral infections are responsible for most cases of medical consultation with the consequence of morbidity, hospitalization and mortality, throughout the world (Abed and Boivin, 2006). Respiratory viral Infections also cause significant socio-economic loss mostly because of lost of productivity or absenteeism. Respiratory viral infections affect any age group. The more severe clinical complications particularly affect immunocompromised patients, children and the elderly (Message and Johnston, 2001).

Respiratory viruses are mostly airborne and tend to have a higher level of circulation during winter in subtropical countries. In the UK, the most common viruses to cause seasonal outbreaks are Respiratory Syncytial Virus (RSV), influenza and parainfluenza, rhinovirus, adenovirus, coronaviruses, coxsackievirus and human metapneumovirus (Tregoning and Schwarze, 2010). This chapter will focus on the infection of 2 of the most common respiratory viruses i.e human parainfluenza virus type 3 (HPIV3) and influenza virus

I.1. PARAINFLUENZA VIRUSES

Human parainfluenza viruses belong to the *Paramyxoviridae* family which also include mumps, measles, and RSV. They account for a major cause of respiratory disease particularly in neonates and infants. The parainfluenza viruses are divided into four different serotypes: HPIV-1, 2, 3 and 4. HPIV-1 and HPIV3 are responsible for roughly

15% cases of colds, croups, bronchitis and pneumonia in children which usually last for 7-10 days (Denny and Clyde, 1986).

I.1.1. STRUCTURAL ORGANISATION

HPIV3 which is described as a pleomorphic enveloped virus, is a single-stranded, nonsegmented, negative strand RNA virus. Its 15,462 base genomic RNA of negative polarity encodes six common structural proteins critical for viral replications i.e. 3'-N-P-C-M-F-HN-L-5' (Bose *et al.*, 2001). The N, P, and L HPIV proteins are the RNA polymerase proteins and nucleocapsid protein, whereas HN (hemagglutinin-neuraminidase) and F (Fusion) proteins are two surface glycoproteins and M protein is the matrix protein which is responsible for viral assembly and budding (Henrickson, 2003).

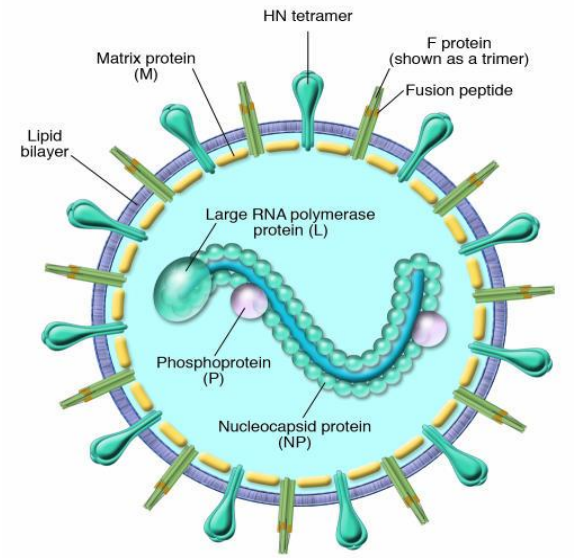


Figure 3.1. Structure of human parainfluenza virus type 3 (Moscona, 2005)

I.1.2. VIRAL REPLICATION

HPIV3 infection is started by viral binding into the target cells. The HN glycoprotein of the HPIV3 interacts and attaches to the sialic acid-containing cell surface receptor. Subsequently, the viral envelope fuses with the plasma membrane of the cells via viral

fusion protein F and then uncoats the genome RNAs. Transcription in the cell cytoplasma is aided by virus-specific RNA-dependent RNA polymerase (L protein) (Henrickson, 2003; Moscona, 2005). The viral mRNAs produced are then translated into viral proteins by the host's ribosomes which directs the replication of the virus genome into a positive-sense RNA strand and then into the negative strand. The single negative strands of RNA which is then encapsidated with NP may be used in further transcription and replication or exported as a new virion (Holtzman *et al.*, 2002).

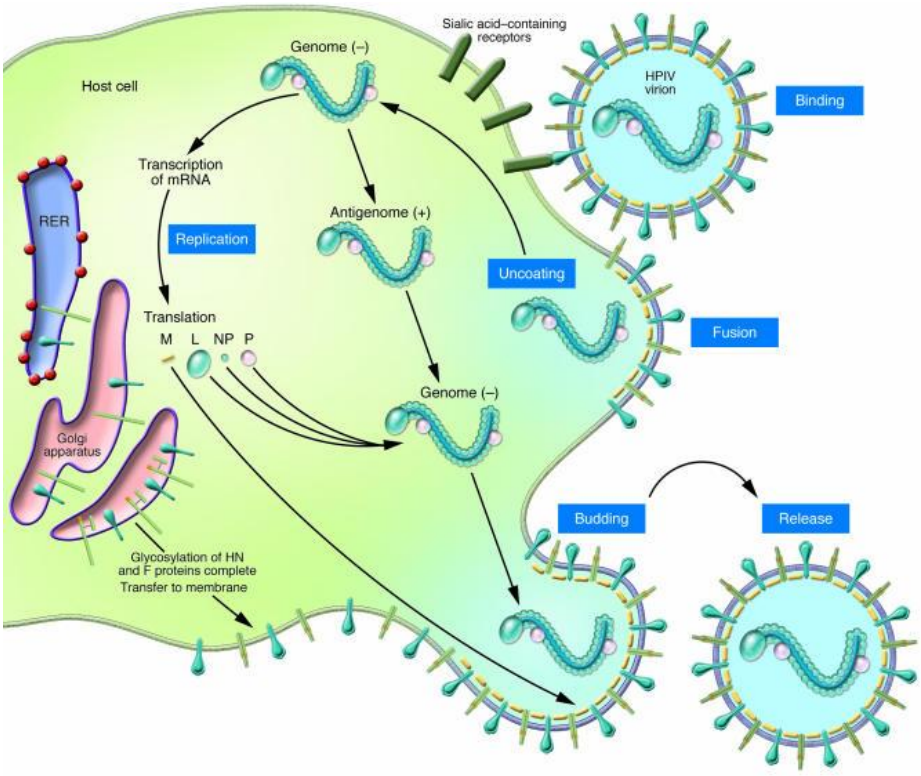


Figure 3.2. Schematic representation of the life cycle of parainfluenza virus (Moscona, 2005).

I.1.3. PATHOGENESIS

Airways epithelium seems to be major site of virus binding and infection. The HPIV replications result in the influx of inflammatory cells into the airways and this seems to be more responsible for the manifestation of clinical symptoms than the cytotoxicity of viral infection. Epithelial damage as a result of the secondary inflammation leads to necrosis of cells, increasing mucus secretion, as well as airway obstruction

accompanied by wheezing and coughing known as bronchiolitis, cold, croups, and pneumonia (Moscona, 2005, Henrickson, 2003).

I.1.4. PREVENTION AND CONTROL

Several compounds including neuraminidase inhibitors (zanamivir), protein synthesis inhibitors (puromycin), and nucleic acid synthesis inhibitors (acyclovir) have been found to have antiviral activity against paramyxoviruses *in vitro* (Henrickson, 2003). However, none of those drugs have been applied clinically. Ribavirin has been proven to have *in vitro* and *in vivo* antiviral activity against HPIV (Browne, 1981). However, it also has been reported that established HPIV3 pneumonias responded poorly to ribavirin (Nichols *et al.*, 2001). So, currently no antiviral drugs have been proven to have clinical efficacy against HPIV3 infections. There is also no licensed HPIV vaccine available at the moment in the market. Although there is a study which shows that the live attenuated HPIV3 vaccine can induce good humoral immune responses in seronegative children (Belshe and Hissom, 1982). Several studies, some of which are meta-analysis, have shown the effectiveness and efficacy of oral or systemic steroids in improving symptoms of croup as early as 6 h after treatment (Somani and Evans, 2001). Therefore the administration of a corticosteroid, particularly dexamethasone, is recommended to be considered even in mild croup (Zoorob *et al.*, 2011).

I.2. INFLUENZA VIRUS

Influenza virus infection is causing a massive global burden of 1 billion cases per year, with an estimated 300,000-500,000 number of mortalities worldwide (Girard *et al.*, 2008; Girard *et al.*, 2010). Influenza virus is highly contagious, spreading via droplets of aerosols from infected people and causing acute respiratory diseases. Epidemics and sporadic pandemics because of the virus are regarded to be a major cause of morbidity and mortality worldwide (Cox and Subbarao, 1999). Influenza-related pneumonia has

been established as a leading cause of infectious disease-related deaths especially in the elderly. The common clinical symptoms of influenza infection are fever and chills, accompanied by dry cough, headache, fatigue, myalgias, and general discomfort (Cox and Subbarao, 1999; Hilleman, 2002).

Influenza viruses i.e. Influenza A, B and C viruses make up 3 of the 5 genera of the *Orthomyxoviridae* family. Influenza A is clinically the most important to humans, as it has the ability to undergo antigenic drift and shift to survive in the human population. As a consequence of this antigenic shift and drift, influenza A viruses are categorised into several subtypes, based on the type of their hemagglutinin (HA) and neuraminidase (NA) proteins. Seventeen different types of hemagglutinin (H1-H17) antigen and 10 different type of neuraminidase antigens (N1-N10) have been identified so far (Das *et al.*, 2010). H1N1 is a subtype of Influenza A which has been declared by the World Health Organization as a pandemic in June 2009. This virus is also endemic in pigs and birds (WHO, 2009). Influenza A H1N1/PR8 is a historic strain which caused a local outbreak of infection in Puerto Rico in 1934 (Hannoun, 2013).

I.2.1. STRUCTURAL ORGANISATION

Influenza A, is an enveloped virus with a genome made of a negative single-stranded segmented RNA. The segmented genome, contains 8 segments which encode for 11 viral genes i.e. HA (hemagglutinin), NA (neuraminidase), M1 (matrix 1), M2 (matrix 2), NP (nucleoprotein), NSP1 (non-structural protein 1), NS2 or NEP (non-structural protein 2), PA (polymerase acidic protein), PB1 (polymerase basic protein 1), PB2 (polymerase basic protein 2) and PB1-F2 (polymerase basic protein 1 – F2) (Zhang et al., 2000).

Most of influenza virus virions are roughly spherical in shape. Three viral transmembrane proteins: HA, NA, and M2 make up the lipid bilayer viral envelope (Deng *et al.*, 2006). The most abundant (80%) envelope protein is HA, while NA protein

makes up around 17 percent and M2 protein is only making up a small proportion of envelope protein in about 16 to 20 molecules per virion. Covered by the viral lipid membrane envelope is the core of the virus, the viral ribonucleoproteins (vRNPs), which are held by a matrix made up from M1 protein. The vRNPs which are viral negative stranded RNAs, are coiled up around NP and a bit of NEP. The three polymerase (3P) proteins (PB1, PB2 and PA) which function as the viral RNA polymerase complex are positioned at one end of the vRNP (Zhang *et al*., 2000).

I.2.2. VIRAL REPLICATION

The surface of the viral envelope is spiked with membrane glycoproteins, hemagluttinin (HA) and neuraminidase (NA) and a small number of M2 (figure 3.3). The HA binds to the host cell sialic acid receptors and initiates receptor mediated endocytosis. The virus is entering the cell via the endocytic vesicle formed. The endosomal vesicle has a low pH which enables the fusion of the viral and endosomal membrane as well as opening up the M2 ion channel. The opening of the M2 ion channel leads to the release the vRNPs from M1 to enter the host cell's cytoplasm (Watanabe *et al.*, 2003). NP, PA, PB1, and PB2 which make up vRNP have nuclear localization signals (NLSs) properties which aid the entry of the vRNP into the nucleus to start transcription and replication. The viral genome then enters the nucleus to begin transcription by performing a 'cap–snatching' mechanism. The viral polymerase complex (PB1, PB2 and PA) binds to the 5' cap of the host mRNA and cleaves the 10-13 nucleotides. So, the capped host mRNA fragments can be used as primers for each of 8 viral mRNA gene segment transcriptions. After being transported into the cell cytoplasm, viral mRNAs subsequently perform a translation process. Each of the 6 newly transcribed mRNAs encodes 1 viral protein, while each of 2 other RNA transcripts are spliced to encode 2 proteins. The viral membrane glycoproteins (HA, NA, and M2) are processed in the endoplasmic reticulum and glycosylated in the golgi apparatus. The viral polymerase complex also primes the old negative sense viral mRNA, through the

production of cRNA (copy RNA) which then translated into the new negative sense viral mRNA. The assembly of the newly formed virions are performed at the host cell membrane where the virus budded out and then released by the help of NA protein to cleave the sialic acid receptor binding to make them free to invade other host cells das (Samji, 2009; Das *et al.*, 2010).

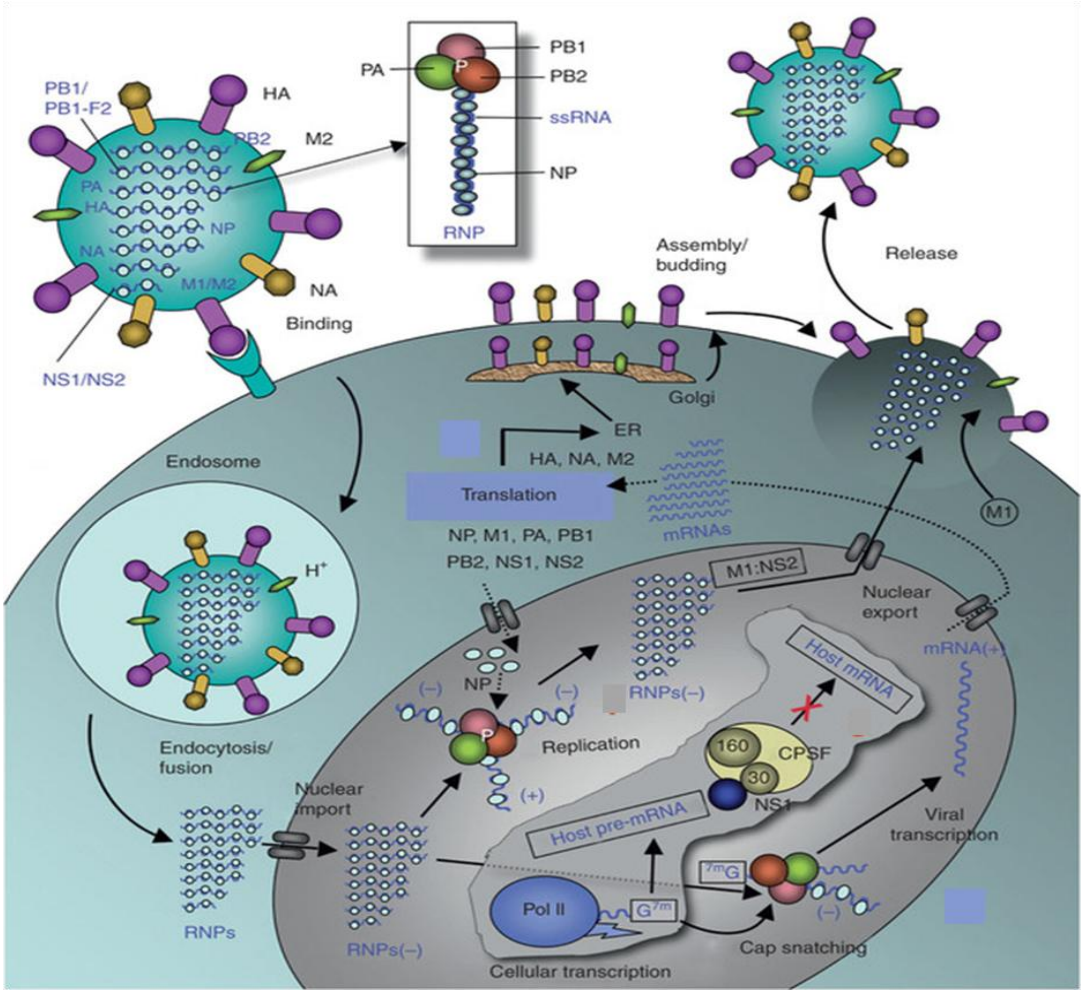


Figure 3.3. Structure and schematic illustration of the life cycle of influenza virus (Das *et al.*, 2010).

I.2.3. PATHOGENESIS

Epithelial cells of the airways are the primary site of influenza virus replication. The infection causes destruction and desquamation of the epithelium in the trachea and bronchi. Oedema and congestion are also often observed. Pathological changes due to the infection occur throughout the respiratory tract, particularly in the lower respiratory tract. The destructed epithelial cells trigger the influx of inflammatory cells into the airways which contributes to the clinical characteristics of the infection, such as fever, myalgia and dry cough. Depending on the host's immune system, influenza infection could recover within a few days but could also develop into primary pneumonitis especially in immunocompromised individuals. The influenza infection might also lead to secondary bacterial infections which could develop into a fatal form of pneumonia (Wright and Webster, 2001; Hilleman, 2002).

I.2.4. PREVENTION AND CONTROL

Currently, vaccines are by far the most effective means of preventing influenza virus infection, either for prevention, or minimising the severity of the disease caused by the influenza virus. However, it is still hard to predict whether vaccine alone would be able to prevent an influenza pandemic break (Nistal-Villan and Garcia-Sastre, 2009).

At the moment, there are four antiviral drugs for approved treatment of influenza virus infections including; M2 ion channel inhibitors (amantadines and rimantadines), which inhibit viral replication of influenza by interfering with the M2 protein and neuraminidase inhibitors (oseltamir and zanamivir). Neuraminidase inhibitors prevent virus release from the host cell by competing with sialic acid to bind with NA consequently the viral cleaving process from the host cells is blocked and the virus would be inhibited to infect the neighbouring cells (Abed and Boivin, 2006). However, albeit being proven to be effective, some studies found that many influenza strains have developed resistance toward M2 inhibitor and there is also increasing evidence of viral resistance towards

oseltamivir (Carr *et al.*, 2002; van der Vries *et al.*, 2010). Although there is an interesting fact that most oseltamivir resistant strain are still sensitive to zanamivir (Hurt et al., 2006). The reason could be because of the different chemical structure between those two. Zanamivir contains a guanidine group, hydrophilic chain and can only be administered via inhalation hence has limited use, while oseltamivir structure contains an amino group, hydrophobic chain, and has a high oral bioavailability hence has been used extensively (Collins et al., 2008).

I.3. THE AIRWAY EPITHELIUM AND RESPIRATORY VIRAL INFECTION

The airway is protected physically and functionally from the external environment by epithelial cells. The cells also play an important role in initiating and regulating innate and adaptive immune responses in the airways. Most if not all respiratory tract viruses enter and replicate within airway epithelial cells. During the infection, the epithelium can be injured, which causes of loss of integrity and protection of both ciliated and non-ciliated respiratory epithelial cells, and leads to necrosis of the airway epithelium, ciliostasis, loss of cilia, and impairment of mucociliary clearance (van der Schans, 2007; Thornton *et al.*, 2008). However, it is likely that the clinical manifestations might be secondary to the release of pro-inflammatory mediators by damaged bronchial epithelial cells rather than a direct cytotoxic effect of the virus (Jackson and Johnston, 2010).

Several cell lines support the growth of HPIV3. The most common are LLC-MK2, Vero, CV-I, primary cynomologus and rhesus monkey kidney, Wish, HMV-II, HEp-2, MDCK, BHK, HeLa, primary human embryo, KB, Am, HEB' L929, HEF and BSC-1 (Genest and Daniel, 1966; Frank *et al.*, 1973; Belshe and Hissom, 1982; Moscona and Galinski, 1990). HPIV3 can also replicate in organ cultures from mouse, guinea pig, ferret, and human fetal respiratory epithelium (Bando et al., 1991). All HPIV demonstrate greater

cytopathic effects on adaption to a particular cell line, with HPIV3 being the most aggressive (Henrickson, 2003).

Apart from the growth in allantoic sac of embryonated egg, influenza A virus can also be grown on primary or continuous epithelial cells. Madin-Darby canine kidney (MDCK) epithelial cells are the most widely used cells in influenza A and B studies (Schepetiuk and Kok, 1993; Reina *et al.*, 1997). Other epithelial cells, such as MEK, Vero, MRC-5, and LLC-MK2 cells also have been reported to support the growth of influenza A virus, although the titres of the virus isolated from these cell lines were significantly less than MDCK (Schepetiuk and Kok, 1993).

In terms of *in vitro* viral studies, cell lines are said to be permissive when they support the replication and multiplication of the virus. Viral infection in permissive cells usually results in cells death, so the infection is known as cytocidal or cytolitic infection. The characteristic of cytocydal infection is morphologic changes of the infected cells defined as cytopathic effects (CPE) which can be visualised in stained cells or in unstained cells under light microscope (Albrecht *et al.*, 1996).

Depending on the type of infection, there are various kinds of CPE such as formation of syncytia which is multinucleated cells formed because of fusion of nuclei, detachment of cells from monolayer and rounding of cells (Albrecht *et. al.,* 1996). In monolayers, most new isolates of the HPIV3 virus produce cytopathic changes including focal cell necrosis and formation of multinucleate syncytia (Craighead and Brennan, 1968). Cytopathic effects produced by human influenza are quite complex. The effects predominantly occur at the columnar epithelial cells in the airways which then result in acute respiratory disease. The influenza viral replication leads to epithelial cell damage because of host cell protein synthesis downregulation and apoptosis or programmed cell death (Kamps *et al.*, 2006). The apoptosis in the influenza infection is characterized by some morphological changes, such as condensation of cytoplasm

and chromatin, cytoskeleton disruption, DNA fragmentation, loss of mitochondrial function, which all lead to the formation of apoptotic bodies which are a small membrane bound particles ready to be cleared by phagocytic cells (Kamps *et al.*, 2006).

I.4. TESTING ANTIVIRAL DRUGS *IN VITRO*

Compounds which have potential antiviral properties usually exert their effect by interfering with viral attachment, inhibiting viral stages of replication, or inhibiting the viral/virion release from host's cells. One of the principles of testing whether compounds have antiviral properties includes the consideration of the type of cell line chosen. The main purpose of testing antiviral compounds *in vitro* is not to simulate human infection but to maximize the opportunity to discover potential lead compounds for their antiviral activities. The infectivity of respiratory viruses can vary on different type of cells. Cells are ideal to be chosen in antiviral assay if they have the ability to support viral entry and produce viable virions or the infectivity of the virus. However, it is not only the type of cells that affect the infectivity of the virus, the procedures performed to infect the cells, such as how long the virus is left in contact with the cells and how many virus should be put on cells (the titer) are also important to be considered. In term of the tested compounds, the success of the antiviral test is also determined by the length of time the drug is allowed to penetrate into cells and reach equilibrium. A positive control known to have antiviral properties should be carefully selected for comparison for antiviral activity (Cann, 1999).

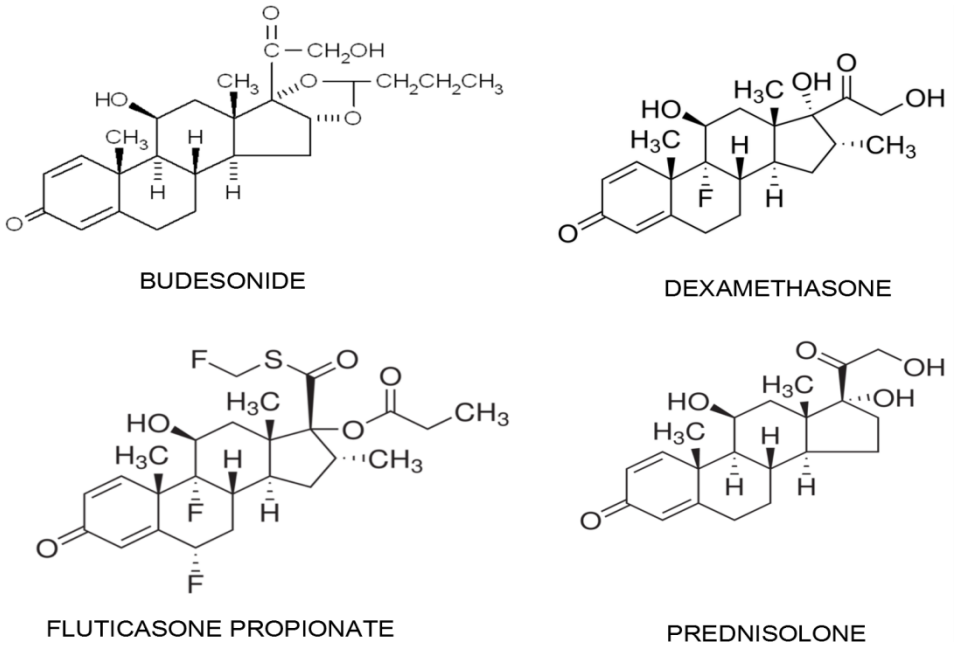


Figure 3.4. Corticosteroids tested in the study

II. AIMS

The aim of this study was to investigate the potential antiviral properties of corticosteroids and compare the effects of corticosteroids on viral replication to the reference antiviral agents effective for the HPIV3 infection (i.e. ribavirin) and influenza A infection (i.e. zanamivir). Corticosteroids: budesonide, dexamethasone, fluticasone propionate, and prednisolone (Fig 3.4) are among the most effective anti-inflammatory agents which are commercially available, clinically important in asthma therapy, and becoming part of treatment in respiratory viral infections**.**

III. METHODS

III.1. ASSAY FOR CELL VIABILITY

III.1.1. MTS ASSAY FOR BSC-1 CELL VIABILITY

Monolayers of BSC-1 cells were grown on 96-well plates with the density of 2.3 x 10^3 cells/well. When cells reach at least 95% confluency, 50 µl of media or 0.5% DMSO in media, or corticosteroids (budesonide, dexamethasone, fluticasone propionate, and prednisolone) in (10^{-8}-10^{-2}mM; 10^{-8}-10^{-1}mM; 10^{-8}-10^{-2}mM; 10^{-8}-10^{-1}mM respectively) were added per well. The cells were then incubated for 24 hours at 37 °C in atmosphere of 5% CO_2. The MTS assay for cell viability on positive control ribavirin was also carried out, 50 µl of ribavirin (1.6x10^{-4}- 3.3x10^{-1}mM) was added on the confluence cells. The cells were incubated with the vehicle or ribavirin for 1 hour at 37 °C. After incubation, 150 µl/well of media was added to each well. All plates were then further incubated at 37 °C in 5% CO_2 for 3 days, after which 40 µl of the tetrazolium-based dye 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt (MTS) was added to each well to determine cell viability and quantify compound toxicity. MTS is metabolized to a soluble formazan product by mitochondrial enzymes of metabolically active cells, thereby allowing the rapid quantitative analysis of cell viability and compound cytotoxicity. The plate was incubated in the dark for 2 hours at 37°C. After incubation, the foil wrapped plate was placed on a plate rocker for 5 minutes to mix the soluble formazan product. The quantity of formazan product was measured at 490 nm in a plate reader and was directly proportional to the number of living cells in culture. The drug concentration was plotted against the optical density of each sample. Corticosteroids and ribavirin MTS assay were carried out in 4 replicates each concentration per plate in 3 different cell passages.

III.1.2. CRYSTAL VIOLET ASSAY FOR MDCK CELL VIABILITY

Crystal violet is a dye that binds electrostatically to nuclear proteins and stains DNA. Lower amounts of crystal violet bound to the cells suggested a decrease in cell number. MDCK cells were plated on 24-well plate at a density of 10 x 10^5/ml (175 µl of cells/well). The corticosteroids (budesonide, dexamethasone, fluticasone propionate, and prednisolone) in (10^{-8}-10^{-1}mM; 10^{-8}-10^{-1}mM; 10^{-8}-10^{-1}mM; 10^{-8}-10^{-1}mM respectively) was then added (175µl/well) to the cells. The plates were incubated for 24 hours at 37°C in 5% CO_2 atmosphere. The next day, the media was replaced by a mixture of fresh media (200 µl /well) and a fresh serial dilution of the corticosteroid (175 µl /well). The crystal violet assay for cell viability on positive control zanamivir (10^{-7}-10^{-1}mM) was also carried out, 175 µl of zanamivir was added to the cells seeded on the previous day. The plates were then incubated at 37°C in 5% CO_2 for 5 hours. After incubation, 300 µl of 1.2% avicel solution (from a mixture of 2.4% avicel stock and MDCK growing media 1:1) was added to the well. The plate was then incubated at 37°C in 5% CO_2 for further 48 hours.

After 48 hours, the media was removed and cells were washed three times with 600 µl of PBS. Cells were then fixed with 400 µl of 4% formaldehyde/well for 45 minutes and washed 3 times with PBS. The cells were then stained with 0.1% of crystal violet in 2% ethanol for 30 minutes. Cells were then rinsed extensively with water until no diluted dye was identified and dried in room temperature overnight. Cell-associated dye was then eluted with 10% acetic acid (1 ml/well) on a rocker for 15 minutes. Extracted dye was then diluted 1:7 with distilled water and transferred into 96-well microtiter plates, and the absorbance was measured at 560 nm using microplate reader.

III.2. PLAQUE REDUCTION ASSAY FOR ANTIVIRAL ACTIVITY

III.2.1. HUMAN PARAINFLUENZA VIRUS TYPE 3 (HPIV3)

The plaque reduction assay was performed according to the standard method described by Cann (Cann, 1999). BSC-1 cells were grown on 96-well plates to reach at least 95% confluency (day 3 after seeding). Fifty microlitres per well of the corticosteroid (budesonide, dexamethasone, fluticasone propionate, and prednisolone) in (10^{-8}-10^{-4}mM; 10^{-8}-10^{-1}mM; 10^{8}-10^{-2}mM; 10^{-8}-10^{-1}mM respectively) was added. The cells were incubated with the vehicle (BSC-1 media for dexamethasone; DMSO for fluticasone propionate, budesonide, and prednisolone) or drug for 24 hours at 37 °C in 5% CO_2. Positive control ribavirin ($1.6x10^{-4}$- $3.3x10^{-1}$mM), was also tested on confluent cells. Fifty microlitres per well of ribavirin was added to each well. The cells were incubated with the vehicle or ribavirin for 1 hour at 37 °C in 5% CO_2. After the incubations, plates were then infected with 50 µl/well of virus in the maintenance medium at 10^5 $TCID_{50}$. The virus was allowed to adsorb for 2 hours at 37 °C in 5% CO_2, after which, a 100 µl/well overlay of 0.6% Avicel RC/CL (FMC BioPolymer) supplemented with maintenance medium was added. After further incubation for 3 days at 37 °C in 5% CO_2, the Avicel overlay was removed by pipetting and cells were fixed and stained with crystal violet.

III.2.2. INFLUENZA A (H1N1/PR8)

MDCK cells were plated on 24-well plates at a density of 10 x 10^5/ml (175 µl /well). The tested corticosteroids: budesonide, dexamethasone, fluticasone propionate, and prednisolone in (10^{-8}-10^{-2}mM; 10^{-8}-10^{-1}mM; 10^{-8}-10^{-1}mM; 10^{-8}-10^{-1}mM respectively) were then added (175 µl /well) to the cells. The plates were incubated for 24 hours at 37°C in 5% CO_2 atmosphere. The next day, the media was replaced by a mixture of fresh media (175 µl /well) and the fresh serial dilution of corticosteroids (175 µl /well). Fifty µl /well influenza A (H1N1/PR8) (1.25 x 10^4 pfu/ml) in the MDCK growing media

was then added to each well. Positive control zanamivir (10^{-8}-10^{-1}mM), was also tested on confluent cells (175 µl /well).The plates were then incubated at 37°C in 5% CO_2 for 5 hours to allow the virus to infect MDCK cells. After incubation, 300 µl of 1.2% avicel solution (from a mixture of 2.4% avicel stock and MDCK growing media 1:1) was added to the well. The plate was then incubated at 37°C in 5% CO_2 for further 48 hours.

After 48 hours, the media was removed and cells were washed three times with 600 µl of PBS. Cells were then fixed with 400 µl of 4% formaldehyde/well for 45 minutes and washed with PBS. To visualise the plaques immunocytochemistry method with aminoethylcarbazole (AEC) was performed. The cells were permeabilised by adding 250µl/well solution containing 0.5% TritonX-100 in PBS for 30 minutes. The residual aldehyde groups were subsequently blocked with 400 µl/well of 10% foetal calf serum (FCS) in PBS for 90 minutes. After being washed with PBS three times, cells were incubated with 200 µl/well of primary antibody (1:500) for 90 minutes, washed with PBS 5 times then followed by 90 minutes incubation with 200 µl/well of antimouse IgG-HRP antibody (1:1000). Finally, a 300 µl/well developing solution of aminoethylcarbazole (AEC) (0.4 mg/ml) in 0.05 M sodium acetate buffer, pH 5.5, containing 0.03% H_2O_2 was added to the well and incubated in the dark for 60 minutes then washed with tap water 3 times. The infected cells will develop pink-reddish colour which can be scanned and analysed.

III.2.3. PLAQUE REDUCTION ASSAY

The concentrations of drug sufficient to reduce plaque number by 50% (IC_{50} values) were calculated by standard methods (Cann, 1999). The percentage of plaque formation was calculated as follows: [(mean number of plaques in test)/(mean number of plaques in control)] × 100

III.3. ICAM-1 ASSAY

BSC-1 cells were seeded onto 24 well plates and then incubated at 37 °C in a humidified atmosphere containing 5% CO_2 until reaching at least 95% confluency. The cell monolayer was then incubated with 294 µl/well of the highest non toxic concentration of corticosteroid or vehicle overnight. The next day 294 µl of HPIV3 at a concentration of 10^5 $TCID_{50}$ units/ml was added to the wells. Avicel overlay was added after 2 hours incubation to let virus infect the cells. After 3 days of incubation, the media was removed and the cells were washed 3 times with PBS before undergo cells lysis.

III.3.1. CELL LYSIS

Infected BSC-1 cells was lysed with cell lysis buffer (20mM Tris-HCl pH 8, 1% Triton X-100, 10% glycerol, 2mM EDTA, 137 mM NaCl) supplemented with 1mM NaVO4, 1mM PMSF, 1µg/ml aprotinin, 10µg/ml leupeptin. Plate on ice was put on rocker for 20 minutes. Cells were then scraped with the tip of a pipette, transferred to eppendorf tubes, and finally centrifuged at 15,000 x g for 5 minutes at 4°C. Supernatant was transferred into an ice-cold 1 ml plastic tube for subsequent protein concentration analysis by BCA assay using bovine serum albumin (BSA) as a standard.

III.3.2. PROTEIN ASSAY

Protein concentration from cell lysate was determined using BCA protein Assay. The BCA protein assay is an assay based on formulation of bicinchonic acid. Serial dilutions of bovine serum albumin (BSA) (2.0, 1.0, 0.5, 0.25, 0.125, 0.0625, 0.01325, 0.0078 mg/ml) were used as standards. Five µl of standard solutions were applied in duplicates to 96-well plates. The BSC-1 cells extracts were added into the 96-well plates. The working solution contained a mixture of 50 parts of BCA reagent A and 1 part of BCA reagent B was made fresh and then added (200 µl) to each well, and subsequently mixed thoroughly on a plate shaker for 30 seconds. The plate then was

incubated for 30 minutes at 37ºC and subsequently left at room temperature for 5 minutes to cool down. The plate was then read at 562 nm wavelength using a plate reader. Using the optical densities of the standards, a graph of optical density against concentration was plotted and extrapolated to calculate the concentrations of the unknown values of the BSC-1 cells extract.

III.3.3. PROTEIN SAMPLES

The remaining protein lysate was then vortexed with one third volume of 3x Laemmli buffer (0.25 M Tris-HCl pH 6.8, 20% (v/v) glycerol, 2% (v/v) SDS, 0.2% (w/v) bromo-phenol-blue, made up to 20 ml with deionised water, plus 4% mercaptoethanol) and frozen at -20°C until assayed.

III.3.4. WESTERN BLOT

Protein samples were electrophoretically size–fractionated using sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). A mixture of 5.27ml H_2O; 3.33ml Acrylamide; 1.25ml Tris.HCl; 100µl SDS; 50 µl APS; and 5 µl TEMED were made and transferred into a cassette (2x3.1ml). Once set, stacking gel (5.77ml H_2O; 1.67 ml Acrylamide ; 2.5ml Tris/SDS; 50 µl APS; 8 µl TEMED) was added with the insertion of a 12 wells comb. Gel in the cassettes then was put into the tank filled with SDS running buffer (Tris-base 30.3 g, glycine 144 g, 20% SDS 50 ml made up to 1 L deionised water). Samples were boiled at 95ºC for 5 min then loaded in each well with volume equals to 10 µg protein. The gel was run at 40-50V until samples have stacked and then run the main gel at 100-130V until the dye front reaches the slot.

III.3.5. PROTEIN TRANSFER

Once the gel has run, the cassette was removed from the tank and opened using a gel knife. The comb like section and bottom of the gel was trimmed off and the gel was placed in transfer buffer (Tris-base 30.3 g, glycine 144 g, up to 1 L deionised water).

The semi dry blotting system was used. The bottom (an anode) of the system was pre-wet before setting up a sandwich consisting of of pre-wet filter paper (20 layers), nitrocellulose membrane, gel, and more filter papers (20 layers). A glass rod was rolled on the sandwich stack before running to remove trapped air bubbles. The blotting system was then run at 39 Amp for a duration of 60 minutes.

III.3.6. ICAM-1 DETECTION

Once transfer was complete, the transferred nitrocellulose membrane was blocked with TBST containing 5% dried skim milk (5% Blotto) for 1 hour under room temperature with shaking. After blocking, ICAM-1 monoclonal antibody was added into the membrane and then incubated overnight at 4°C.

The next day, membrane was briefly rinsed in 1 x TBST, then 2 x 5 minutes, and 1 x 15 minutes. After washing completed, the membrane was then incubated with anti rabbit HRP-conjugated secondary antibody in 5% Blotto on the roller for 60 min at room temperature. In order to remove excess secondary antibody, the membrane was then washed again briefly in 1 x TBST, then 2 x 5 minutes, and 1 x 15 minutes. Then it was developed by ECL development reagent after washing, and exposed to hyperfilm in a darkroom.

After treating with stripping buffer, the membrane was reprobed with GAPDH primary antibody to confirm equal protein loading. Primary antibody stripping was performed by incubating membranes with stripping buffer at room temperature for 15 minutes with shaking. After washing with TBST, 2 x 5 minutes, then 1 x 15 minutes, nitrocellulose membrane was incubated again with blocking solution before new protein detection

The film scanned and then analysed with densitometry using ImageJ software. The area obtained from the blot was then normalised with the corresponding area of GAPDH as a housekeeping protein.

IV. RESULTS

IV.1. CELL VIABILITY ASSAY

The main purpose of assaying for cell viability under treatment with corticosteroids and antiviral agents was to ensure that the compound concentrations would not affect the host cell viability. Only the compound concentration which does not have any significant different viability to untreated cells will be used for subsequent antiviral assays. Cell Viability assay was also carried out to ensure that the antiviral effects observed are not due to the tested compounds' cytotoxicities.

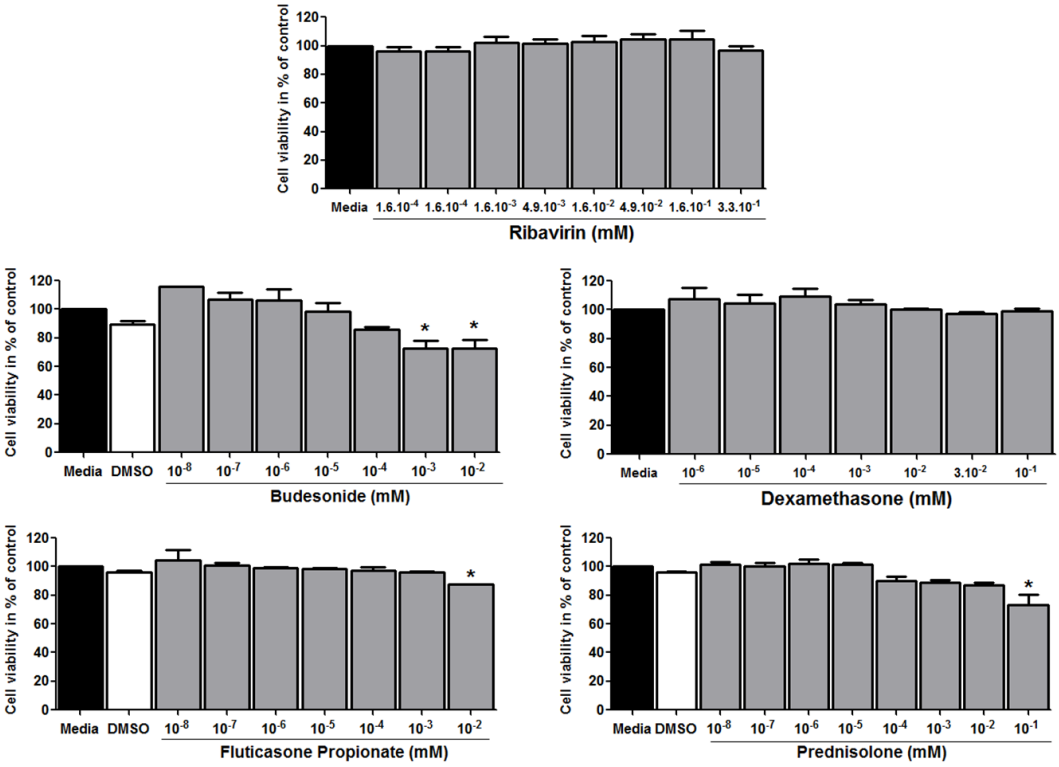


Figure 3.5. *In vitro* cell viability assay of ribavirin and corticosteroids determined by MTS assay. The effect of DMSO 0.5% as budesonide, fluticasone, and prednisolone vehicle on cell viability was also determined, to eliminate the possible cytotoxic effect of the solvent. Each point represents average value ± SEM of 3 independent experiments.

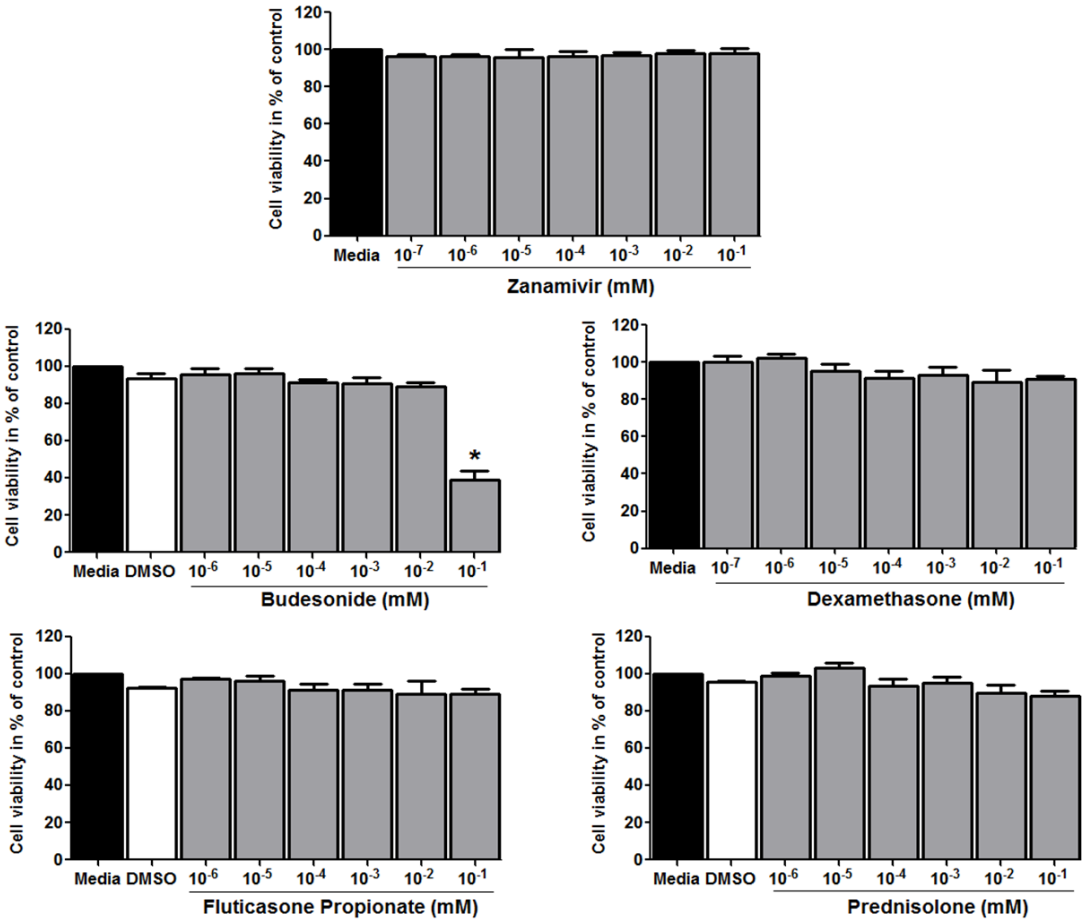
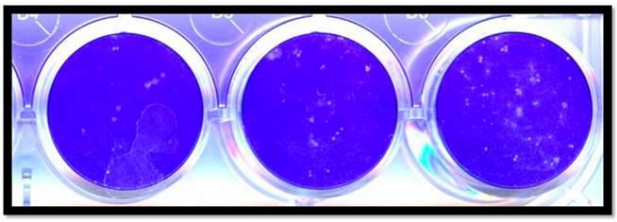


Figure 3.6. *In vitro* cell viability assay of zanamivir and corticosteroids determined by crystal violet assay. The effect of DMSO 0.5% as budesonide, fluticasone, and prednisolone vehicle on cell viability was also determined, to eliminate the possible cytotoxic effect of the solvent. Each point represents average value ± SEM of 3 independent experiments.

IV.2. ANTIVIRAL EFFECT OF CORTICOSTEROIDS

The inhibitory effect of corticosteroids on HPIV3 and influenza A infections were investigated by plaque reduction assays. Dexamethasone significantly decreased HPIV3 and Influenza A (H1N1/PR8) titre compared to other steroids (Fig 3.8 and Fig 3.10).



A B C

Figure 3.7. Example of plaque reduction assay for HPIV3 on BSC-1 cell lines. After 2 hours of virus adsorption, Avicel™ overlay were added. After 3 days cells were fixed with 2% formaldehyde and stained with 0.1% crystal violet solution. A: cells were incubated with 0.1 mM of Dexamethasone B: cells were incubated with 0.01 mM of Dexamethasone C: Control wells where cells were incubated with media.

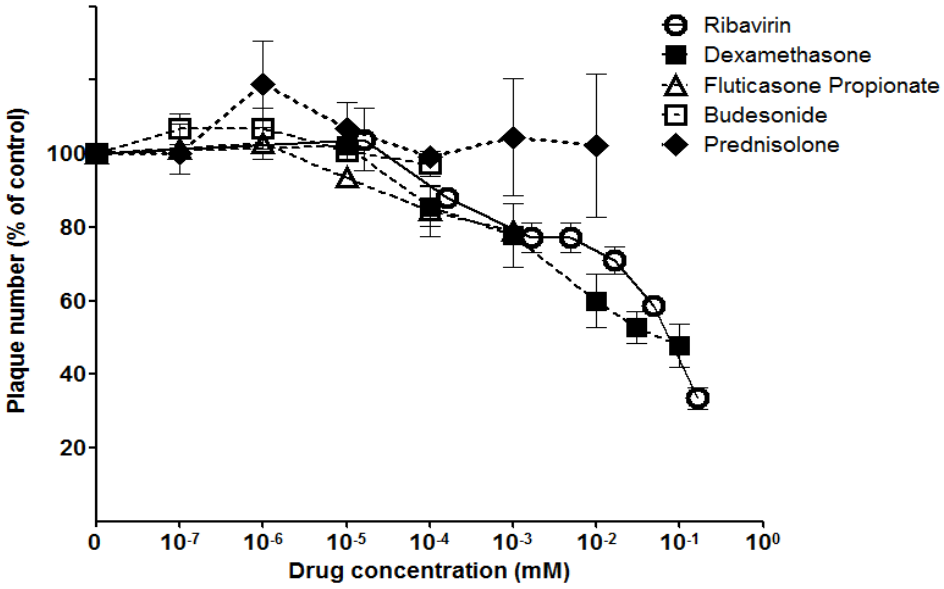
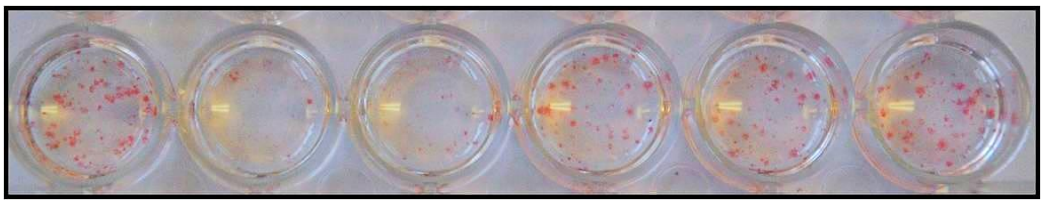


Figure 3.8. Concentration-response curve of corticosteroids and the positive control ribavirin of HPIV3 infection on BSC-1 cells. Each point represents average value ± SEM of 3 independent experiments.

Dexamethasone was the only corticosteroid which reached 50% inhibition of viral replication. The IC_{50} of dexamethasone was $0.5\pm0.3.10^{-1}$mM compared to $0.7\pm0.2.10^{-1}$mM for ribavirin. Budesonide and fluticasone propionate had a negligible antiviral effects but their activity did not reach 50% inhibition in plaque formation over the tested range of concentrations. Prednisolone did not show any inhibition of HPIV3 replication.



A B C D E F

Figure 3.9. Example of plaque reduction assay for influenza A (H1N1/PR8) on MDCK cell lines. After 5 hours of virus adsorption, Avicel™ overlay were added. After 48 hours of further incubation, cells were fixed with 2% formaldehyde and immunostained with aminoethylcarbazole (AEC). A: Control well where cells were incubated with media; B: cells were incubated with 0.1 mM of Dexamethasone; C: cells were incubated with 0.01 mM of Dexamethasone C: cells were incubated with 0.001 mM of Dexamethasone; D: cells were incubated with 0.0001 mM of Dexamethasone; E: cells were incubated with 0.00001 mM of Dexamethasone.

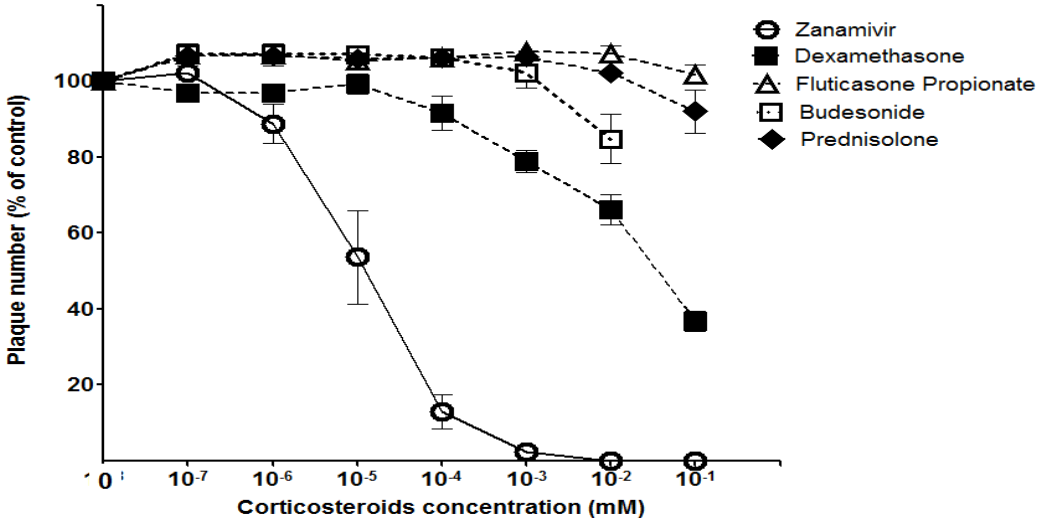


Figure 3.10. Concentration-response curve of corticosteroids and the positive control zanamivir of influenza A (H1N1/PR8) infection on MDCK cells. Each point represents average value ± SEM of 3 independent experiments.

As expected, zanamivir inhibited influenza A replication with an IC_{50} value of $1.5\pm1.2.10^{-5}$mM. Dexamethasone also demonstrated a significant inhibitory effect on influenza A replication with an IC_{50} value of $0.3\pm0.01.10^{-1}$mM. In contrast, budesonide and prednisolone showed negligible antiviral properties against influenza A (H1N1) infection. Fluticasone propionate, budesonide and prednisolone antiviral activities did not reach 50% inhibition over the tested range of concentrations.

IV.3. EFFECT OF CORTICOSTEROIDS ON ICAM-1 EXPRESSION

HPIV3 infection on BSC-1 cells increased the level of ICAM-1 expression although this may not statistically significant. Treatment with dexamethasone, fluticasone propionate or prednisolone reduced the expression of ICAM-1 but again the reduction was not statistically significant (fig 3.11).

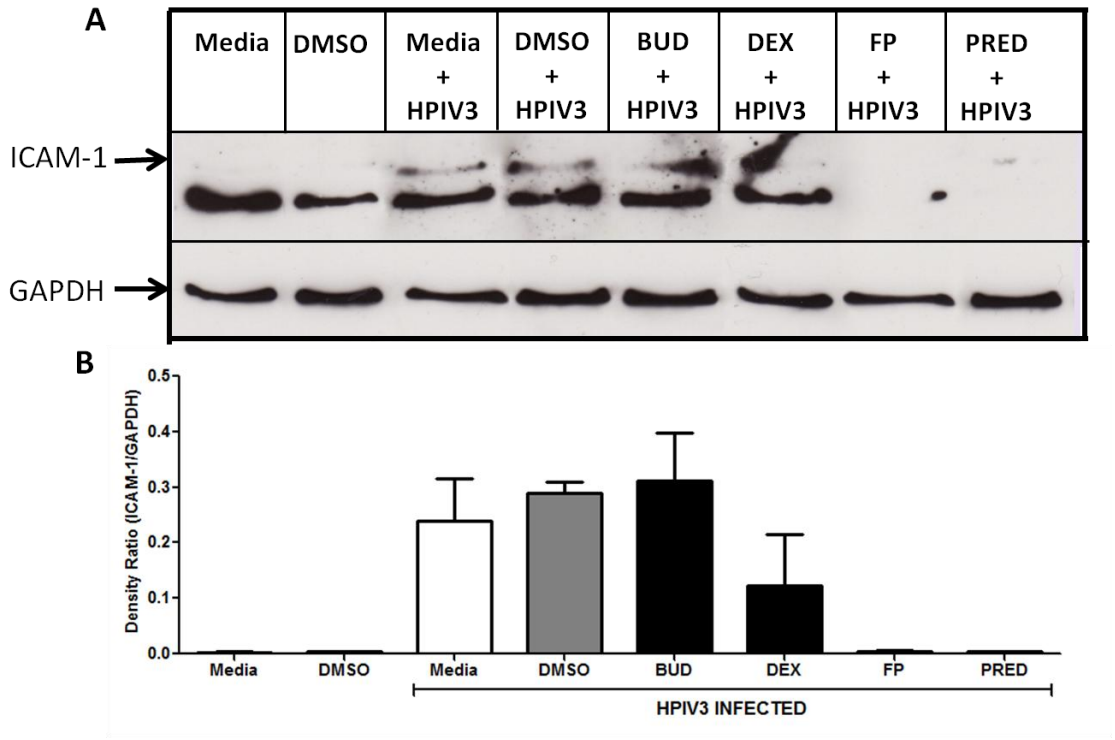


Figure 3.11. A. A western blot analysis for intercellular adhesion molecule (ICAM)-1 level in BSC-1 cells infected with HPIV3 and incubated with corticosteroids (BUD=Budesonide; DEX=Dexamethasone; FP=Fluticasone Propionate; PRED=Prednisolone). GAPDH was used as housekeeping protein. B. The density ratio of ICAM-1 expression normalised to GAPDH. Results are expressed as mean±S.E.M. Data were analysed using one-way ANOVA, followed by post hoc Bonferroni's test ($P < 0.05$; n=3).

V. DISCUSSION

Respiratory viral infection places an exceptional challenge to the host immune system for not only to rapidly eliminate the culprit but also control the excessive inflammation to prevent acute respiratory failure and support tissue repair to restore the normal respiratory function. Corticosteroids are anti-inflammatory agents whose activities are to suppress potentially damaging excessive proinflammatory immune responses, but at the same time might also suppress the necessary immune responses to eliminate the virus (Webster *et al.*, 2002).

Corticosteroids (dexamethasone) are recommended for treatment of croup which is usually due to HPIV3 infection. Corticosteroid treatment in croup patient has shown to reduce relapse and improve symptoms (Bjornson *et al.*, 2004). The role of corticosteroid in influenza infection however is still controversial, although, some guidelines recommend the use of a corticosteroid in the case of influenza infection in patient with asthma (World Health Organisation, 2010).

Currently, little has known about the contribution of corticosteroid treatments to HPIV3 and influenza A replications. Therefore, in order to study the effect of corticosteroids on viral replication and to further evaluate if there is any potential antiviral effect of corticosteroids, viral inhibition assays using plaques reduction assays were carried out in this study.

Epithelial cells may be one of the most important targets for corticosteroid. Some previous studies have been carried out to evaluate the role of corticosteroid in viral infections. There is some evidence which shows the possible antiviral effects of corticosteroids. Dexamethasone and hydrocortisone have shown to inhibit the infection and reduce the viral titers of RV14 (Rhinovirus 14) (Suzuki *et al.*, 2000). Dexamethasone has also been shown to posses antiviral activity by reducing the

release of avian sarcoma virus progeny *in vitro* (Wainberg *et al.*, 1982). However, some studies while showing alteration of proinflammatory cytokines by corticosteroid intervention did not show any reduction in viral release and replication (Noah *et al.*, 1998). In the current studies, dexamethasone was shown to have antiviral properties against HPIV3 infection on BSC-1 cells. Fluticasone propionate and budesonide were also shown to inhibit HPIV3 replication although both drugs failed to reach 50% plaque reduction. There was no inhibition of HPIV3 replication by prednisolone.

Respiratory viruses enter into and replicate within epithelial cells lining the airways. Entry is dependent on interaction of specific virus with specific receptors, for example some group of rhinoviruses exploit intercellular adhesion molecule (ICAM)-1 to aid the virus entrance to the host cell (Hofer *et al.*, 1994). Influenza viruses bind sialic acid residues on the surface of the host cell *via* hemmaglutinin to enter the host cell. Previous study suggested that the mechanism by which dexamethasone inhibits RV14 infection in human tracheal epithelial cells is most likely via reducing the expression of ICAM-1 (Suzuki *et. al*., 2000). This study was supported by the finding which demonstrated that corticosteroids e.g. hydrocortisone, dexamethasone and mometasone furoate also inhibits RV16-induced increases in ICAM-1 surface expression albeit showing no alteration of virus infectivity or replication (Papi *et al.*, 2000).

Gao *et al.* (2000) showed that HPIV3 infection upregulates ICAM-1 expression on airway epithelial cells, an effect that has been thought would facilitate viral cell attachment and entry (Gao *et al.*, 2000). In this present study, while some corticosteroids investigated were shown to reduce ICAM-1 production , the antiviral effect of corticosteroids did not have any correlation with ICAM-1 expression on BSC-1 cells. Therefore, the inhibition of HPIV3 replication by dexamethasone might not only be mediated by inhibition of ICAM-1.

In the current study, dexamethasone significantly reduced the replication of influenza A (H1N1/PR8) on MDCK cells, while other corticosteroids tested i.e. budesonide, fluticasone propionate, and prednisolone, were shown to have negligible antiviral activity against influenza A infection.

One of corticosteroid mechanism of actions is to suppress proinflammatory mechanism, via interaction with transcription factors, including nuclear factor (NF)-*κ*B which is known to regulate the expression of many inflammatory genes and play an important role in the inflammatory processes (Barnes and Adcock, 2003). The NF-κB signaling pathway plays a significant role in generating immune response and mediating inflammation especially in the case of infection (Bonizzi and Karin, 2004). When inactive, NF-kB is complexed with an inhibitory protein subunit (IkBα). NF-kB is activated by signal either originating from the cytoplasm (i.e TNF-α and IL-1β) or nucleus due to DNA damage. The signals trigger the degradation of IkBα and then release the NF-kB to activate transcription factor of responsive genes including those which encode pro-inflammatory cytokines (Adenuga and Rahman, 2007). Many of the anti inflammatory actions of corticosteroids are suggested to be mediated by inhibition of transcription factor NF-kB which leads to transcriptional repression of many pro-inflammatory mediators (Barnes and Adcock, 2003).

Some groups of viruses have the ability to exploit the NF-kB pathway for their own benefits i.e. facilitate replication and escape from immune system surveillance. Dengue and Sindbis virus activate NF-kB to induce apoptosis of host cells which lead to viral spread. NF-kB is also suggested to be involved in retrovirus, herpes simplex virus, adenovirus, and CMV genes expression (Hiscott *et al.*, 2006; Kumar *et al.*, 2008).

Influenza virus life cycle also relies on the NF-kB signalling pathway. Host cells with low NF-kB activity are resistant to influenza infection (Flory *et al.*, 2000). Therefore, activation of NF-kB is a prerequisite for effective influenza infection. The activation of

NF-kB pathway has been suggested to be caused by over expression of influenza viral proteins such as HA, NP and M1 (Flory *et al*., 2000). The NF-kB activation is essential to increase the synthesis of vRNA of influenza virus during replication to enhance virus production. Influenza virus also induces apoptosis of the host cells via NF-kB-dependent induction of pro-apoptotic factors tumor necrosis factor-related apoptosis-inducing ligand to enhance viral spreading and propagation (Wurzer *et al.*, 2004).

The NF-kB signalling pathway activation seems to be a trade off in influenza infection. In one hand, NF-kB activation enhances viral propagation and spreading, but on the other hand, NF-kB activation means activating host immune response against the infection. To counter innate immune response activated by NF-kB during viral infection, influenza virus evolves to encode NS1 protein which is also an antagonist to interferon cellular responses by which the influenza virus is able to counteract the antiviral response from the NF-kB pathway and at the same time exploit the same pathway to enhance viral replication (Garcı *et al.*, 1998). If the antiviral action of dexamethasone is mediated via NF-kB transrepression, in this study dexamethasone seems to have the ability to combat the influenza A (H1N1/PR8) virus counteraction in favour of reducing viral replication.

HPIV1 or Sendai virus and HPIV3 are also known to increase NF-kB activation during the start of infection although little has known as why there is a prominent NF-kB activation (Algarte *et al.*, 1999). It is possible that NF-kB activation could be exploited by the viruses for their own benefits i.e. facilitate replication or it could be an aggressive immune response to eradicate the viruses. So there is an open possibility which requires further study that the inhibition of NF-kB activation by dexamethasone might also be proposed as one of dexamethasone antiviral mechanisms against HPIV3 infection on BSC-1 cells.

The data from the studies comparing the specific agonistic properties of synthetic corticosteroid are still quite elusive to define as they are generated from diverse *in vivo* and *in vitro* studies under various conditions with different parameter measured. In addition, corticosteroids are covering a very large group of compounds. Therefore, the studies on corticosteroids are usually only carried out only on some of the member of the group which are relevant to the study. For that reasons, it is quite a challenge to compare the activity of corticosteroid tested in this study on their transactivation and transrepression activities to further evaluate as why one corticosteroid i.e. dexamethasone has antiviral properties while others (i.e budesonide, fluticasone propionate, and prednisolone) do not.

Hofmann *et al*. (1998) evaluated a series of corticosteroids including dexamethasone and prednisolone. The study revealed that hydrocortisone and predisolone have a lower reduction on NF-kB activation on L929 cells compared to the rest of the corticosteroid group tested including dexamethasone (Hofmann *et al.*, 1998). Albeit carried out in a different cell line which could have cell-specific NF-kB activity. This study therefore underlines the fact that antiviral activity of dexamethasone could be mediated by several mechanisms, not only by reducing ICAM-1 expression but also by NF-kB transrepression.

VI. CONCLUSION

Dexamethasone has antiviral activity against HPIV3 and influenza A (H1N1/PR8). While other corticosteroids tested (i.e. budesonide, fluticasone propionate and prednisolone) do not have or have negligible antiviral activities. The antiviral activity against HPIV3 is not only mediated by inhibition of ICAM-1 expression. Dexamethasone, a potent anti inflammatory drug, reduces the excessive pro inflammatory mediators via transrepression of NF-kB activation. Both Influenza A and HPIV3 has been known to increase NF-kB activity during infection, therefore, suppression of NF-kB activation by dexamethasone could be proposed as one of mechanism by which it reduce viral replication.

CHAPTER IV

IN VIVO SCREENING OF MURINE RESPIRATORY INFECTIVE AGENTS

I. INTRODUCTION

The respiratory tract has a vast surface area with a direct contact with the environment. The lung is always in direct and continuous exposure to air from the surrounding environment. However, most respiratory pathogens are recognized quickly by the immune surveillance which then defends the airway and eradicates the pathogens by combined functions of the innate and adaptive immune responses (Vareille *et al.*, 2011).

Respiratory viruses, such as respiratory syncytial virus (RSV), rhinovirus, influenza virus, parainfluenza virus, and adenovirus cause acute localized respiratory tract infections. During primary respiratory viral infection, both adequate innate and adaptive immunity are needed to eradicate the viruses. The study of these immune responses has demonstrated that the dysfunction of any single immune mechanism results in devastating consequence for the host. During secondary infection, the adaptive immune mechanism developed from the first infective agent encounter, facilitates faster response to eradicate the virus (Vareille *et al.*, 2011).

I.1. VIRUS DETECTION BY THE HOST

The mucosal surface and airway epithelial cells are the main portal for a wide range of respiratory pathogens including respiratory viruses. Epithelial cells, macrophages and dendritic cells carry out a continuous surveillance to detect the pathogen or virus invasion. The early detection of the viruses by the immune system is a critical step in controlling the invasion as well as their replication and initiating the immune response to facilitate their clearance. The host has a set of receptors which recognise the basic viral component initiating the immune response. The basic component of the viruses is defined as PAMPs (pathogen-associated molecular patterns). The PRRs-(pattern-recognition receptors) bearing cell such as epithelial cells, alveolar macrophages and

dendritic cells recognise the PAMPs triggering a cascade of signals to produce cytokines and chemokines, which orchestrate the immune response to establish antiviral activity in the airways. Chemokines attract circulating leukocytes to the site of infection. The cytokines and PRRs also trigger the development of adaptive immune response by initiating the maturation and migration of dendritic cells (Kohlmeier and Woodland, 2009).

Among the most studied PRRs are those of the TLR (Toll-like receptor) family. Currently there are 10 TLRs (TLR1 to TLR10) identified to be expressed in human respiratory tracts. Invading viruses can be detected by TLR either on the cell surface or in cytoplasmic endosome. In term of respiratory viral infections, TLR3, TLR7, TLR8, and TLR9, which found in endosomes recognise viral nucleic acids i.e. dsRNA (double-stranded RNA), ssRNA (single-stranded RNA), and CpG DNA, respectively. TLR4 which is expressed on the surface of cells also has been shown to recognise the RSV's F (Fusion) protein. The interactions of TLRs and PAMP's then induce the expression of type I IFNs (interferons) (Kawai and Akira, 2006) (fig 4.1).

Viral RNA is also detected by several RNA helicases which reside in the cytosol such as retinoic acid-inducible gene I (RIG-I), and melanoma differentiation-associated gene 5 (MDA-5). The viral RNA recognition by RNA helicases also induces transcription factor IRF-3 (Interferon Regulatory Factor -3) which leads to the synthesis of Type I and III interferons. In addition, NOD-like receptors (NLRs) particularly NLRP3 have also been shown to have the ability to recognise and initiate an immune response to respiratory viruses (fig. 4.1) (Tregoning and Schwarze, 2010).

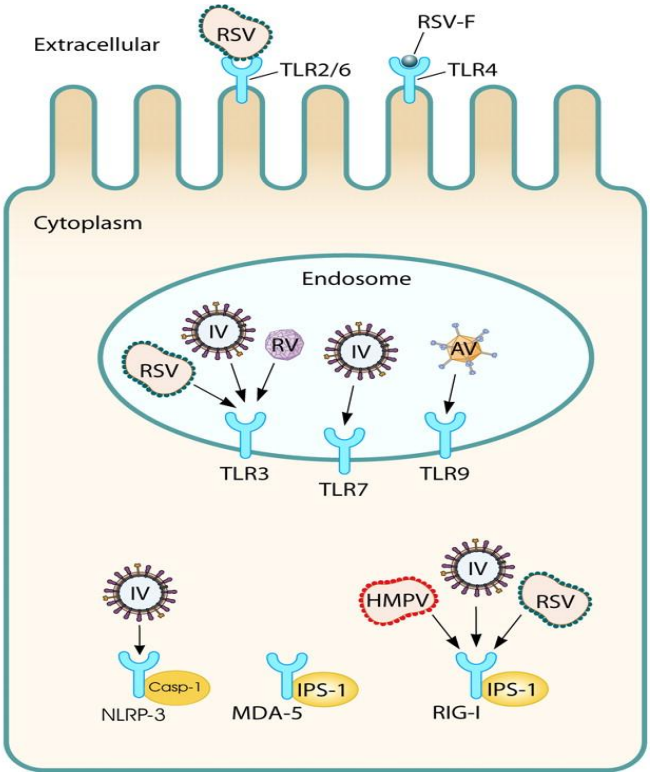


Figure 4.1. Respiratory viruses are detected by host innate immune system, in extracellular compartment by TLR2, TLR4, and TLR6, in endosome by TLR3, TLR7, and TLR 9, and in cytoplasm by RIG-1, MDA-5, and NLRP3. IV: influenza virus, PIV: parainfluenza virus, RSV: respiratory syncytial virus, HMPV: human metapneumovirus, RV:rhinovirus (Tregoning and Schwarze, 2010).

I.2. VIRUS-INDUCED INNATE IMMUNE RESPONSE

The detection and recognition of basic viral components via PRRs initiates a cascade of immune responses which aim to defend against the viral infection by releasing cytokines and chemokines which then recruit inflammatory cell including neutrophils, eosinophils, NK cells, and macrophages to the site of infection (Vareille *et al*., 2011).

Dendritic cells (DC) which circulate in 2 forms i.e. cDCs (conventional DCs) and pDCs (plasmacytoid DCs) have an important role in triggering adaptive immunity as well as producing type I IFNs respectively after their PRRs interact with PAMPs. After antigen internalisation, cDCs undergo maturation and migration to lymph nodes where they mature into antigen presenting cells (APCs) bearing major histocompatibility complex

(MHC) class I and II molecules which then interact and stimulate CD8+ and CD4+ cells respectively (Wu and Dakic, 2004).

Prompted by type I IFNs, DCs, macrophages, and epithelial cells initiate a cascade of chemokines and cytokines storm to recruit inflammatory cells such as neutrophils, NK cells, monocytes and T-cells. Neutrophils are recruited to the site of infection as a response to the production of IL-8, CXCL1 and CXCL5. Neutrophils protect the host from respiratory virus by releasing antiviral proteins i.e. lactoferrin and defensin. There is a thought that neutrophils only play a minor role in viral infection as inhibition of neutrophils has no significant effect on influenza clearance (Vareille *et al*., 2011).

Eosinophils are recruited and activated by GM-CSF (granulocyte-macrophage colony-stimulating factor), eotaxin-1/CCL11, eotaxin-2/CCL24, and RANTES/CCL5. Once activated, eosinophils secrete ECP (eosinophil cationic protein) and eosinophil-derived neurotoxin which have antiviral activities (Gleich, 2000).

NK-cells play a significant role in eradicating invading virus as these cells are the producers of IFN-γ which has strong antiviral properties as well as has essential role in activating antigen presenting cells. NK-cells are recruited and activated by IFN-α, IFN-β, and MIP-1α/CCL3 (Biron *et al.*, 1999).

Macrophages or monocytes release IL-12 and IL-10 which are important in NK cell activation, Th1 responses regulation, and B-cell survival and proliferation and antibody production. Macrophages are recruited and activated by IL-1β, MIP-1α/CCL3, MCP-1 (monocyte chemo attractant protein 1)/CCL2, and TNF-α (tumour necrosis factor alpha). The cells phagocytes, processes, and present viral antigen therefore have a significant role in priming adaptive immunity (Vareille *et al*., 2011).

T-cell and B-cell lymphocytes are the hallmarks of adaptive immune response to virus infection. The Th1-cells are trafficked to the site of infection through the production of

RANTES/CCL5 and IP-10/CXCL10 whereas the Th2 cells via production of IL-1β. The antiviral substances IFN-γ, IL-2, IL-12, and TNF-α are produced by Th1 cells hence Th1 cells play a key role in immune responses against viral infection. Th2 cells to a lesser extent also contribute in the humoral immunity against the invading viruses by the production of IL-4, IL-5, and IL-13 (Ramshaw *et al.*, 1997).

I.3. VIRUS INFECTIONS AND AIRWAY RESPONSIVENESS

Respiratory viral infections increase airway responsiveness even in non asthmatic persons. In the airway, the neural control of the smooth muscle is primarily provided by the vagus nerve. The bronchoconstriction due to parasympathetic nerve activity is initiated by the release of acetylcholine (ACh) from the nerve endings which then binds to and stimulates the M3 subtype excitory muscarinic receptors. This action leads to smooth muscle contraction. ACh also feeds back to M2 subtype muscarinic receptors on the nerve ending to decrease further Ach release (fig 4.2). Therefore the M2 receptors inhibit or limit vagally induced bronchoconstriction (Jacoby and Fryer, 1999).

Viral respiratory infection induces airway hyperresponsiveness in human via the vagus nerve. The dysfunction of the M2 receptor has been thought to mediate the increase of airway responsiveness in viral infection. Guinea pigs infected with HPIV3 which were experiencing airway hyperresponsiveness to inhaled histamine were identified to have M2 dysfunctions (Jacoby and Fryer, 1991). The M2 dysfunction and airway hyperresponsiveness was also observed in virus infected rat (Sorkness *et al.*, 1994). Loss of negative feedback leads to increased parasympathetic activity.

The M2 receptor is highly glycocylated. The M2 glycocylation particularly sialation plays an important role in the interaction of the receptor with acetylcholine. Influenza and parainfluenza virus exert neuraminidase enzyme which interact with sialic acid residue from glycol protein on the cell membrane to facilitate entrance of the virus into targeted cells. The viral neuraminidase is thought to play a role in causing an M2 receptor

dysfunction by cleaving the sialic acid residues on the receptor (Gies and Landry, 1988; Fryer *et al.*, 1990).

Viral infection and exposure of IFNγ to the parasymphatetic neurons in culture have also been shown to cause the M2 receptors dysfunction as well as causing 10 folds reduction in the M2 receptor gene expression. Therefore, the virus itself and the inflammatory response to the virus contribute to the lost of functions and gene expression which lead to potentiate the airway responsiveness (Jacoby and Nadel, 1989).

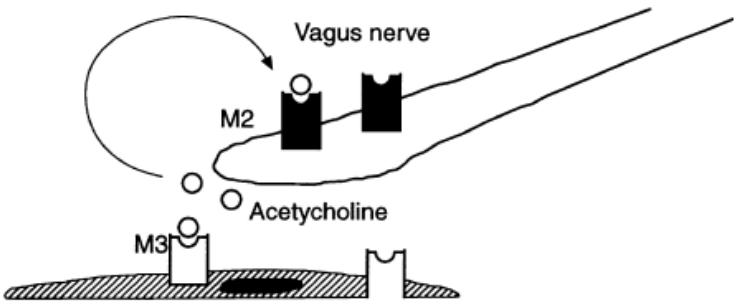


Figure 4.2. Airway smooth muscle contraction and bronchoconstriction as a ersult of acetylcholine binding to M3 receptors. Acetylcholine also binds to M2 receptor on the nerve ending to give negative feedback to reduce the further release of acetylcholine (Jacoby and Fryer, 1999).

I.4. SMALL ANIMAL MODEL TO STUDY RESPIRATORY VIRUSES

The type of respiratory viruses which infect humans is extensive. In infancy and children parainfluenza virus type 3 (HPIV3) is thought to be an important cause of bronchiolitis and croup which are seen in endemic and epidemic patterns. The influenza viruses have a greater role in older children and adults (Clyde, 1980).

Animal models of viral respiratory infection have given better understanding of the pathophysiology of respiratory viral infection as well as the mechanism of virus-host interaction. *In vivo* models also give a better simulation on how viral respiratory infection involves in and affects other respiratory diseases such as asthma, COPD, and cystic fibrosis. The use of small animal models in respiratory viral infection can also provided an insight into the dynamic of host immune response, which has central role for the successful resolution of infection. Rodents, especially mice, rats, and guinea pigs, are the most commonly used as an animal model for respiratory infection because they usually have lower purchase costs, wide availability, and have a relative extensive availability of reagents and antibodies to study immunological pathways essential for the infection (Mohtasham *et al.*, 2004).

Murine models of respiratory infection have been developed for many viruses. The model has provided an important contribution to the understanding of viral life cycle in the airway as well as the role of inflammatory cells and cytokines/chemokines. The mouse models of influenza and parainfluenza are probably the best characterised small animal model at the moment. Those models usually have robust inflammatory responses particularly T-cells and B-cells response similar to human infection. Although findings from such studies cannot be extrapolated directly to the human situation, they can provide indicators to whether the model employed is pointing in the right direction in the simulation of disease states or health outcomes sought (Kohlmeier and Woodland, 2009).

Some careful considerations have to be taken into account before developing animal model of respiratory viral infection as there is significant variation on how different species responds to the given infecting virus. A wide variety of host defence mechanisms toward pathogens have been identified so the most appropriate study parameters should be chosen with the consideration for both the virus and the host. Some of the factors that have to be concerned including the typical host immune response, the amount of virus infected, the pattern of viral replication, and the time course of infection. The animal models have also represented human infection in the similarity of clinical symptoms, histopathological changes, viral replication and also viral transmission (Mohtasham *et al.*, 2004).

I.4.1. PARAINFLUENZA TYPE 3 VIRUS INFECTIONS MODELS

HIPV3 is a major cause of lower respiratory syndrome in young children, infant, elderly, and immunocompromised patients. The clinical syndromes of HPIV3 infection in humans include acute laryngotracheobronchitis (croup) with symptoms such as fever, cough, and laryngeal obstruction; bronchiolitis, tracheal bronchitis, and pneumonia with symptoms such as fever, wheezing, and rales; and to a lesser account neurologic disease such as multiple sclerosis, Guillain-Barré syndrome, and adults with demyelinization syndromes (Henrickson, 2003).

HPIV3 infect the host (human and animals) both naturally and under experimental conditions. The natural host of this virus includes man, guinea pigs and monkeys (Henrickson, 2003). Guinea pig is a natural host of HPIV3. In our laboratory, HPIV3 infection of guinea pig has proven to be successful. The infected animal showed increase in airway inflammation, airway oedema, and airway hyperresponsiveness to histamine provocation (Toward *et al.*, 2005; Ford *et al.*, 2013) . The HPIV3 model developed in our laboratory could be a good basis to elucidate the mechanism of viral

induced asthma exacerbation in mice and their response to asthma treatment i.e. corticosteroids. It will initially be determined if HPIV3 will infect mice.

I.4.2. INFLUENZA INFECTION MODELS

In humans, influenza virus primarily infects the respiratory tract causing fever, chills, headache, myalgia, lethargy and anorexia. Respiratory symptoms include dry cough, pharyngeal pain and nasal congestion. Acute onset of symptoms occurs within one or 2 days after infection (Cate, 1987).

Several animal species have been exploited as a host in influenza studies, each with their own merits. Ferret, guinea pigs, hamster, cotton rats, and non human primates are natural host of influenza viruses without the need of viral adaptation with human influenza strain. However, mice are apparently resistant to human influenza infection therefore the virus would need prior adaptation in order to make the murine model to be more useful. Virus adaptation to the aimed host is usually done by passaging the virus in the lung of the aimed species until it shows expected viral growth kinetics and signs of disease. Some animals like ferrets and macaques have shown symptoms of influenza infection resembling those of humans such as fever, nasal discharge, lethargy, myalgia, anorexia, and sneezing. The infection of influenza in mice and cotton rats although less symptomatic than the other mentioned models, still show hypothermia and weight loss (Bouvier and Lowen, 2010).

I.4.3. POLY (I:C) MODELS

TLR3 activation by the virus is thought to be an important step in inducing a cascade of immune responses by releasing of cytokines and chemokines. DsRNA is the ligand for TLR3. There are 8 families of dsRNA viruses identified including rotaviruses. Poly (I:C) [Polyinosinic:polycytidylic acid] is a a synthetic double-stranded RNA (dsRNA) analogue. As a dsRNA analog of dsRNA virus, poly (I:C) is able to interact with toll-like receptor 3 (TLR3) in the membrane of dendritic cells, macrophages, and B-cells

mimicking certain effects which occur in the context of dsRNA virus infections (Alexopoulou *et al.*, 2001).

Poly (I:C) is an immunostimulant which induces the production of interferon (IFN) . *In vivo*, Intraperitoneal injection of Poly (I:C) in mice caused IFN-α/β production and NK cells activation (Djeu et al., 1979). *In vitro*, in lung epithelial cells, stimulation with poly (I:C) was able to induce the expression of C-jun-amino-terminal kinase (JNK) and nuclear factor kappa B (NF-kB) signaling pathways which are important in the inflammatory response. Therefore understanding the effect of TLR3 activation by poly (I:C) might give a better insight to the mechanism underlying a virus infection (Takeda and Akira, 2004). Poly (I:C) could also be a useful substitute for viral infection in animals model as it avoids the risk of infecting the experimenter.

II. AIMS

The aim of this study was to develop an animal model of viral infection in the airways of mice which expresses moderate inflammatory features of viral infection such as influx of inflammatory cells into the airways and airway hyperresponsiveness towards methacholine provocation. The model has to be able to provide a good basis for further investigation of roles of the respiratory virus in asthma exacerbation. Three approaches will be used: HPIV3, Poly (I:C), and influenza A (H1N1/PR8).

The objective of this study was to determine a dose of the infective agents which produced a significant increase in airway cellular inflammation and airway hyperresponsiveness but which did not affect survival of the animals.

III. METHODS

III.1. DEVELOPMENT OF MICE MODEL OF HPIV3 INFECTION

III.1.1. INFECTION OF MICE

Male BALB/c mice 20-25 grams (n=6) were infected on day 1 and day 2. On the day of infection, a vial of HPIV3 virus was thawed and retained on ice. Mice were intranasally infected with 50 µL of virus stock ($2.12x10^9$ HPIV3 particles per ml). Each mouse was inoculated with 12.5 µL of virus stock in one nostril and then 12.5 µL was given to another nostril twice to give 50 µL of virus stock in total. After nasal inoculation, the mice were held in upright position for 2 minutes to facilitate the delivery of the virus to the airways. The procedure was repeated twice giving the animal 50 µL of virus stock in total. The second viral inoculation was also carried out in similar procedures as the first inoculation. The 50 µL volume was decided to be given for intranasal applications in mice based on a previous study which suggested that giving 50 µL was able to achieve relative distribution of more than 50% instilled substance in the lungs compared to 5 µL which seems to be undistributed. The same study also revealed that instillation of substance in anaesthetised mice result in 6 folds more distribution in the airways compared to intranasal application in conscious mice with the same volume (Southam *et al.*, 2002).

Therefore, as the intranasal infection of HPIV3 in conscious mice did not result in the expected response, and in order to assess the effect of anaesthesia on the HPIV3 infection, an intranasal inoculation of HPIV3 in anaesthetised mice (n=6) was also carried out. Mice were lightly anesthetized by isoflurane (Oxygen 40%: isoflurane 60%). While anesthetized, the mice were intranasally infected with 50 µL of virus stock. The second viral inoculation was carried out in conscious unanaesthetised animals. Control mice (n=6) were also anesthetized and subjected to intranasal administration of

conditioned media (sham). Inoculation of HPIV3 and sham were performed in a class II safety cabinet

Following infection, HPIV3 infected mice were returned to their cages, and then housed in a mice isolator for a further 4 days. On 4 days post second infection, mice were sacrificed. The time of sacrificing was selected based on the HPIV3 infection in the guinea pigs in our laboratory (Toward *et al.*, 2005; Ford *et al.*, 2013).

III.1.2. AIRWAY RESPONSIVENESS AND LAVAGE

Airway hyperresponsiveness provoked by increasing dose of methacholine was measured on day 0 and day 6 using unrestrained whole body plethysmography (Buxco) as described in CHAPTER 2. Baseline airway functions and body weight were also monitored every day until the procedures were completed.

Mice were euthanized by intraperitoneal injection with pentobarbitone sodium overdose on day 6. The tracheas were exposed surgically, tracheal cannula was inserted, and the lung was lavaged *in situ* with 1 ml of sterile PBS as described in CHAPTER 2. The left upper lobe of the lung tissues from each mouse was bisected after being lavaged and then the lung quickly frozen at −80°C until assayed. Frozen lung was used for detection of virus by reverse -transcription polymerase chain reaction (RT-qPCR and plaque assay. HPIV3 infection protocol is shown in figure 4.3.

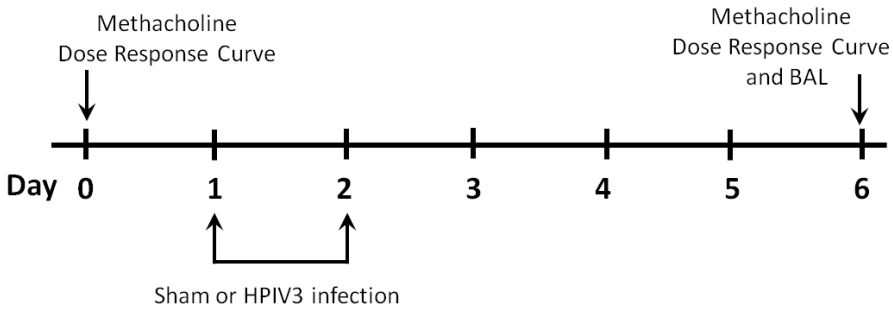


Figure 4.3. Experimental protocol of HPIV3 infection in BALB/c mice

III.1.3. DETERMINATION OF VIRAL TITRES

III.1.3.1. QUANTITATIVE DETECTION OF HPIV3 RNA BY LIGHT CYCLER RT-QPCR

Unless stated otherwise, the detailed procedure is detailed in CHAPTER 2

III.1.3.1.1. Nucleic Acid Extraction

The 100 mg thawed lung was transferred into a homogenization tube containing 1 ml sterile phosphate buffered saline. The lungs were homogenized two times at 224 G for twenty seconds with 5 seconds pause between the intervals with a tissue homogenizer. The homogenate was then centrifuged at 170 G for 6 minutes. Lung supernatant was then aliquoted and frozen at −80°C until assayed. On the day of assay, RNA was extracted from 200 µL lung supernatant and the subjected to RT-qPCR with lightcycler as described in CHAPTER 2.

III.1.3.1.2. Determination of HPIV3 titre on by Plaque Assay

The BSC-1 media in this experiment was supplemented with 1% penicillin streptomycin, to prevent any contamination from BALF. BSC-1 cells were seeded onto 96 well plates and then incubated at 37 °C in a humidified atmosphere containing 5% CO_2 until reaching at least 95% confluency. The cell monolayer was then infected with 50 µl/well of fresh BALF of each animal, and then 100 µl/well of Avicel monolayer was added. HPIV3 virus stock was used as control. After 3 days of incubation, the cell monolayer was fixed with formaldehyde and stained with crystal violet as detailed in CHAPTER 2.

III.2. DEVELOPMENT OF MICE MODEL OF POLY (I:C) INOCULATION

III.2.1. INSTILLATION OF MICE

Mice were lightly anesthetized as previously described with isoflurane. Under anaesthesia, mice were administered 30 µg poly (I:C) or 100 µg poly (I:C) in 50 µL PBS, via the nares. Each mouse was given 12.5 µL of poly (I:C) stock in one nostril and then 12. 5 µL was given to the other nostril twice to give 50 µL poly (I:C) stock in total. After intranasal administration, the mice were held in upright position for 2 minutes to facilitate the delivery of the poly (I:C) to the airways. The procedure was repeated. Mice received three administrations of poly (I:C) with a 24 hour rest period between each administration (day 1; day 2; and day 3). In the control group, mice were instilled with PBS. After administration, mice were allowed to recover and returned to their cage. Baseline respiratory function reading was taken everyday. Poly (I:C) administration protocol is shown on the following diagram (fig. 4.4).

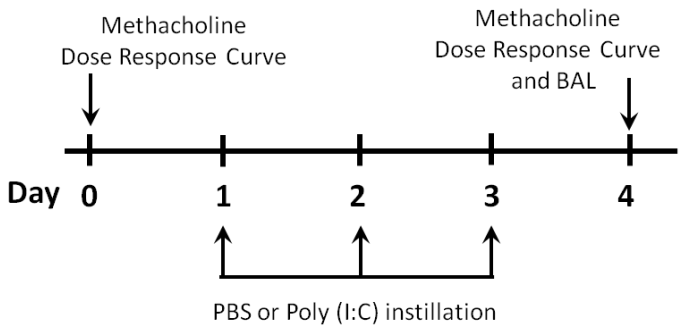


Figure 4.4. Experimental protocol of poly (I:C) instillation in BALB/c mice

III.3. AIRWAY RESPONSIVENESS AND LAVAGE

Airway hyperresponsiveness to methacholine was measured on day 0 and day 4 using unrestrained whole body plethysmograph obtained from Buxco as described in CHAPTER 2.

III.4. DEVELOPMENT OF MICE MODEL OF INFLUENZA A (H1N1/PR8) INFECTION

Mouse-adapted influenza A H1N1/PR8 stock was grown in embryonated egg and kept at −80°C as described in CHAPTER 2.

III.4.1. INFECTION OF MICE

The animals were anaesthetized lightly with isoflurane. Influenza A (H1N1/PR8) 10 or 25 pfu in 50 µl PBS per mouse was inoculated intranasally. Each mouse was given 12.5 µL of virus stock in one nostril and then 12.5 µL was given to the other nostril twice to give 50 µL virus stock in total. After nasal inoculation, mice were held in upright position for 2 minutes then were put back in the transfer cage in prone position to facilitate the virus to spread evenly to the right and the left lungs. Control mice were treated the same and subjected to intranasal administration of PBS. All procedures were conducted in class II biosafety hood/cabinet. Mice were then returned to the mouse isolator once the infection finished.

III.4.2. AIRWAY RESPONSIVENESS AND LAVAGE

Airway responsiveness on methacholine provocation was measured on day 0 and on the specified sacrificed day using unrestrained whole body plethysmography described in CHAPTER 2. On days 4, 7, and 10 after infection, 8 mice (4 mice infected with 10 pfu and 4 mice infected with 25 pfu of virus) were sacrificed and bronchoalveolar lavage was performed. Control mice were sacrificed on day 10 after PBS instillation. Baseline respiratory functions and body weight were also monitored every day until the procedures was complete. Left lobe of the lung tissues from each mouse was used for histologic analysis and immersed in phosphate buffered formaldehyde solution. The remaining lungs were quickly frozen at −80°C for later detection of virus plaque forming assay. Influenza A infection protocol is shown in figure 4.5.

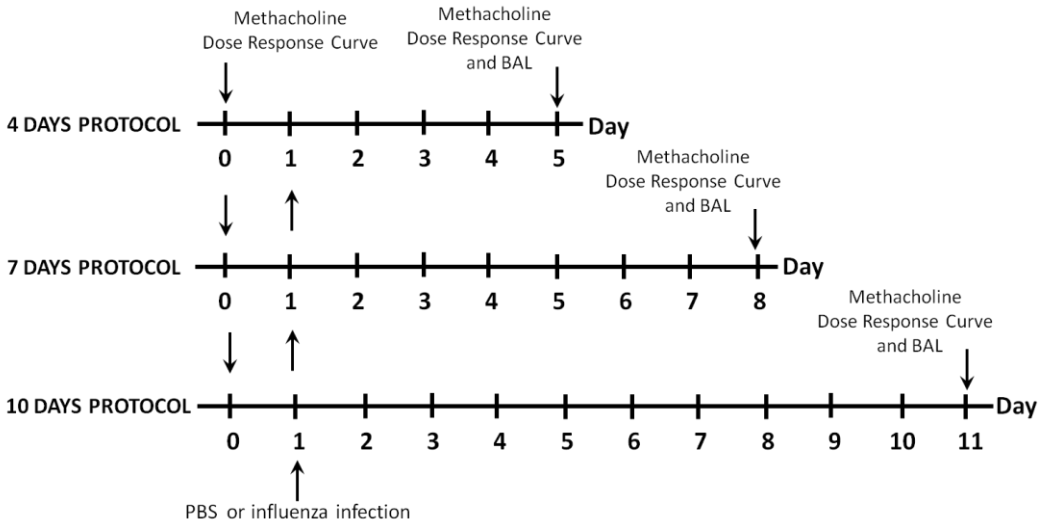


Figure 4.5. Experimental protocol of influenza virus infection in BALB/c mice

III.4.3. INFLUENZA VIRUS TITRES

Plaque forming assays were performed to measure virus loads in the lung of infected mice. The frozen lungs were thawed and, transferred into homogenisation tube containing 1 ml of MDCK medium without foetal calf serum. Using a Precellys beaded tube, the lungs were homogenised in a tissue homogeniser 2 times at 224 G for 20 seconds with 5 seconds pause between the spin and then clarified by centrifugation at 224 G for 10 minutes at 4°C. The clear supernatants containing virus were diluted serially using MDCK media, 1:2000 then 1:3 for 6 times. The diluted viruses were infected to a confluent MDCK monolayer, incubated for 5 hour at 37°C to facilitate infection, then were overlaid with 1.2% Avicel in MDCK media and incubated at 37°C for a further 48 hours. The monolayer cells were then immunostained with AEC as described in CHAPTER 2.

III.4.4. LUNG HISTOLOGY

Lung was stained with H and E and was analysed with histopathology scoring as described in CHAPTER 2.

IV. RESULTS

IV.1. HPIV3 INFECTION IN MICE

IV.1.1. BASELINE BODY WEIGHT AND RESPIRATORY FUNCTIONS

In order to study HPIV3 respiratory infection, 6 BALB/C mice were intranasally inoculated with $2.12x10^9$ HPIV3 particles per ml of HPIV3. The baseline respiratory functions and body weight were monitored until the day they were sacrificed (day 4 post second infections). Significant change of body weight was absent in all HPIV3 or sham infected mice (fig. 4.6). At no time point did mice infected with HIPV3 or sham (media) exhibit clinical signs suggesting respiratory distress (fig. 4.7).

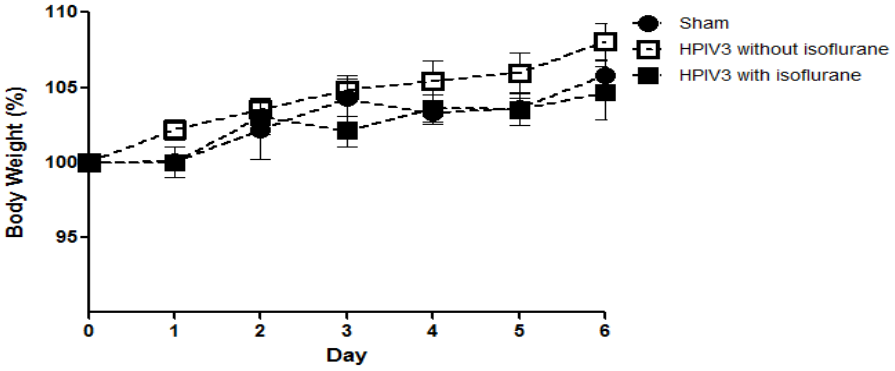


Figure 4.6. Body weight changes of mice infected with HPIV3 or instilled with sham. Results are expressed as means ± SE of a percentage of the baseline values.

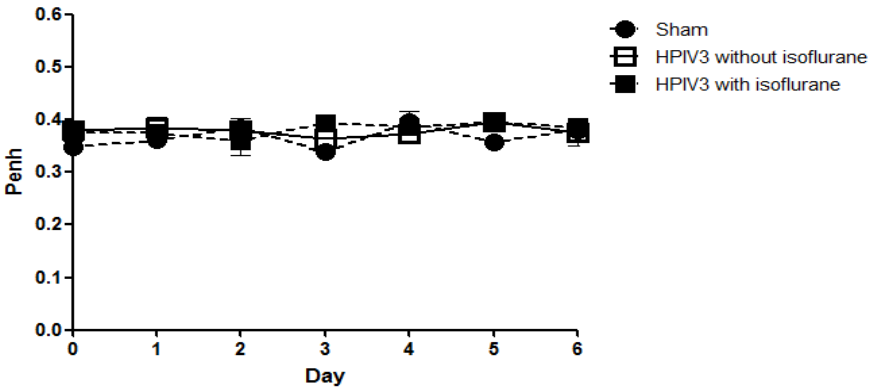


Figure 4.7. Changes in the baseline airway function expressed as means ± SE of absolute Penh in mice infected with HPIV3 or instilled with sham.

IV.1.2. AIRWAY RESPONSIVENESS

HPIV3 infection in either conscious or anaesthetised mice did not result in significant increase of airway responsiveness as shown in figure 4.8.

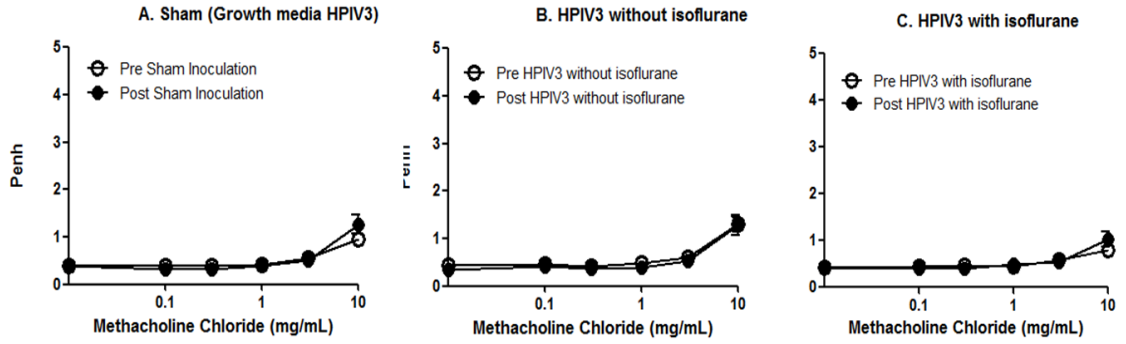


Figure 4.8. Dose response curve of HPIV3 infection on conscious (B) and isoflurane anaethetised (C) mice. Mice were instilled intranasally with HPIV3 on day 1 and day 2 while they were anaesthetised or conscious. Sham instilled anaesthetised mice were used as control (A). Mice were challenged with increasing concentration of methacholine chloride (0.1 mg/ml; 0.3 mg/ml; 1 mg/ml; 3 mg/ml; and 10 mg/ml). Baseline lung function and responses to increasing doses of methacholine was measured by whole body plethysmography**.** Results are expressed as mean ± SEM of absolute Penh. Data were analysed with unpaired two tailed t-test (* P< 0.05; n=6).

IV.1.3. CELLULAR INFLAMMATORY RESPONSES TO HPIV3 INFECTION

Inflammatory cells recruitment in the airway and cell recovery in bronchoalveolar lavage (BAL) was used to characterize the airway inflammatory response to HPIV3. Compared to sham instilled animals, there was no significant increase in either total cells influx or in individual inflammatory cells as shown by figure 4.9.

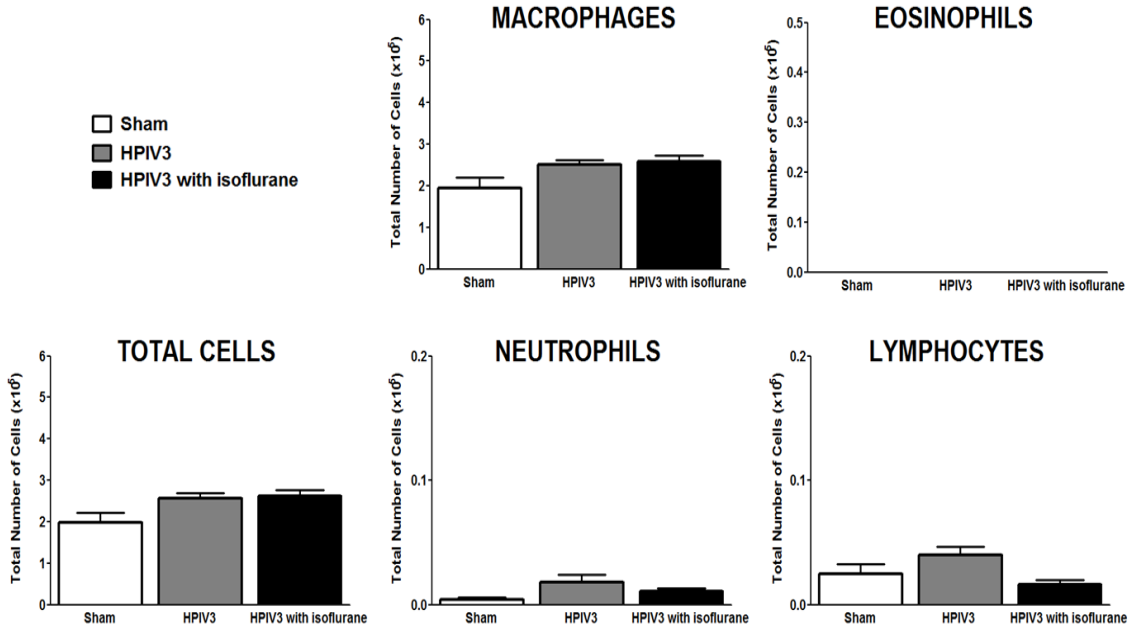


Figure 4.9. The number of total cells, macrophages, eosinophils, lymphocytes and neutrophils found in the bronchoalveolar lavage fluid of sham or HPIV3 infected mice. Results are expressed as mean ± SEM. Data analysed for each differential cell type and total count using one-way ANOVA, followed by post hoc Bonferroni test to determine the difference in cell counts (*P < 0.05, n=6).

IV.1.4. HPIV3 TITRES IN THE BALF AND LUNGS

The animal was infected with 50 µl of $2.12x10^9$ HPIV3 particles per ml. The titre of the virus in anaesthetised animals was determined by qRT-PCR compared to those of sham treated animals. However, there was no difference in the count of RNA particle in HPIV3 infected animal compared to those instilled with sham. The results are presented in table 4.1.

In order to make sure that there is no live infectious virus in the BALF, the fresh BALF from HPIV3 infected animals were also infected on HPIV3 sensitive cells (BSC-1 cells). After 3 days incubation, there is no cytopathic effect (CPE) observed which shows no sign of infection in the cells.

Table 4.1. RNA was extracted from fresh BALF and lung from HPIV3 or sham infected animals. Virus titres were determined using qRT-PCR and are expressed as particles per ml, β-actin was used as a housekeeping gene. Statistical analysis (unpaired t test, two tailed) shows no significant difference in the titre of sham and HPIV3 infected animals.

Group	Virus titre in particles mL^{-1}	β-actin concentration mL^{-1}
HPIV3 infected BALF	29.4±6.3	26.6±12.0
HPIV3 infected lungs	27.9±8.4	157.7±35.9
Sham infected BALF	42.0±10.8	18.2±8.4
Sham infected lungs	30.4±5.4	117.0±23.0

IV.2. POLY (I:C) INSTILLATION

IV.2.1. BASELINE BODY WEIGHT AND RESPIRATORY FUNCTIONS

Both concentrations of poly (I:C) (100µg/50µl and 30µg/50µl) on mice had no effects on either baseline airways function or body weight. Mice's body weight and baseline respiratory functions which were monitored throughout the study did not show significant change (fig. 4.10 and fig 4.11 respectively).

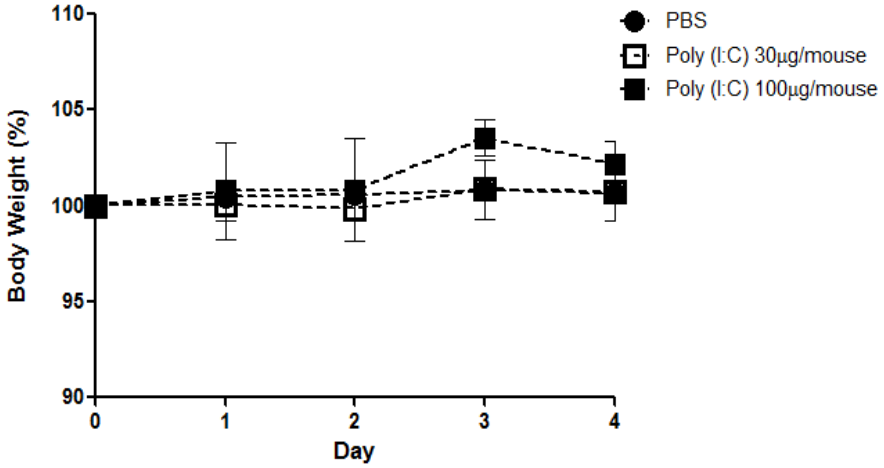


Figure 4.10. Body weight changes of mice instilled with poly (I:C) or PBS. Results are expressed as means ± SE of a percentage of the baseline values.

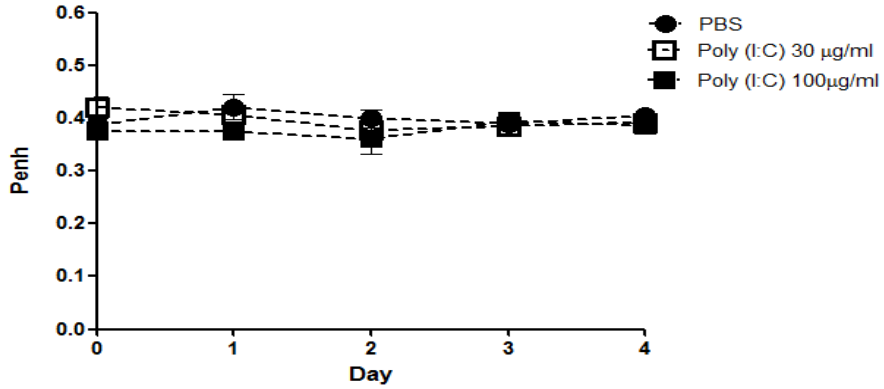


Figure 4.11. Changes in the baseline airway function expressed as means ± SE of absolute Penh in mice instilled with poly (I:C) or PBS.

IV.2.2. AIRWAY RESPONSIVENESS

Low concentration of poly (I:C) (30µg/50µl) did not result in significant increase of airway responsiveness. So, a higher dose (100µg/50µl) of poly (I:C) was delivered which revealed airway hyperresponsiveness provoked by the highest dose of methacholine chloride (Fig 4.12).

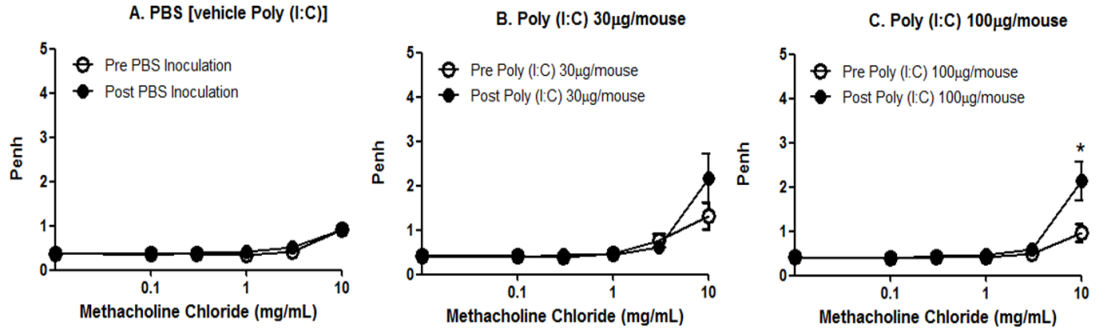


Figure 4.12. - Mice were instilled with PBS (A), 30µg/50µl (B), or 100µg/50µl (C) of poly (I:C) intranasally every 24 h for three days (day 1-3). On day 0 and 24 h after the last poly(I:C) administration, mice were challenged with increasing concentration of methacholine chloride (0.1 mg/ml; 0.3 mg/ml; 1 mg/ml; 3 mg/ml; and 10 mg/ml). Baseline lung function and responses to increasing doses of methacholine was measured by whole body plethysmography**.** Results are expressed as mean ± SEM of absolute Penh. Data were analysed with unpaired two tailed t-test (* P< 0.05; n=6).

IV.2.3. CELLULAR INFLAMMATORY RESPONSES TO POLY (I:C) INSTILLATION

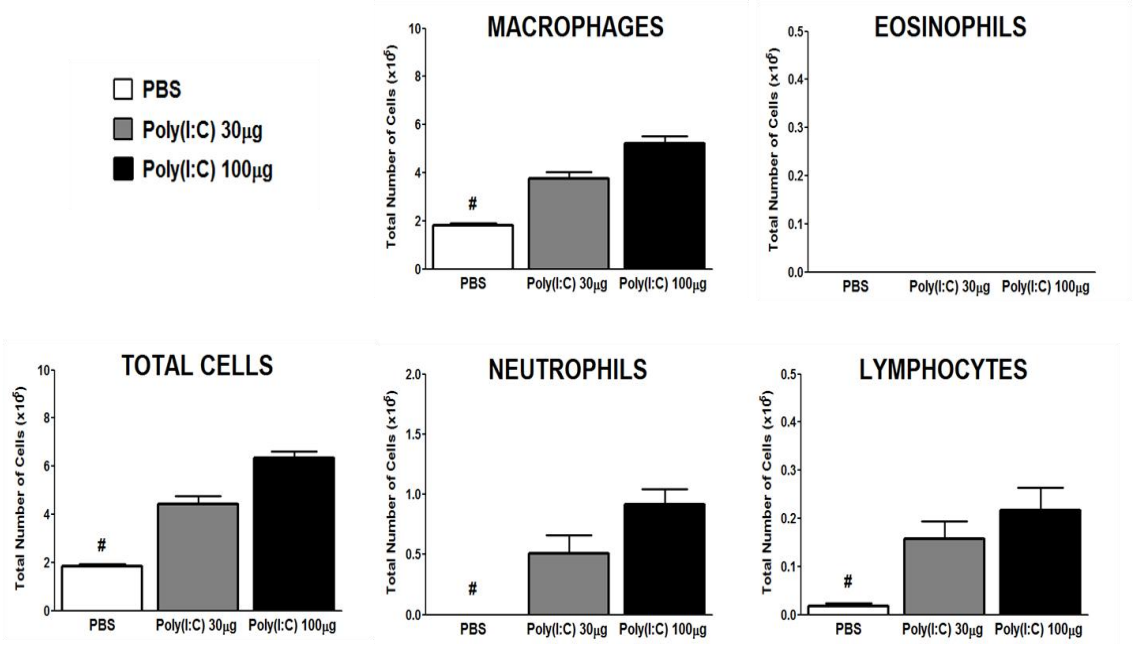


Figure 4.13. The number of total cells, macrophages, eosinophils, lymphocytes and neutrophils found in the bronchoalveolar lavage fluid of PBS or poly (I:C) instilled mice. Results are expressed as mean ± SEM. Data were analysed for each differential cell type and total count using one-way ANOVA, followed by post hoc Bonferroni test to determine the difference in cell counts. $^{\#}$significantly different from all other groups (P < 0.05; n=6).

Intranasal administration of three once-daily doses of poly (I:C) resulted in inflammatory cell influx into the lung. There was a significant increase in total cells in the BALF samples at 100µg/50µl and also with 30µg/50µl of poly (I:C) compared to PBS instilled mice (Figure 4.13). There were significant increase in the numbers of macrophages, neutrophils, and lymphocytes; eosinophils were not detected in either PBS or poly (I:C) treated mice.

IV.3. INFLUENZA A (H1N1/PR8) INFECTION

The PR8 (H1N1) influenza A virus was initially isolated from human and subsequently adapted to mice.

IV.3.1. BASELINE BODY WEIGHT AND RESPIRATORY FUNCTIONS

BALB/c mice were infected with 10 pfu/50µl and 25 pfu/50µl of H1N1/PR8 virus via the intranasal route. Baseline lung functions and weight loss was recorded for the following 10 days after infection. The weight loss kinetics was not significantly different between mice infected with 10 or 25 pfu of virus. There was a trend of weight loss starting from day 6 after infection which slowly recovered on day 10 after infection in mice infected with influenza A virus. Control mice instilled with PBS did not experience any weight lost on those time points (fig. 4.14). However, due to the small number of animal used in the experiment, the difference of the mice weight lost was not significantly different between H1N1/PR8 infected mice and PBS instilled mice at any time point.

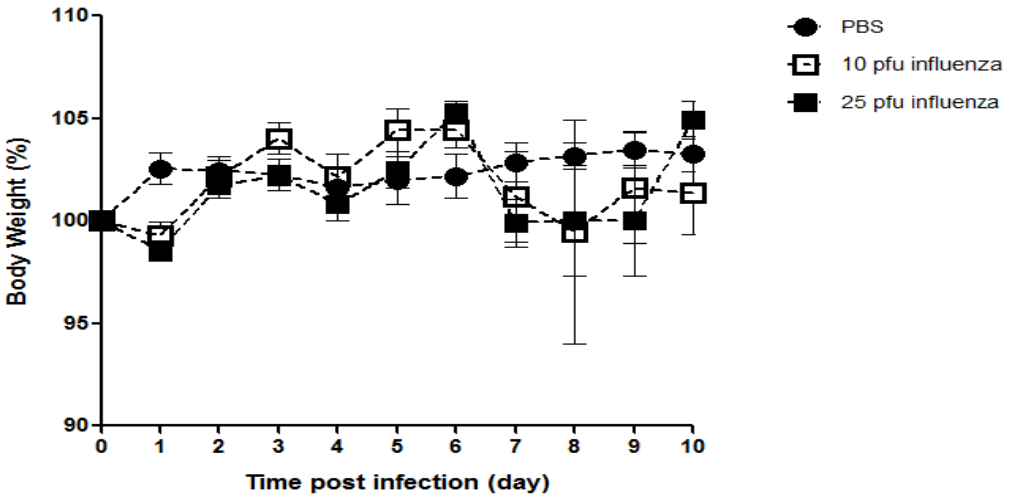


Figure. 4.14. Body weight changes of mice infected with influenza A (H1N1/PR8). Results are expressed as mean ± SEM of a percentage of the baseline values.

Baseline lung functions of infected mice, expressed in Penh, had similar kinetics to those of body weight. The baselines Penh were starting to escalate on day 6 after infection and then persistently increased and peaked on day 8 for mice infected with 10 pfu of virus, while baselines Penh of the group of mice infected with H1N1/PR8 25 pfu peaked on day 7. Penh subsequently decreased and recovered on day 10. The peaked baselines Penh on virus infected group were significantly different compared to mock (PBS) instilled group of mice as depicted in figure 4.15.

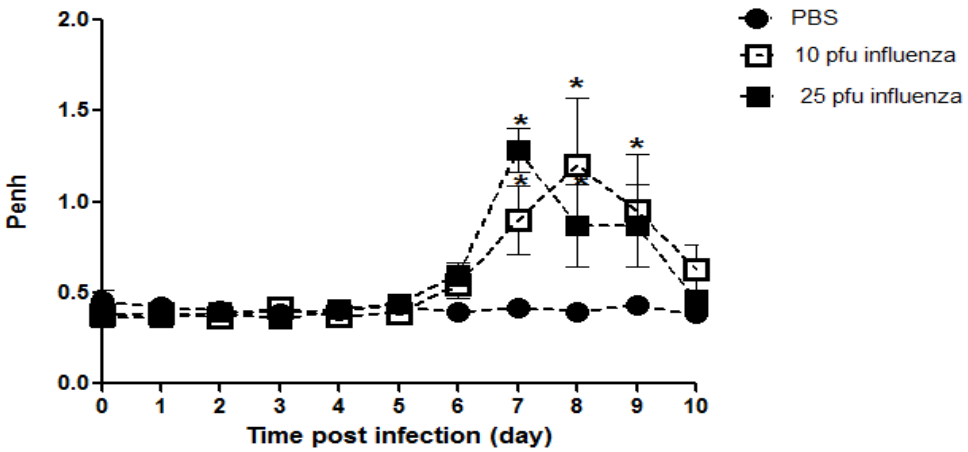


Figure 4.15. Changes in the baseline airway function expressed as means ± SE of absolute Penh in mice infected with influenza virus. Data were analysed with unpaired two tailed t-test (*P< 0.05).

IV.3.2. AIRWAY RESPONSIVENESS

Airway responsiveness to inhaled methacholine increased following H1N1/PR8 infection with 10 and 25 pfu in mice starting at day 4 after infection with an increase in the maximal response at a methacholine concentration of 10 mg/ml. A significant increase in baseline lung functions (Penh) was detected on day 7 in the group of mice infected with 25 pfu of virus. The airway responsiveness was returning to control levels by day 10. Infected mice were more sensitive to methacholine compared to PBS instilled mice, requiring lower concentrations to elicit the same Penh (fig 4.16).

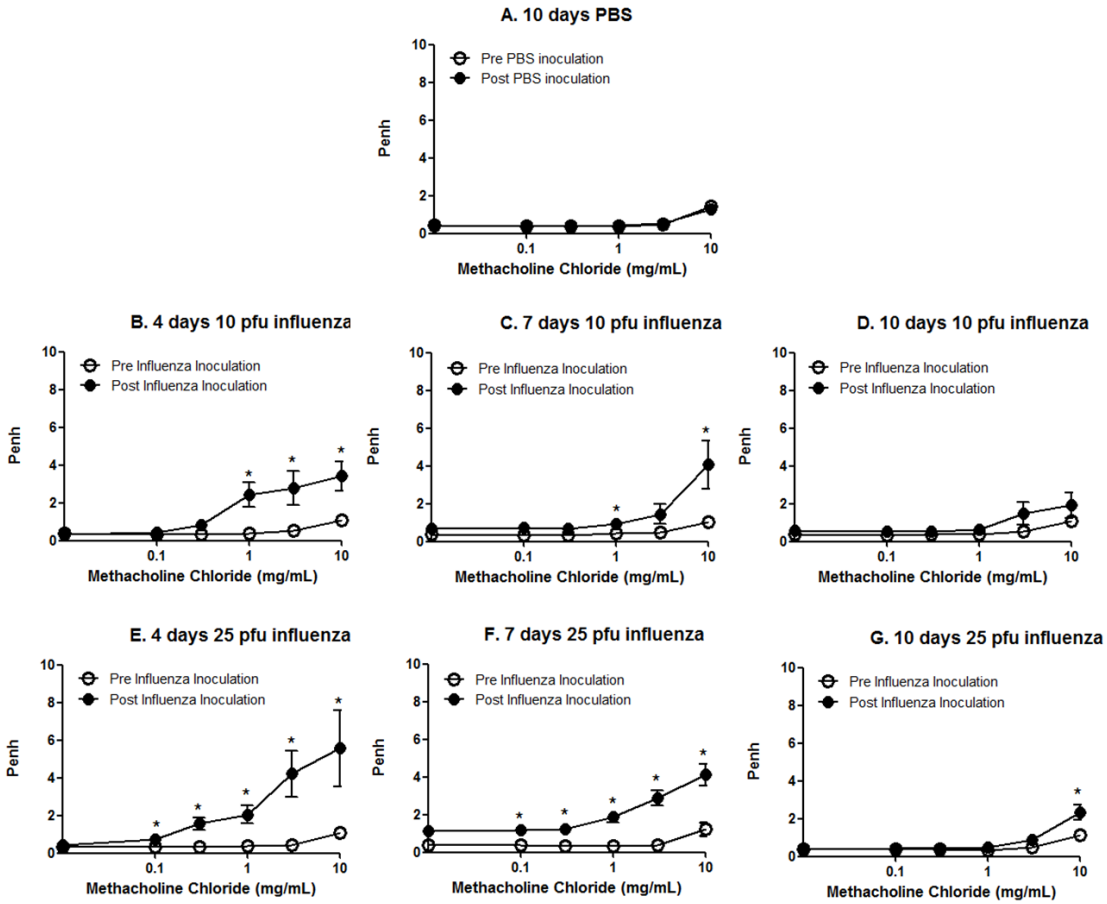


Figure 4.16. - Mice were infected with 10 pfu (B, C, and D) or 25 pfu (E, F, and G) of influenza A virus (H1N1/PR8) intranasally. On day 4 (B and E), 7 (C and F), and 10 (D and G) days after infection, mice were challenged with increasing concentration of methacholine chloride (0.1 mg/ml; 0.3 mg/ml; 1 mg/ml; 3 mg/ml; and 10 mg/ml). Baseline lung function and responses to increasing doses of methacholine was measured by whole body plethysmography**.** Results are expressed as mean ± SEM of absolute Penh. Data were analysed with unpaired two tailed t-test (* P< 0.05; n=4 except for group of mice infected with 25 pfu of H1N1 for 7 days (F), n=3).

IV.3.3. CELLULAR INFLAMMATORY RESPONSES TO INFLUENZA A (H1N1/PR8) INFECTION

The recruitment of inflammatory cells in the airways recovered in BAL fluid was also monitored on day 4, 7 and 10 after infection to characterise the inflammatory response. Inflammation was markedly increased. On day 4 after infection with influenza A virus, there was an increasing trend of total cell count compared to controls even though not statistically significant. The cell count was not significantly different from PBS instilled animals probably because the small number of animals used. On day 7 and 10 after infection of influenza A virus, the number of cells significantly increased compared to PBS instilled animals (fig 4.17 and 4.18)

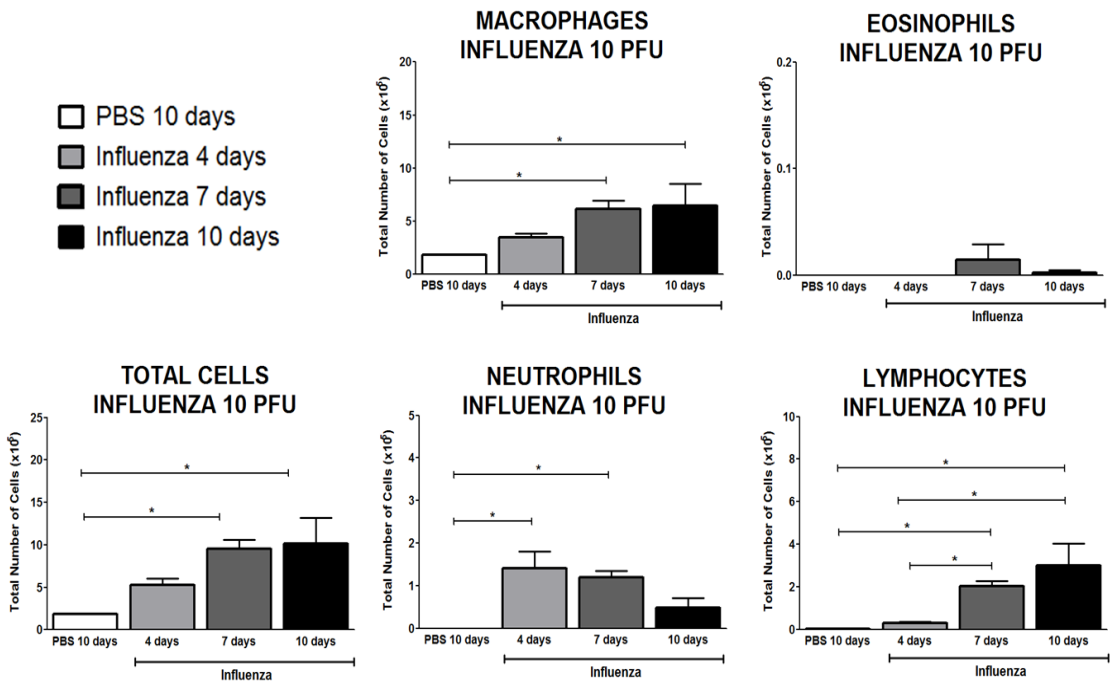


Figure 4.17. The number of total cells, macrophages, eosinophils, lymphocytes and neutrophils found in the bronchoalveolar lavage fluid of of mice infected with 10 pfu of influenza A virus. Results are expressed as mean ± SEM. Data were analysed for each differential cell type and total count using one-way ANOVA, followed by post hoc Bonferroni test to determine the difference in cell counts. *connected with corresponding bar is significantly different from each other ($P<0.05$; n=4).

Differential cell counts show a considerable influx of both macrophages and neutrophils on day 7 after virus infection. Neutrophil counts decreased gradually whereas lymphocyte counts progressively increased from day 4 until day 10 post infections. Eosinophils were not identified or were in very low counts.

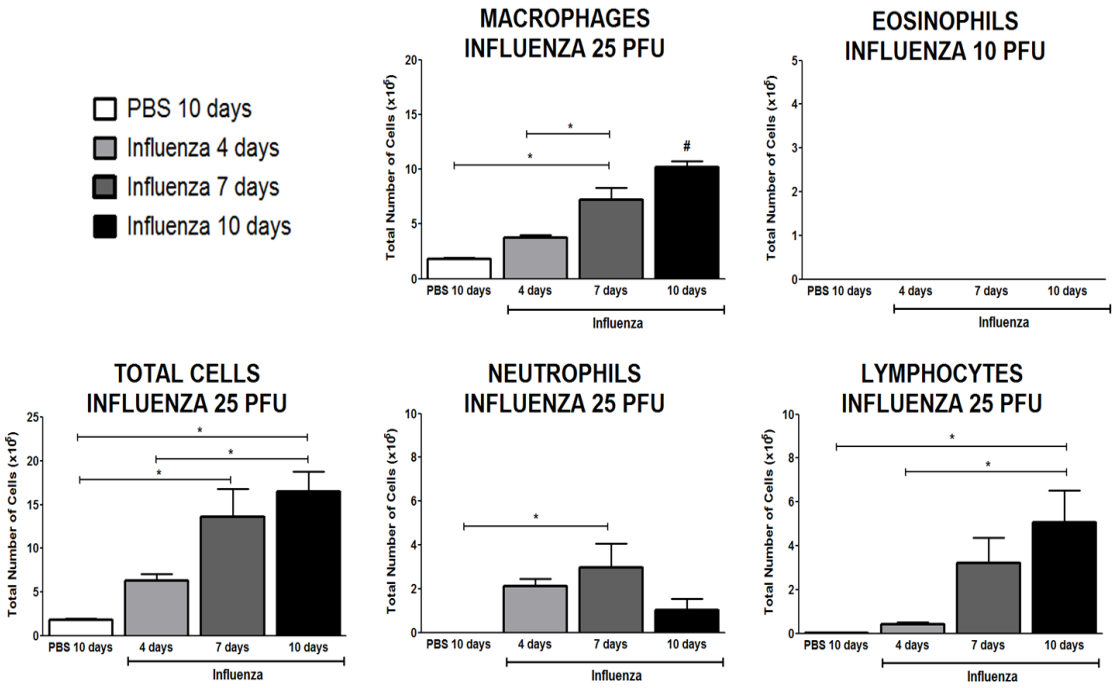


Figure 4.18. The number of total cells, macrophages, eosinophils, lymphocytes and neutrophils found in the bronchoalveolar lavage fluid of of mice infected with 25 pfu of influenza A virus. Results are expressed as mean ± SEM. Data were analysed for each differential cell type and total count using one-way ANOVA, followed by post hoc Bonferroni test to determine the difference in cell counts. #significantly different from all other groups; *connected with corresponding bar is significantly different from each other (P<0.05; n=4 except for group of mice infected with 25 pfu of H1N1 for 7 days, n=3).

IV.3.4. INFLUENZA VIRUS TITRES IN THE LUNGS

Influenza infection in the lungs was confirmed with plaque assay. Influenza virus was recovered and peaked on day 4 after infection but was below the level of detection on day 10 after infection. Even though there was a trend of decreased viral titres after day 4 post infection, the viral titres on different time points were not significantly different (Fig 4.19).

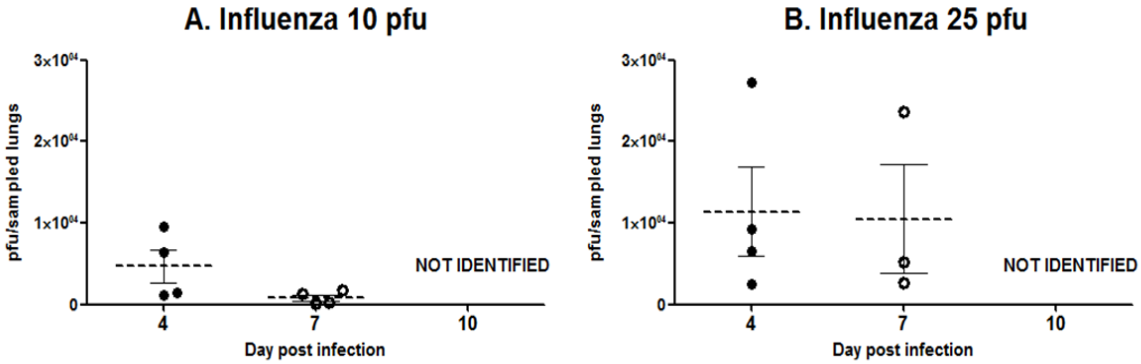


Figure 4.19. Viral titres of lungs of mice infected with influenza A 10 pfu and 25 pfu. The lungs were harvested on day 4, 7, and 10 after infection. Virus titres were determined by plaque assay on MDCK cells. Virus titres are expressed as mean ± SEM of pfu per sampled lungs. Data were analysed using one-way ANOVA, followed by post hoc Bonferroni's test to determine the difference in viral titres (*P < 0.05, n=4 except for group of mice infected with 25 pfu of H1N1 for 7 days, n=3).

IV.3.5. LUNG HISTOLOGY

Histological analysis of influenza infected lungs showed evidence of bronchiolitis and perivascularitis (Fig 4.20). By day 7 post infection, infected lungs showed a severe cellular inflammation which characterised by a massive amount of cellular infiltrates in peribronchial and perivascular area. Epithelial cell shedding, alveolar collapse, and pulmonary haemorrhage were also observed on day 7 after influenza virus infection (Fig 4.21). There was no significant difference in the mean of pathology score of mice infected with 10 or 25 pfu of influenza A which killed at 4, 7, or 10 days after infection. Only the histopathologycal score from mice infected with 25 pfu of influenza virus (5.5±1.7) was significantly different from un-infected animals (1.2±0.3) (Fig 4.22).

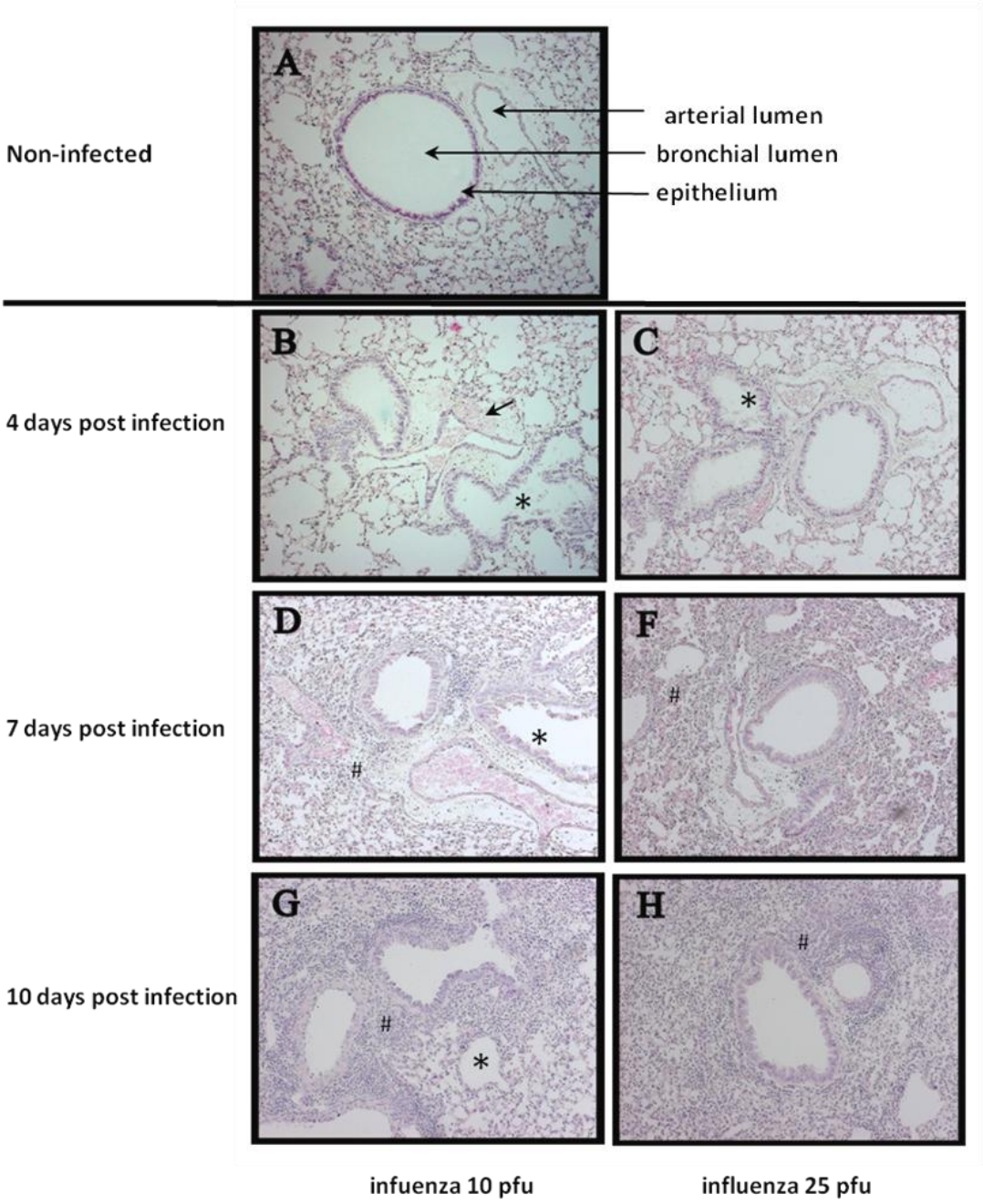


Figure 4.20. Histopathological changes of lungs of un-infected mice (A) or mice infected with 10 pfu (B, D, and G) or 25 pfu (C, F, and H) influenza A virus. The lungs were harvested on day 4 (B and C), 7 (D and F), and 10 (G and H) after infection. The lungs were stained with haematoxylin and eosin to display general morphology (100x magnification). Arrowhead (→) = pulmonary haemorrhage: * = debris of epithelium shedding: # = perivascular and peribronchial cellular inflammation.

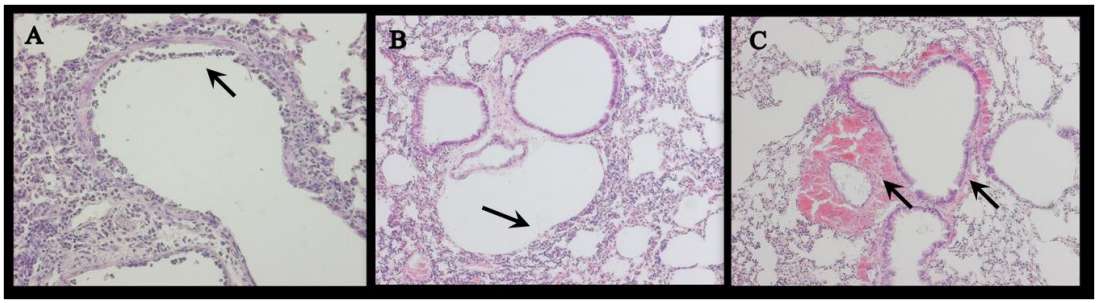


Figure 4.21. Histopathological features of lungs of mice infected with influenza A virus on 7 days after infection. Arrowheads show A. Epithelial cells shedding; B. Alveolar collapse; C. Pulmonary haemorrhage. The lungs were stained with haematoxylin and eosin to display general morphology (100x magnification).

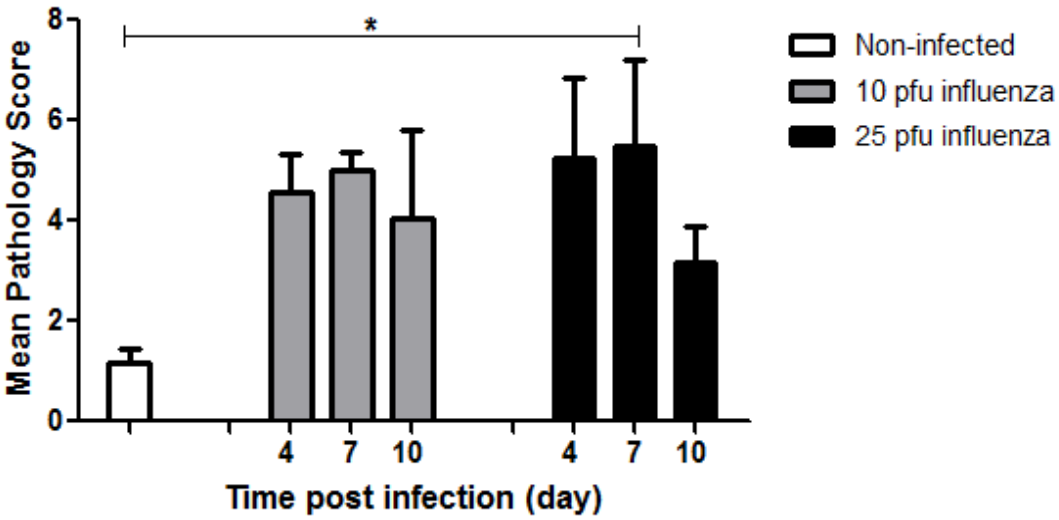


Figure 4.22. The mean lung pathology scores of influenza A infected mice. Results are expressed as mean±S.E.M. Data were analysed using one-way ANOVA, followed by post hoc Bonferroni's test to determine the mean pathology score (*P < 0.05).

IV.4. INFLUENZA A (H1N1/PR8) INFECTION: 5 DAYS 10 PFU

This study was planned to be followed by a series of experiments in which introducing the respiratory infective agent was added to the murine model of ovalbumin-induced allergic airway inflammation in order to observe viral associated exacerbation. For that reason, moderate doses of virus were needed so that when allergen (ovalbumin) challenge was delivered, the animal would survive without severe pneumonia but still showed considerable inflammatory cell recruitment into the airways and airway hyperresponsiveness.

Based on the above results, 4 days infection with 10 pfu of influenza A virus seems to be capable of fulfiling the desired effects on the allergen challenge day. However, the logistic of the experiment required that mice would need to be culled on the day after the allergen challenge day (day 5). An experiment to ensure that on day 5 after infection with 10 pfu of influenza A, mice still have the desired inflammatory state was therefore conducted.

On day 5 after inoculation with 10 pfu of influenza A, there was no increase in respiratory function baseline. There was a significant increase in airway responsiveness and inflammatory cell influx as depicted on figure 4.23 and figure 4.24.

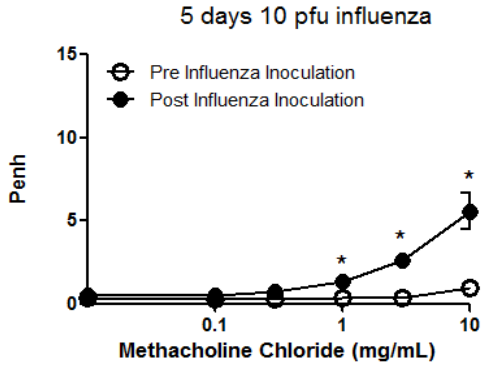


Figure 4.23. Mice were infected with 10 pfu of influenza A virus (H1N1/PR8) intranasally. On day 0 and 5 days after infection, mice were challenged with increasing concentration of methacholine chloride (0.1 mg/ml; 0.3 mg/ml; 1 mg/ml; 3 mg/ml; and 10 mg/ml). Baseline lung function and responses to increasing doses of methacholine was measured by whole body plethysmography**.** Results are expressed as mean ± SEM of absolute Penh. Data were analysed with unpaired two tailed t-test (* P< 0.05; n=6).

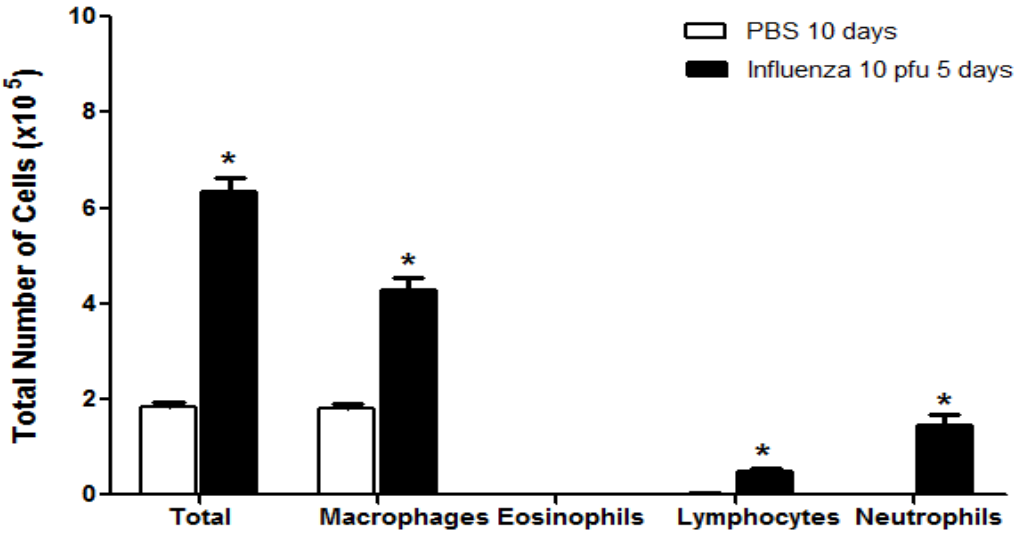


Figure 4.24 The number of total cells, macrophages, eosinophils, lymphocytes and neutrophils found in the bronchoalveolar lavage fluid of mice infected with 10 pfu of influenza A virus for 5 days or PBS for 10 days. Results are expressed as mean ± SEM. Data were analysed for each differential cell type and total count using one-way ANOVA, followed by post hoc Bonferroni test to determine the difference in cell counts (*P < 0.05; n=6).

V. DISCUSSION

This study was conducted to understand better the pathogenesis of respiratory viral infection and associated host responses during acute infection.

V.1. HPIV3 INFECTION IN MICE

The design of the experiment by infecting the animals on day 1 and day 2 and then terminating on day 6 was based on successful *in vivo* experiments in our laboratory carried out by Toward *et al. (*2005) and Ford *et al.* (2013) who infected guinea pigs with the virus. In addition, *in vitro* work carried out previously (CHAPTER 2; Fig. 2.5) also showed that HPIV3 reached the highest titre on day 3-7 post infection.

The present study demonstrates that HPIV3 in the titres previously used for guinea pigs, does not infect mice. Despite two sets of HPIV3 infection experiments performed with and without anaesthesia, there was no reduction of lung functions throughout the study. There was also no increase in airway responsiveness as well as in inflammatory cells influx in the airways of the infected animals compared to sham instilled animals. Viral titres, determined with RT-PCR, from the lungs and BAL fluids of HPIV3 infected animal were not significantly different compared to those of sham instilled mice.

The RT-PCR is a quick and very sensitive method to determine viral titre. However, RT-PCR is not able to differentiate between infectious and non-infectious viral particles. Therefore, a cell culture method was also conducted to test the infectivity of the recovered virus from HPIV3 infected mice. There is no sign of viral replication when the BALF of the infected mice was applied to sensitive cells (BSC-1 cells) whereas cytophathic effects were observed on cells incubated with virus stock.

This model was tried to be developed as it has been successfully conducted in guinea pigs and it also successfully induced exacerbation in OVA induced allergic airway inflammation in guinea pigs. However, the results of the current experiment show that there is no considerable benefit in continuing to develop the HPIV3 in mice model for subsequent studies.

V.2. POLY (I:C) INSTILLATION IN MICE

The failed HPIV3 experiment lead the search for an effective alternative respiratory infective agent and next to be used was poly (I:C), a synthetic dsRNA which also a TLR3 ligand. Activation of TLR3 by dsRNA resulted in impairments in lung function marked by AHR to methacholine and inflammation. The changes described are similar to those of mice infected with Respiratory Syncytial Virus (RSV) (Schwarze and Johnston, 2004; Schwarze and Schauer, 2004).

In the current study, administration of a high dose (100µg/50µl) of poly (I:C), intranasally induced a significant increase in airway responsiveness towards increasing dose of methacholine challenge which was accompanied by an increase in airway inflammation. All leukocyte types were increased except eosinophils. This is not the first study showing that the administration of poly (I:C) in mice caused airway inflammation accompanied by airway hyperresponsiveness. Some studies have reported that acute or chronic poly (I:C) instillation induced airway hyperresponsiveness and cellular inflammation (Stowell *et al.*, 2009; Starkhammar *et al.*, 2012).

The TLR family has been found in the lung especially in smooth muscle cells and primary bronchial epithelial cells (Sha *et al.*, 2004). TLR are also expressed by monocytes and neutrophils (Zarember and Godowski, 2002). The activation of TLR by dsRNA *in vivo* and *in vitro* results in a significant increase in the expression of pro-inflammatory cytokines, chemokines, and growth factors (Ritter *et al.*, 2005). The

increase in expression of the cytokines and chemokines leads to the infiltration of inflammatory cells into the respiratory airways and the lung. Both low dose (100µg/50µl) and high dose (100µg/50µl) of poly (I:C) were able to induce inflammatory cells influx into the airways.

In summary this study has shown that administration of poly (I:C) increases airways responsiveness and inflammatory cells influx similar to viral infection. Therefore, poly (I:C) is worth to be considering as a potential agent to be exploited in subsequent studies as it may trigger an exacerbation if applied to allergic mice. In addition, poly (I:C) as a synthetic agent bears no risk of infection to the investigator.

However, being a synthetic agent, poly (I:C) is not able to replicate as viruses. Poly (I:C) might also only activate TLR 3 while viral infection activates a plethora of PRRs (pattern recognition receptors), therefore it might fail to completely simulate the pathogenesis induced by respiratory viral infection.

V.3. INFLUENZA A (H1N1/PR8) INFECTION

The consideration that poly (I:C) is a synthetic agent which may not represent the complete response expressed by respiratory viral infection led us to search for a respiratory infective agent and we selected influenza A (H1N1/PR/8) virus. Several studies have shown that the mouse is a useful model to study the virulence of influenza virus particularly the H1N1 influenza A virus (Kash *et al.*, 2006; Inn *et al.*, 2013; Shi *et al.*, 2013). The models have been proven to be able to be utilised to study the infectivity, pathogenesis, as well as subsequent application such as in the evaluation of the role of viral infection in asthma.

Infection of influenza H1N1/PR8 virus in mice in the present experiment increased the cellular inflammation. On day 4 after infection the total cell number recovered in BAL fluid was increased in both infective dose 10 and 25 pfu per mouse, even though not statistically significant. On day 7, the airway cellular inflammation was augmented

compared to that of day 4. The airway inflammation was marked with neutrophilia. The recruitment of neutrophils in the early course of influenza infection, has been shown in some animal models of influenza infection, including mouse, ferret, and macaque (Smith and Sweet, 1988; Baskin *et al.*, 2007; Wareing *et al.*, 2007). Related to the neutrophilia, the upregulation of several genes associated with neutrophil recruitment and activation are also observed in macaque model on day 4 after infection (Kobasa *et al.*, 2004).

The number of macrophages was also gradually increased from day 4 to day 7 after the H1N1/PR8 infection. Apart from neutrophils, the recruitment of macrophages is also regarded as an important response to acute virus infection (Papadopoulos *et al.*, 2007). Macrophages have important roles to kill and perform phagocytosis to viruses/virions and virus infected cells (Hashimoto *et al.*, 2007). Neutrophils and macrophages contribute to viral elimination in animal models of influenza infection cells (Hashimoto *et al*., 2007).

Lymphocytes, particularly CD8+ T (cytotoxic T cells = CTL) and CD4+ T (T helper 1) cells are also essential in the process of resolution from influenza infection (Doherty *et al.*, 1997). In the current study, the number of lymphocytes was progressively increased started from day 7. The number of lymphocytes on day 7 was 6 folds higher than the number of lymphocytes on day 4 in mice infected with 10 pfu of virus. The increase was 8 folds in mice infected with 25 pfu of virus. It needs 5-7 days after initial influenza infection for the T-lymphocytes to present in the lungs to eliminate the virus (White *et al.*, 2008). In the current study, the highest level of lymphocytes number occurred on day 10 after infection when viral titre was unidentified in the lung homogenate. This is in agreement with Flynn et al. study which reported that the CTL number peaked at day 9-10 coincided with viral elimination (Flynn *et al.*, 1998).

Infection of mice with 10 or 25 pfu of influenza virus per mouse also increased airway hyperresponsiveness. Some suggestions can be proposed to explain the enhancement of the airway responsiveness. The first suggestion is the defect of M2 receptors due to viral infection as previously discussed in the INTRODUCTION section in this chapter. The second hypothesis involves macrophages and neutrophils. *In vitro*, viral infected macrophages released reactive oxygen species and inflammatory mediators including prostaglandin E2, platelet activating factors, and thromboxane X2 (Folkerts *et al.*, 1998). Neutrophils have been reported to be activated by viral infection which then increases the release of superoxide enzymes (Folkerts *et al*., 1998). Those processes were noted to contribute to the enhancement of airway hyperresponsiveness (Matsumoto *et al.*, 1999). However, in the present study, on day 10 after infection the baselines respiratory function was returned to normal and airway hyperresponsiveness was also resolved even though cell influx in the airway was still markedly high. This suggests that the inflammatory cell influx in the airway in this influenza A infection was not the only key factor in determining the pulmonary function and airway responsiveness. A study of influenza A infection in cotton rat suggested that epithelial cell damage plays a more crucial role in inducing pulmonary function deterioration rather than inflammation as the tachypnea of the cotton rats had resolved even though the evidence of pneumonitis, peribronchiolitis, alveolitis, and perivascularitis still persisted (Trias *et al.*, 2009).

As formerly mentioned, influenza virus is known to target epithelial cells lining the airways. Decreased baseline lung function or increased Penh and increase in airway responsiveness coincided with epithelial damage (Trias *et al*., 2009). Bozanich *et al.* (2008) reported that the increase of airway hyperresponsiveness in influenza A infected mice provoked by methacholine was associated with the increase of protein level in BAL fluid. They then proposed that the alteration of epithelial barrier permeability might

increase the exposure of metacholine to the nervous system which lead to the increase of airway hyperresponsiveness (Bozanich *et al.*, 2008).

Recent study identified IL-33 – IL-13 pathway to contribute to the development of airway hyperresponsiveness in influenza infection (Chang *et al.*, 2011). This pathway is independent from the Th2 pathway which is responsible for the development of airway hyperresponsiveness in asthma. Influenza infection increases the level of IL-33 expression in the lungs of mice (Le Goffic *et al.*, 2011). IL-33 has been thought to play a role as a danger signal and being released when cells undergo apoptosis or necrosis (Zhao and Hu, 2010; Borish and Steinke, 2011). In the acute influenza infection, IL-33 is released from both macrophages (Chang *et al*., 2011) and viral infected epithelial cells (Le Goffic *et al*., 2011). In the milieu of IL-33, natural helper (NH) cells, an innate lymphoid cells which bear ST2, the receptor for IL-33, secrete a high level of IL-13 which contribute to the development of airway hyperresponsiveness (Chang *et al*., 2011)

The data from this current experiment suggests that there was a clear dose-dependent relationship of the dose of inoculum on the severity of influenza A (H1N1/PR8) infection in mice. The influenza A (H1N1/PR8) virus showed a significant replication in the respiratory tract of the infected mice. After an intranasal application, virus titres found in lung homogenates from 4 days infected mice were the highest. The titres were then gradually decreased and becoming undetected on day 10 after infection. The reduced viral replication after day 4 post infection might probably be because either most of the targeted epithelial cells have become shed and necrotic and therefore did not allow further increase in viral replication or because of the effectiveness of inflammatory cells along with their respective inflammatory mediators to eradicate the virus.

Histopathological analysis showed a gradual increase in lung inflammation form day 4 and peaked on day 7 after infection. In a study of mice infected with LC_{50} of the same virus (influenza A H1N1/PR8), a marked lung cellular inflammation was started on day 2 after infection (Fukushi *et al.*, 2011). In the same study, histopathological features such as peribronchial and perivascular inflammation, epithelial cells shedding, alveolar collapse, and pulmonary haemorrhage were also observed as they were in the current study.

The plan of subsequent studies to induce exacerbation in respiratory allergic mice has made a moderate viral respiratory infection which not lethal to mice a necessity. To determine the dose of the influenza A (H1N1) virus and the length of infection required to cause moderate inflammation without apparent pneumonia, 2 doses of intranasal instillation i.e. 10 pfu and 25 pfu were inoculated and 3 time points were determined. Moderate infection was verified by observing the pattern of baseline lung functions, kinetics of weight loss, inflammatory cell influx, and airway hyperresponsiveness at 4, 7, or 10 days after inoculation.

Moderate infection with no increase in baseline respiratory function but marked airway hyperresponsiveness was obtained by infecting mice with 10 pfu of influenza A virus for 4 days. Therefore the subsequent experiment by introducing this respiratory viral infection model into ovalbumin induced allergic mice will be conducted by infecting the mice with 10 pfu of influenza A virus, then challenged with ovalbumin on day 4 after infection. The subsequent experiment will require the mice to be sacrificed on day 5 after infection. Therefore an additional experiment infecting mice with 10 pfu of virus for 5 days was conducted, and the results which showed moderate inflammation ensuring the following experiments to be executed as planned.

VI. CONCLUSION

HPIV3, Poly (I:C), and influenza A (H1N1/PR8) inoculation of mice has been screened and studied. HPIV3 infection in mice did not result in expected inflammatory responses such as an increase in airway responsiveness as well as influx of inflammatory cells into the airways. To the best of my knowledge, this is the first study reporting the infection of HPIV3 in mice. The lack of demonstrable HPIV3 infection explains why this has not been published elsewhere.

Poly (I:C) was able to produce some inflammatory responses. A high concentration of poly (I:C) (100µg/50µl) increased airway responsiveness and inflammatory cell influx in the airway with neutrophilia as seen in respiratory viral infection. However, poly (I:C) is a synthetic dsRNA and is not a virus and therefore does not replicate and induce an immune response as complete as viral infection.

Influenza A (H1N1/PR8) infection resulted in marked increase in airway responsiveness and inflammatory cell recruitment into the airway. This study extended previous published work (Julander *et al.*, 2011) by investigating the infectious dose of the virus and the duration of the infection. The infection of mice with 10 pfu of influenza A (H1N1/PR8) for 4/5 days was able to produce a moderate infection with moderate inflammation yet marked airway hyperresponsiveness as required to be integrated with the murine model of ovalbumin induced allergic disease to cause an exacerbation. Therefore, influenza A (H1N1/PR8) was selected to be used in subsequent studies.

CHAPTER V

MURINE MODEL OF OVALBUMIN INDUCED ALLERGIC AIRWAYS INFLAMMATION

I. INTRODUCTION

Asthma is a complex syndrome which is characterised by many clinical phenotypes most commonly are chronic inflammation with reversible airway limitation accompanied by airway hyperresponsiveness. Animal models of asthma have provided a significant contribution to better understand the pathophysiology of asthma particularly to study the pathogenetic pathway and to suggest therapeutic targets for intervention (treatment by drug).

The mouse is the most widely used animal to model asthma as it offers numerous advantages compared to other animal models. Strain BALB/c is the most used among mouse strains as genetically it has a well characterised immune response which bears a Th2 dominant immune mechanism in response to allergic stimulation. An ideal animal model has to have a close resemblance to human asthma. Most of the mouse models show some characteristics which make them suitable for evaluating the pathophysiology and immunology of allergic asthma. Several characteristic of the mouse model such as inflammatory cell infiltration, production of antigen specific IgE, elevation in Th2 type immune cytokines such as IL-4, IL-5, and IL-13 resemble the features of human asthma (Wills-Karp, 2000). Mouse also exhibits airway hyperresponsiveness and some murine models also demonstrate the early and late phase allergic reactions after encounter with specific allergen. Lung function monitoring is also possible with unrestrained conscious mice, hence it is easy to assess the alteration in an airway function which have cardinal features of asthma. Some mouse chronic models of asthma also show some evidence of airway remodelling with smooth muscle hyperplasia, collagen deposition, mucus hypersecretion, and membrane fibrosis (Kannan and Deshpande, 2003; Stevenson and Birrell, 2011).

As in any other animal models, the mouse asthma model does not fully simulate human asthma. Therefore it needs a judicious approach to extrapolate the findings into

human. Asthma does not spontaneously develop in mice (Taube *et al.*, 2004). It needs to be induced. The most widely used model is acute allergic response to inhaled allergen. There are also anatomical and physiological differences between mouse and human. Mouse is quadrupeds which may have influence on the lung response to airflow limitation as a result of different interaction between forces acting on the lung and the gravity (Bettinelli *et al.*, 2002). Branching pattern of the airways of mouse and human is also different. Mouse has less branching airways than human which has an impact in aerosol deposition (Wenzel and Holgate, 2006). Unlike human eosinophils, mouse eosinophils seem to be insensitive to many stimuli which degranulate human eosinophils (Gelfand, 2002). Inflammatory cells distribute in vascular and perivascular areas of mice which does not occur in human asthma (Wenzel and Holgate, 2006). Mouse acute models of asthma lack chronicity features such as development of airway remodelling which is seen in human asthma (Zosky and Sly, 2007) although chronic allergen challenge does lead to remodelling in mice (Evans, 2009). However, the presence of those differences does not mean that mouse models are not able to give a valuable insight to study human asthma, because every model has its own merits, its strength and weaknesses.

The basic protocol of asthma induction in mice includes multiple sensitisations with allergen in conjunction with adjuvant which is followed by a repeated inhaled or intranasal exposure of allergen challenge. Allergens including OVA (ovalbumin), house dust mite (Lambert *et al.*, 1998), cockroach antigens (Warner *et al.*, 2004), Aspergillus fumigatus (Kurup et al., 1997), and ragweed extracts (Chapoval et al., 2002) are the most popular.

At present, mice acute ovalbumin (OVA) models have been used in most studies. OVA, an antigen from chicken egg white is relative cheap and has a well established immune characterisation. Some may say that OVA lacks of clinical relevance because it is not the normal antigen which triggers asthma in humans. However, the OVA model offers

more detailed endpoints of the experiments as many choices of antibodies-specific to OVA are commercially available (Schröder and Maurer, 2007). Murine models of OVA-induced allergic respiratory inflammation are also able to robustly reflect many of human asthma cardinal features such as IgE production elevation, Th2 immunity responses, airway inflammation with eosinophilia, airway hyperresponsiveness, EAR, LAR, increased mucus production and oedema (Nials and Uddin, 2008).

Mice are usually sensitised with 1 – 8000 ug of OVA, commonly by multiple intra peritoneal injections. OVA is usually injected in combination with adjuvant. The most commonly used adjuvant is aluminium hydroxide which is added in the sensitisation to skew the immune development towards the Th2 phenotype. Adjuvant-free protocols are also possible to be performed even though it needs more antigen injections to achieve complete sensitisation. Animals are challenged with ovalbumin usually about 2-4 weeks after completion of sensitisation. The challenge models are mostly short term (1-8 days).The endpoint assessments are usually performed 24 hours after challenge (Kumar et al., 2008).

Currently, there is no standardised protocol of an OVA model of asthma. Most laboratories have their own protocol. Protocols vary according to the endpoints evaluated in the study. As a result, it is also difficult to directly compare results from different mouse OVA model. Our laboratory also has developed a mouse acute ovalbumin model that demonstrated some important features in asthma including elevation of IgE and OVA-specific ovalbumin IgG, eosinophilia, EAR, LAR, and airway hyperresponsiveness (Fernandez-Rodriguez et al., 2008). Therefore this model will be used in this and subsequent chapters.

II. AIMS

This chapter's aim was to study the effects of acute exposure of OVA in OVA sensitised mice. The endpoints investigated were lung function expressed as Penh, airway responsiveness to methacholine provocation, and influx of inflammatory cells into the airways.

III. METHODS

The details of the methods used are described in **CHAPTER 2.**

III.1. ANIMAL

Six Male BALB/c mice (20-25g) per group were used for all studies

III.2. SENSITISATION

Mice were sensitised on day 0 and 5 by intraperitoneal injections (0.25 ml) of the mixture of OVA (50 µg/mouse) and $Al(OH)_3$ (50 mg/mouse) in saline (fig 5.1).

III.3. OVALBUMIN CHALLENGES

On day 17, mice were exposed to aerosolised OVA (0.5%) or saline (in a control group) inhalation challenges in a perspex box for 2 x 1 hour, with a 4 hour gap between exposures.

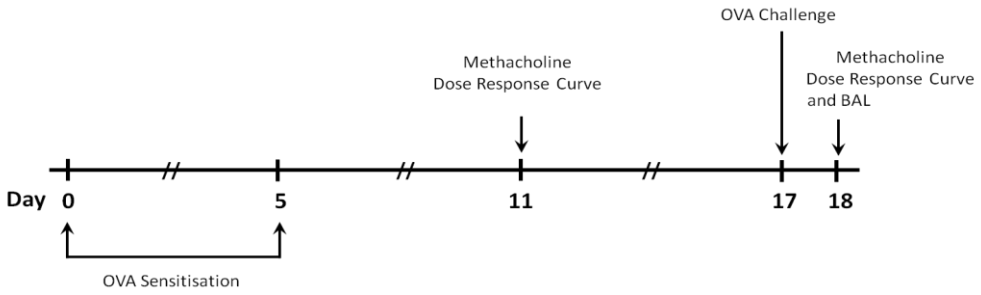


Figure 5.1. Experimental protocol of ovalbumin sensitised ovalbumin induced allergic airway inflammation in mouse

III.4. EARLY PHASE AND LATE PHASE DETERMINATION

Measurement of the lung function of unrestrained mice was recorded as Penh using a Buxco system. Values of Penh after allergen challenge were recorded at 0, 20, 40, 60, 90 and 120 minutes, then hourly until 10 hours followed by a final reading at 19 hours after the second ovalbumin challenge.

III.5. AIRWAY RESPONSIVENESS MEASUREMENTS

Airway responsiveness in mice was measured after methacholine (acetyl-β-methylcholine chloride) provocation. Respiratory activity was recorded for 5 min, to establish baseline value for Penh. Mice were subsequently exposed to increasing doses of aerosolized methacholine dissolved in saline (1, 3, 10, 30, 100 mg/ml). The procedures were started with preselected flow rate at 1.0 min; with 1.5 minutes of intermittent methacholine exposures (at 25% duty: 1.5 second exposure followed by 4.5 seconds without exposures on a continuous cycle), and 2 min dryer time. Plethysmographic data were recorded for another 5 minutes after aerosol administration. The Penh values measured during each 5 minutes sequence were averaged and are expressed for each methacholine concentration as absolute Penh values. Airway responsiveness measurements were performed on day 11 of experimental protocol and 19 hours after the final ovalbumin exposure.

III.6. TOTAL AND DIFFERENTIAL CELL COUNTS

After the final airway responsiveness measurements, the mice were administered a lethal dose of sodium pentobarbitone, the lungs were then removed and lavaged. Using the BAL fluid, recovered total and differential cell counts were then carried out using the methods described in CHAPTER 2. A naive, non-sensitised, group of mice was also included as a reference to establish the normal cell counts in a mouse.

IV. RESULTS

IV.1. EARLY AND LATE-PHASE REACTIONS

The bronchoconstriction in ovalbumin (OVA) and saline challenged mice were monitored every hour for 19 hours. OVA sensitised and challenged mice showed an early marked bronchoconstriction (Early asthmatic responses - EAR) (27.82±4.3%), 1.5 hours after the second challenge. The brochoconstriction resolved on hour 4 and then peaked again between 6 and 12 hours (44.9±7.9%). This second brochoconstriction was a late phase reactions (LAR) which recovered to baseline after 19 hours. In contrast with ovalbumin challenged group, saline challenged group showed bronchorelaxation at most of the time points monitored. The detailed comparison of the mean time course changes in Penh between OVA challenged and saline challenged group was depicted on fig 5.2.

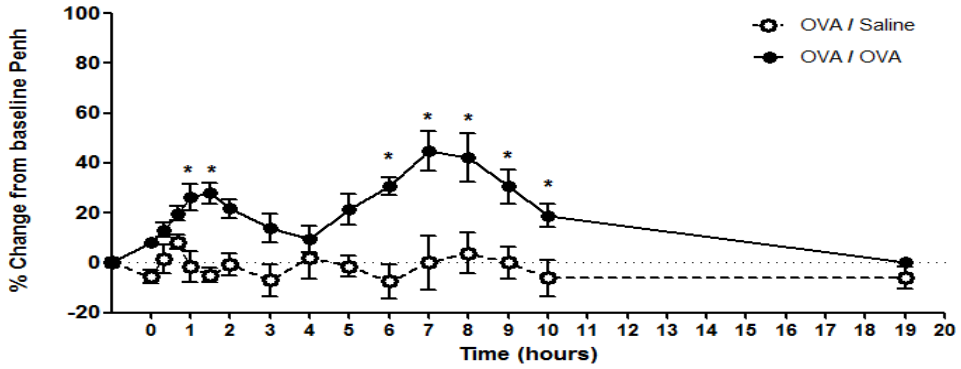


Figure 5.2. – Mean time-course values of Penh in OVA sensitised mice that were challenged with saline or 0.5% OVA. Mean changes in Penh are expressed as mean±S.E.M. percentage change from baseline. Data were analysed with unpaired two tailed t-test (* P< 0.05; n=6).

IV.2. AIRWAY RESPONSIVENESS TO METHACHOLINE

Enhanced airway responsiveness is a well-established characteristic of asthma. Saline challenged mice showed no significant increase in responsiveness toward increased dose of methacholine inhalation, compared to the values before saline challenge (Pre-Saline challenge). In contrast, there was a remarkable AHR increase in OVA

challenged mice from the airway responsiveness before OVA challenge (Pre-OVA challenge). Figure 5.3 depicts the response of saline and OVA challenged mice toward increasing dose of methacholine chloride.

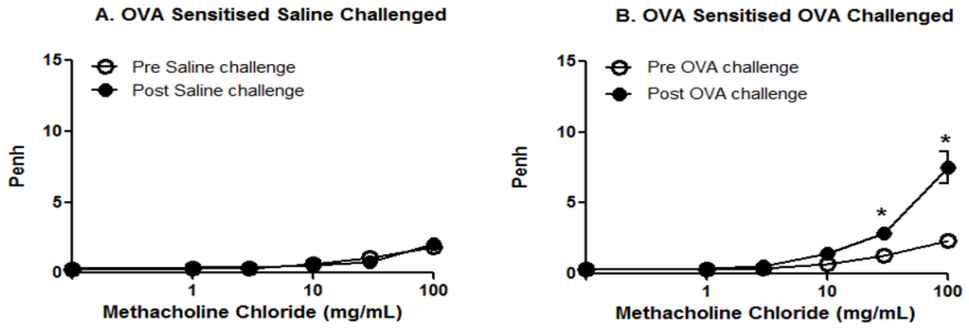


Figure 5.3. Mice were challenged with saline or 0.5% OVA on day 17. On day 11 and 24 h after the challenges (day 18), baseline lung function and AHR to increasing doses of methacholine were measured by whole body plethysmography**.** OVA challenge increased Penh levels from the Penh values before OVA exposures, while there is no significant increase of Penh on the saline challenged group. Results are expressed as mean ± SEM of absolute Penh. Data were analyzed with unpaired two tailed t-test (*P<0.05; n=6).

IV.3. TOTAL AND DIFFERENTIAL CELL COUNTS

OVA challenge led to a significant increase in total and differential cell counts compared to saline challenged group as shown on figure 5.4. The number of individual leukocytes number, particularly eosinophils, in OVA challenged group are significantly greater than in the saline challenged group.

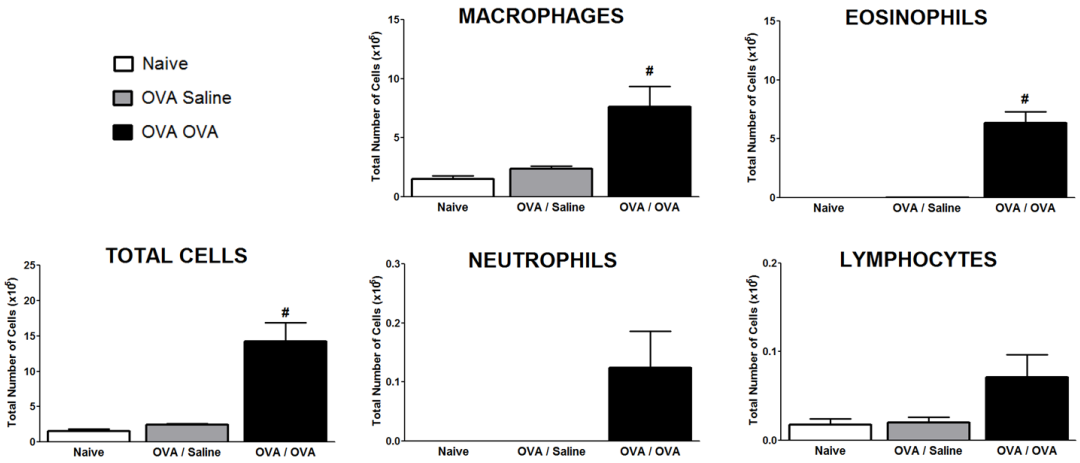


Figure 5.4. The number of total cells, macrophages, eosinophils, lymphocytes and neutrophils found in the bronchoalveolar fluid of naïve (non-sensitised), OVA and saline challenged mice. Results are expressed as mean±S.E.M. Data were analysed with one-way Analysis of Variance followed by a Bonferroni post-test. #significantly different from all other groups (P<0.05; n=6).

V. DISCUSSION

The model of acute asthmatic mice was performed based on the study carried out by Fernandez-Rodriguez *et al.*, (2008). Models of asthma should be able to consistently replicate most, if not all, of the key features of asthma. These are inflammatory cell migration into the airway with eosinophilia, airway hyperresponsiveness, early and late asthmatic responses, and airway remodelling. The acute mouse model developed by Fernandez-Rodriguez *et al.,* (2008) demonstrated most of these features.

An elevation of inflammatory cells influx was observed in these models which imitate a feature of human asthma. A significant increase in the percentage of eosinophils present in the BAL fluid of OVA challenged mice was also observed. Eosinophils influx has been thought to play an important role in determining the LAR. Eosinophil products which are released on degranulation also led to epithelial injury which may further contribute to the development of airway hyperresponsiveness (Barnes, 2008).

Few mice models of asthma were able to show clear early and late asthmatic response. Both early and late asthmatic response will only expressed with multiple challenge, so in this study, OVA challenge was given twice, 4 hours apart. EAR peaked at 1-2 hours and LAR peaked 7-8 hours after the second OVA challenge.

Airway hyperresponsiveness was assessed using the increased dose of a non-selective muscarinic receptor agonist, methacholine. Many factors have been suggested to contribute to the development of airway hyperresponsiveness in asthma, one of them is the accumulation of inflammatory cells (Meurs *et al.*, 2008) which might be the case in this present study.

Eventhough acute models of asthma successfully replicate many cardinal features of asthma, there is a major drawback as the acute model with short term exposure to allergen would not exert the key features seen in chronic human asthma, such as

airway remodelling. The exhibition of the central features of asthma also tends to be temporary which usually resolve within a week of the allergen challenge (Mcmillan and Lloyd, 2004). However, many of these robust and consistent acute models of ovalbumin-induced allergic inflammation in mice have given a significant contribution to provide a better understanding in the patho-immunology of asthma and will continue to do so.

VI. CONCLUSION

The developed acute murine model of ovalbumin induced airway inflammation replicates most of central features in human asthma such as increase in inflammatory cell influx, eosinophilia, airway hyperresposiveness, early and late asthmatic responses. This model would be a good base for subsequent studies in modelling asthma exacerbation and investigating the effect of corticosteroid on the developed model.

CHAPTER VI

INFLUENZA INFECTION IN A MURINE MODEL OF ALLERGIC AIRWAYS INFLAMMATION

I. INTRODUCTION

Respiratory viral infection has a close association with asthma. There is an implication that childhood respiratory viral infection has been involved in the inception of asthma (Busse *et al.*, 2010). A substantial body of evidence also suggests that viral infection triggers wheezing and exacerbation in both children and adults with asthma (Cypcar *et al.*, 1992; Nicholson *et al.*, 1997) . Viral infection was responsible for most of childhood asthma exacerbation accounting for up to 80-85% of all cases and roughly 44% of asthma exacerbations in adults (Johnston, 1995; Nicholson *et al.*, 1997).

It has been suggested that there is a synergistic interaction between exposure to high concentration of sensitising allergen and viral infection, causing a severe asthma exacerbation, often requiring hospitalisation (Tunnicliffe *et al.*, 1999; Green, 2002). The synergistic relationship might happen because the airway epithelium is damaged as a consequence of both viral infection and allergic inflammation. In the presence of viral infection, the epithelial shedding could facilitate and enhance allergen absorption which then increases inflammation (Sakamoto *et al.*, 1984). On the other hand, the epithelial damage as a result of pre-existing airway allergic inflammation could increase viral replication which leads to more severe clinical consequences (Jakiela *et al.*, 2008). In addition, several studies show that asthmatics might have impaired antiviral immune system which may lead to more severe and persistent viral infection and enhancement of airway allergic inflammatory responses (Contoli *et al.*, 2006; Forbes *et al.*, 2012).

In one study by Message *et al.* (2008) who investigated the responses of both asthmatic and non asthmatic patient toward human rhinoviruses showed that asthmatics had significantly more severe respiratory symptoms, reduction in lung functions, and increase in airway hyperresponsiveness as well as eosinophilia compared to non asthmatic patients. They also found that only in asthmatic patients, was the viral load highly associated with more severe deterioration. In asthmatic

patients, neutrophilic and eosinophilic inflammation was also an important determinant for the degree of lung function impairment (Message *et al.*, 2008).

Rhinovirus was identified as the most frequent cause of acute asthma exacerbation (Johnston, 1995). Rhinoviruses has been show to increase the histamine release, eosinophil recruitment and increasing the possibilities of late phase asthmatic reactions development in people with asthma (Gern *et al.*, 1997; Green, 2002). Although not as common as rhinovirus in causing acute asthma exacerbation, influenza viruses were responsible for a quite high proportion of severe asthma exacerbation. Influenza A is the second most identified virus after rhinoviruses in adults admitted to hospital for an acute asthma exacerbation (Teichtahl *et al.*, 1997).

Influenza infection in people with asthma has been suggested to increase hospitalisation due to exacerbation (Glezen *et al.*, 2000). During the influenza season, asthmatics which constitute nearly 79% of all high risk group in proportion had significantly more cases of hospitalisation and outpatient clinic visit‘s compared to non asthmatic healthy people (O'Brien *et al.*, 2004). In 2009, when the pandemic of H1N1 emerged, asthma has even been associated more complications such as ICU admission (O'Riordan *et al.*, 2010).

I.1. IMMUNOLOGY OF VIRAL INDUCED ASTHMA EXACERBATION

Respiratory viruses target epithelial cells lining in the airways. The injured epithelial cells release a plethora of inflammatory mediators including IL-1, IL-6, IL-8, IP-10, RANTES, GM-CSF, and eotaxin which lead to activation and migration of inflammatory cells such into the airway (fig 6.1) (Dougherty and Fahy, 2009).

The nature of virus-induced asthma exacerbation is different from allergen induced asthma. When there is a combination of viral infection and allergen exposure in allergen sensitised individuals, neutrophils appear to play more important role while in allergen induced exacerbation, eosinophils is more dominant (Fraenkel, 1995).

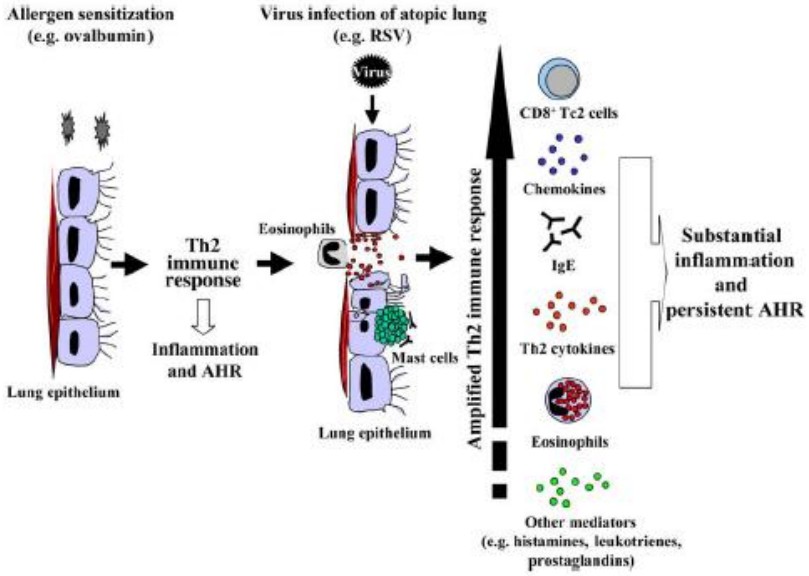


Figure 6.1. Immunological mechanisms of viral infection in allergic individuals exposed to allergen which induce exacerbation of airway pathology (Tauro *et al.*, 2008)

In experimental rhinovirus infection in asthmatic patients, there was evidence of increased IL-8, a neutrophil chemoattractant, as well as neutrophil influx in BAL fluid, sputum and nasal secretion (Message *et al.*, 2008). The upregulation of neutrophils, the release of the products of activated neutrophils, and IL-8 might also involve in the enhancement of AHR. IL-8 has been noted to be a determinant of increasing AHR in viral induced-exacerbation of asthma. The level of TNF-alpha released by neutrophils is also correlated with the degree of AHR (Nagarkar *et al.*, 2009).

Eosinophils, their products and chemoattractants were also shown to be more up-regulated following rhinovirus infection in asthmatic subjects compared to normal non asthmatic subjects (Heymann *et al.*, 1995). In the asthmatic adults with rhinitis, the eosinophil cationic protein content in sputum was also correlated to the level of AHR (Grunberg, 1997).

Macrophages also play an important role in viral infection. As the most dominant leucocytes in the airway, in the case of viral infection and replication, macrophages release some inflammatory mediators such as TNF-alpha, IL-1, IL-8, and MIP-1 alpha.

In addition macrophages also produce type I interferon as an antiviral agent (Laza-Stanca *et al.*, 2006).

However, some studies show that asthmatics seem to have an abnormal innate immune response and produce less interferon than non asthmatics in response to rhinovirus infection (Wark, 2005; Contoli, 2006; Contoli *et al.*, 2006; Bullens *et al.*, 2008). The deficiency of interferon in asthmatics has been associated with increasing pathogenesis of asthma exacerbation including the increasing airway symptoms, lung function impairment, and increasing viral load (Contoli *et al.*, 2006).

T lymphocytes appears to be important in viral infection and asthma but when both cases occur concurrently, the balance between the typical Th2 cytokines environment in asthma and Th1 predominant responses in viral infection would determine the severity of exacerbation. In asthmatics, the inflammation is characterised by Th2 type cytokines such as IL-4, IL-5, and IL-13, while in respiratory viral infection, Th1 type immune response which lead to the production of IFN-gamma for viral clearance is more dominant (Contoli *et al*., 2006).

Evidence shows that in the asthmatic environment rich in Th2 cytokines, the response to viral infection might skew toward Th2 response which leads to inhibition of normal Th1 immune response. The Th1 deficiency would reduce the ratio between Th1 and Th2 cytokines production in asthmatics compared to non asthmatics (normal subjects). Some studies demonstrated that IFN-gamma/IL-5 ratio and IFN-gamma/IL-4 ratio is negatively related to asthma severity, asthma symptoms, and time needed for viral clearance (Papadopoulos *et al.*, 2002; Brooks *et al.*, 2003). Therefore, a stronger Th1 immune response is vital to ameliorate viral induced asthma exacerbation.

I.2. ANIMAL MODELS OF VIRAL ASSOCIATED ASTHMA EXACERBATION

Animal models are useful in investigating underlying immune responses involved in the interaction between respiratory viral infection and airway allergic inflammation. The studies on the interaction between respiratory viral infection, allergen sensitisation, and exposure to high concentration of sensitised allergen mostly evaluate the underlying mechanism of interaction between respiratory virus infection with allergen sensitisation or the role of respiratory viral infection in the established allergic airway inflammation. This chapter will only focus in the interplay between respiratory virus infection and high exposure of allergen in established allergen sensitisation. The term 'exacerbation' in this and subsequent chapters is defined as the worsening of allergic airway inflammation and respiratory function demonstrated in the animal model.

Several animals have been exploited in the model to evaluate the co-administration of respiratory virus infection and high concentration of allergen administration, with mouse the most utilised. Some murine models have been used as a tool to investigate a range of different respiratory viruses, such as rhinovirus, respiratory synctial virus, parainfluenza virus, metapneumovirus, and influenza virus.

Most of current our understanding on how respiratory virus infection might exacerbate asthma is derived from studies on the major asthma exacerbations caused by virus, rhinovirus (Papadopoulos *et al.*, 2000). Bartlett *et al* (2008) have been successfully developed a model of murine model of rhinovirus exacerbation in mice sensitised and challenged with ovalbumin (Bartlett *et al.*, 2008). The infection of rhinovirus during the ovalbumin challenge resulted in the enhancement of airway hyperresponsiveness, elevation in Th1 and Th2 cytokines, as well as increase of inflammatory cells influx in the airways particularly neutrophils, eosinophils, and lymphocytes. However, consideration must be given to comparisons between different viral respiratory infection

and airway allergic inflammation models because the genetics of the animal, the type of virus, the time of viral infection, the viral and allergen concentration/load may all contribute to the complex mechanism of interaction between respiratory viral infection and asthma. The studies by Barends *et al* (2004) underlined the previously mentioned premises. Barends *et al* conducted a comparative study on the effect of three different respiratory viruses; RSV, pneumonia virus of mice (PVM), and influenza A virus during allergen challenge in sensitised mice (Barends *et al.*, 2004). Both RSV and influenza A are identified as the trigger of asthma exacerbation in human (Teichtahl *et al.*, 1997; O'Donnell and Openshaw, 1998). Both viruses also increased the effect of allergen sensitisation in mice by increasing Th2 type cytokines (O'Donnell and Openshaw, 1998; Suzuki *et al.*, 1998). Barends *et al.* (2004) reported that theTh1 cytokine expression (IFN-γ and IL-12) induced by individual virus infection was not affected by ovalbumin sensitisation or challenge. When Barrends *et al* evaluated the Th2 cytokines (IL-4, IL-5, and IL-13) expression in the lungs they found that RSV and PVM increased the production of IL-4, IL-5, and IL-13 as well as causing perivasculalitis and eosinophilia in OVA sensitised and challenged mice. In contrast to the two other viruses, influenza A has no effect on the expression of Th2 cytokines even though the infection was still causing eosinophilia. Different findings were reported by Marsland *et al.* (2004) who reported that influenza infection increased the Th2 lymphocytes and upregulated IFN-γ (Marsland *et al.*, 2004). Different from Barends *et al*. (2004) who administered ovalbumin challenge in the mice in the recovery phase of infection, Marsland *et al.* (2004) challenged the animal in the acute phase of infection.

II. AIMS

The aims were to investigate the role of influenza infection on already established allergen sensitisation in mice when the allergen challenge is given during the acute phase of viral infection. The endpoints of the study included the enhanced early and late phase asthmatic reactions, inflammatory cells infiltration into the airway, airway hyperresponsiveness and protein leakage, and change in histological features.

III. METHODS

The methods used in the studies are described in more detail in CHAPTER 2

III.1. ANIMAL

Six Male BALB/c mice (20-25g) per group were used for all studies

III.1.1. SENSITISATION

Mice were sensitised by intraperitoneal injections (0.25 ml) of the mixture of OVA (50 µg/mouse) and $Al(OH)_3$ (50 mg/mouse) in saline on day 0 and 5.

III.1.2. INFLUENZA VIRUS INFECTION

Mice were anaesthetised lightly with isoflurane and then intranasally infected with 10 PFU in 50 µl PBS per mouse on day 13 (fig.6.2). The virus solution was inoculated intranasally. Each mouse was inoculated with 12.5 µL of virus stock in one nostril and then 12.5 µL was given to another nostril twice to give 10 pfu/50 µL of virus stock in total. The animal was held in upright position for 2 minutes then was put back in the transfer cage in prone position to facilitate the virus to spread evenly to the right and the left lungs. Control mice were treated the same and subjected to intranasal administration of sham. Sham is allantoic fluid from PBS instilled embryonated egg treated similarly with influenza virus infected embryonated egg. All procedures were conducted in class II biosafety hood/cabinet. Mice were then returned to their cage in mouse isolator once the infection finished.

III.1.3. OVALBUMIN CHALLENGES

Mice were challenged with aerosolised OVA (0.5%) or saline (in a control group) in a perspex box for 2 x 1 hour, with a 4 hour gap between exposures.

III.1.4. EARLY PHASE AND LATE PHASE DETERMINATION

The lung function of unrestrained mice was measured as Penh using a whole body plethysmography system from Buxco. Values of Penh after allergen challenge were measured at 0, 20, 40, 60, 90 and 120 minutes, then hourly until 10 hours with a final reading at 19 hours after the second ovalbumin challenge.

III.1.5. AIRWAY RESPONSIVENESS MEASUREMENTS

Airway responsiveness in mice was measured after methacholine (acetyl-β-methylcholine chloride) provocation. Respiratory activity was recorded for 5 min, to establish baseline value for Penh. Mice were subsequently exposed to the increasing doses of aerosolized methacholine dissolved in saline (1, 3, 10, 30, 100 mg/ml). The procedures were started with preselected flow rate at 1.0 min; with 1.5 minutes of intermittent methacholine exposures (at 25% duty: 1.5 second exposure followed by 4.5 seconds without exposures on a continuous cycle), and 2 min dryer time. Plethysmographic data were recorded for another 5 minutes after aerosol administration. The Penh values measured during each 5 minutes sequence were averaged and are expressed for each methacholine concentration as absolute Penh values. Airway responsiveness measurements were performed on day 11 and 19 hours after the final ovalbumin exposure.

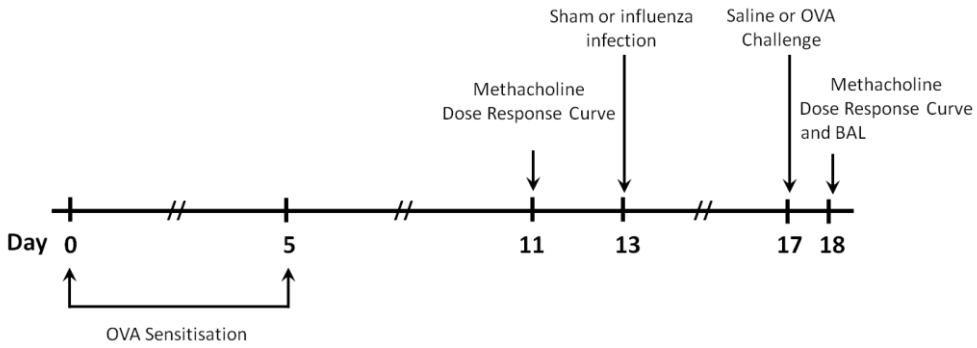


Figure 6.2. Experimental protocol of influenza A (H1N1) infection in OVA model of allergic airway inflammation.

III.2. TOTAL AND DIFFERENTIAL CELL COUNTS

After the final airway responsiveness measurements, the mice were administered a lethal dose of sodium pentobarbitone, the lungs were then removed and lavaged. Using the BAL fluid, recovered total and differential cell counts were then carried out using the methods described in CHAPTER 2.

III.3. BAL FLUID PROTEIN CONTENT

Total Protein content of BAL fluid was analysed with BCA protein assay as described in CHAPTER 2.

III.4. VIRUS TITRE DETERMINATION

The virus titre was quantitated by plaque assay with AEC immunocytochemistry as described previously in CHAPTER 4.

III.5. LUNG HISTOLOGY

Lung was stained with H and E and was analysed with histopathology scoring as described in CHAPTER 2.

IV. RESULTS

IV.1. THE EFFECTS OF SHAM ON THE MURINE MODEL OF ALLERGIC AIRWAY INFLAMMATION

The influenza virus was propagated in embryonated chicken egg. The virus stock was harvested, aliquoted, and kept in allantoic fluid from the egg before dilution to the desired concentration with PBS to be administered intranasally to infect the animals. Allantoic fluid contains ovalbumin, the protein used as an allergen to induce airway allergic inflammation in the current series of experiments. Even though ovalbumin content reduced as the chicken embryo develops (Sugimoto, 1999), and the virus stock was diluted as 1 part in 599,249 part of PBS, the virus dilution delivered in the mouse might still contain small traces of ovalbumin which might alter the features of the previously developed allergic airway inflammation model in mice. Therefore 2 groups of 6 mice were sensitised with OVA, instilled with sham (allantoic fluid from embryonated egg instilled with PBS instead of influenza virus), then 1 group was challenged with saline and the other with ovalbumin to investigate whether the allantoic fluid content in the virus stock might increase the allergic inflammatory responses in the developed murine model.

OVA sensitised mice instilled with sham and challenged with saline did not show any EAR or LAR. The airway function recorded overtime after saline challenge was not significantly different from mice without sham instillation (fig. 6.3). Sham instillation in OVA sensitised mice also did not significantly increase the inflammatory cells recruitment in the airway compared to un-instilled mice.

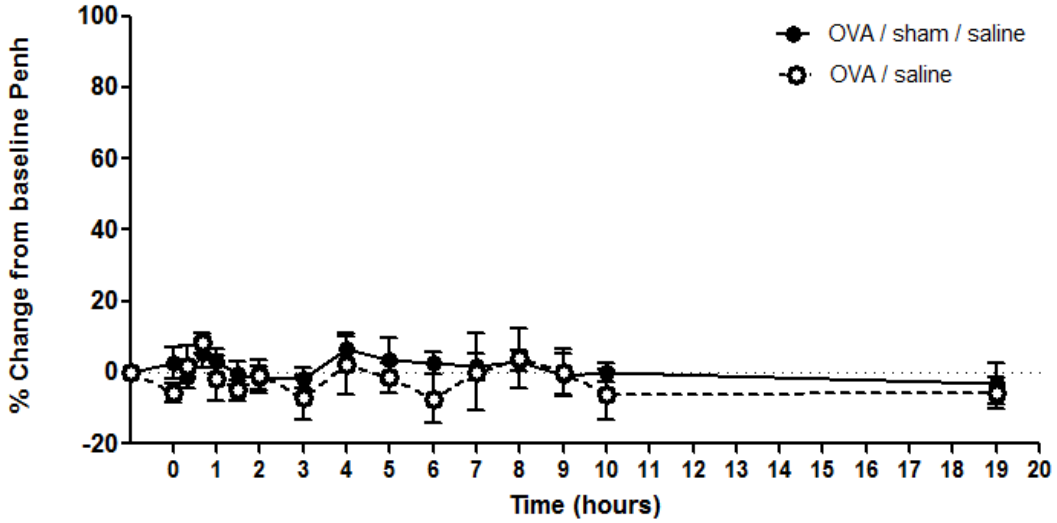


Figure 6.3. Mean time-course values of Penh in OVA sensitised mice that were un-instilled or instilled with sham and then challenged with saline. Mean changes in Penh are expressed as mean±S.E.M. percentage change from baseline. Data were analysed with unpaired two tailed t-test (* P< 0.05; n=6).

Early and late asthmatic responses were observed in the sham instilled mice challenged with OVA. The peak of EAR in sham instilled mice was not significantly different from un-instilled mice. The peak LAR between two groups was also not significantly different, 62.7±14.5% increase in baseline Penh in sham instilled mice and 44.9±7.9% in un-instilled mice. Therefore it can be said that allantoic fluid contained sham did not have any significant effect on the EAR and LAR as described in figure 6.4

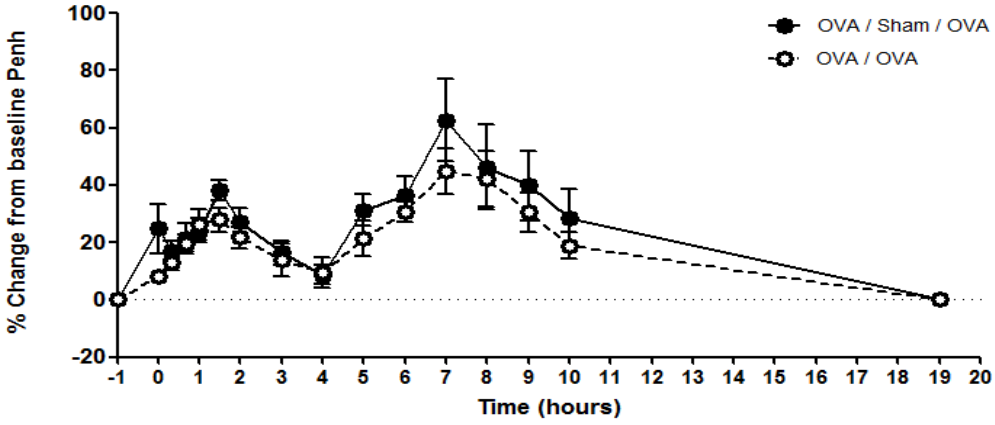


Figure 6.4. – Mean time-course values of Penh in OVA sensitised mice that were challenged with 0.5% OVA. Mean changes in Penh are expressed as mean±S.E.M. percentage change from baseline. There was no significant different between un-instilled or sham instilled animals. Data were analysed with unpaired two tailed t-test (* P< 0.05; n=6).

Figure 6.5 represents the inflammatory cells influx in the airway of the groups mice instilled with sham compared to un-instilled mice. Sham did not significantly affect the total number of inflammatory cell in the BAL fluid as well as the individual leukocytes counts in all compared groups (naive vs sham, OVA/Saline vs OVA/Sham/Saline, and OVA/OVA vs OVA/Sham/OVA). There were no eosinophils detected in the BAL fluid in sham group just similar to the un-instilled group when they were challenged with saline.

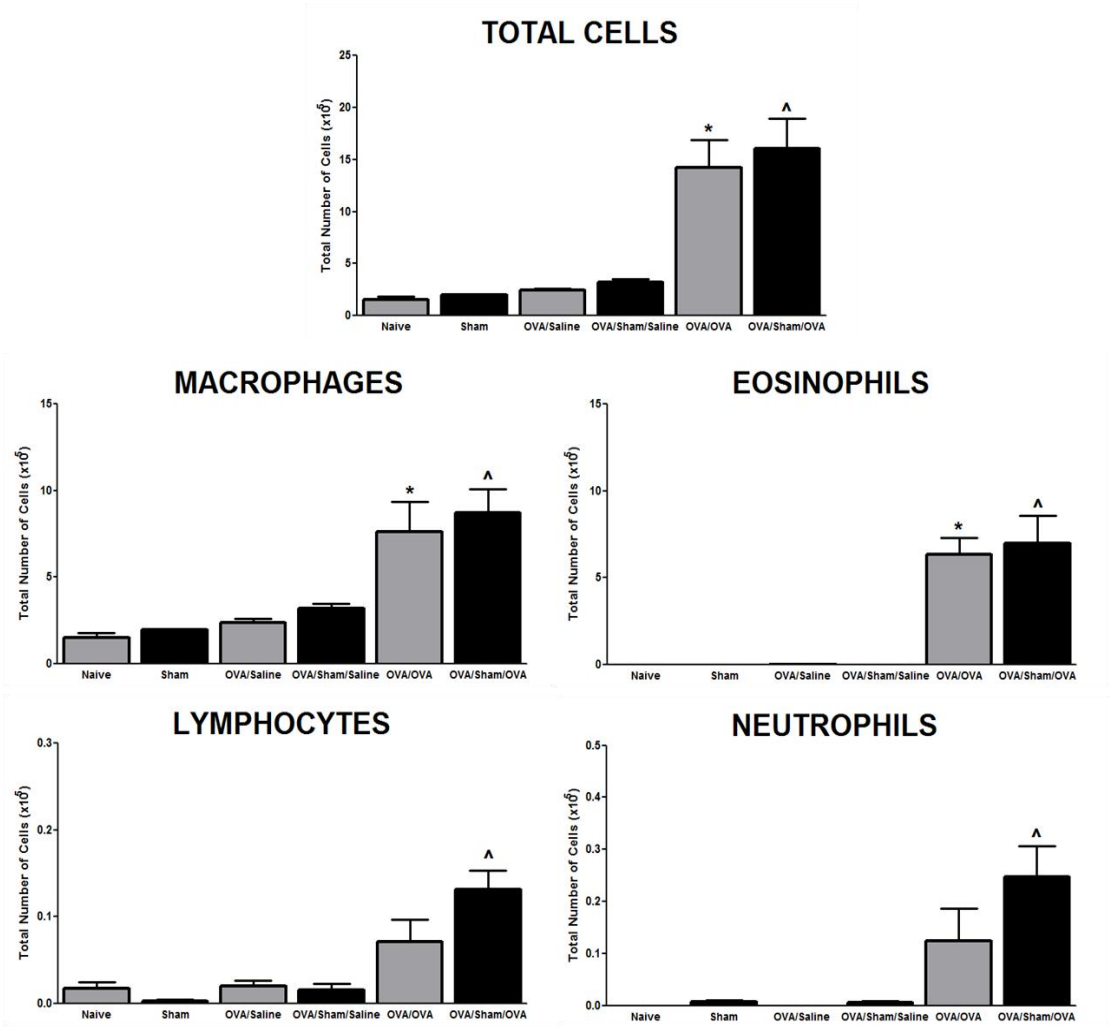


Figure 6.5. The number of total cells, macrophages, eosinophils, lymphocytes and neutrophils found in the bronchoalveolar fluid of naïve (non-sensitised), sham instilled, OVA and saline challenged mice. Results are expressed as mean±S.E.M. One-way Analysis of Variance followed by a Bonferroni post-test. ^significantly different from OVA/Sham/Saline; *significantly different from OVA/Saline ($P<0.05$; n=6 except for sham instilled animals n=4).

Airways reactivity to methacholine was not affected by sham instillation as there is no significant different of the bronchoconstriction between un-instilled and sham instilled

mice. There was no significant increase in the AHR after saline challenge compared to that before saline challenge. However, when comparing the pre-OVA challenge and post-OVA challenge airway responsiveness, there was a significant increase in airway responsiveness in either sham instilled or un-instilled mice which challenged with OVA. The post-challenged airway increase in sham instilled-OVA challenged mice was not significantly different from the post challenged increase in un-instilled mice (fig 6.6).

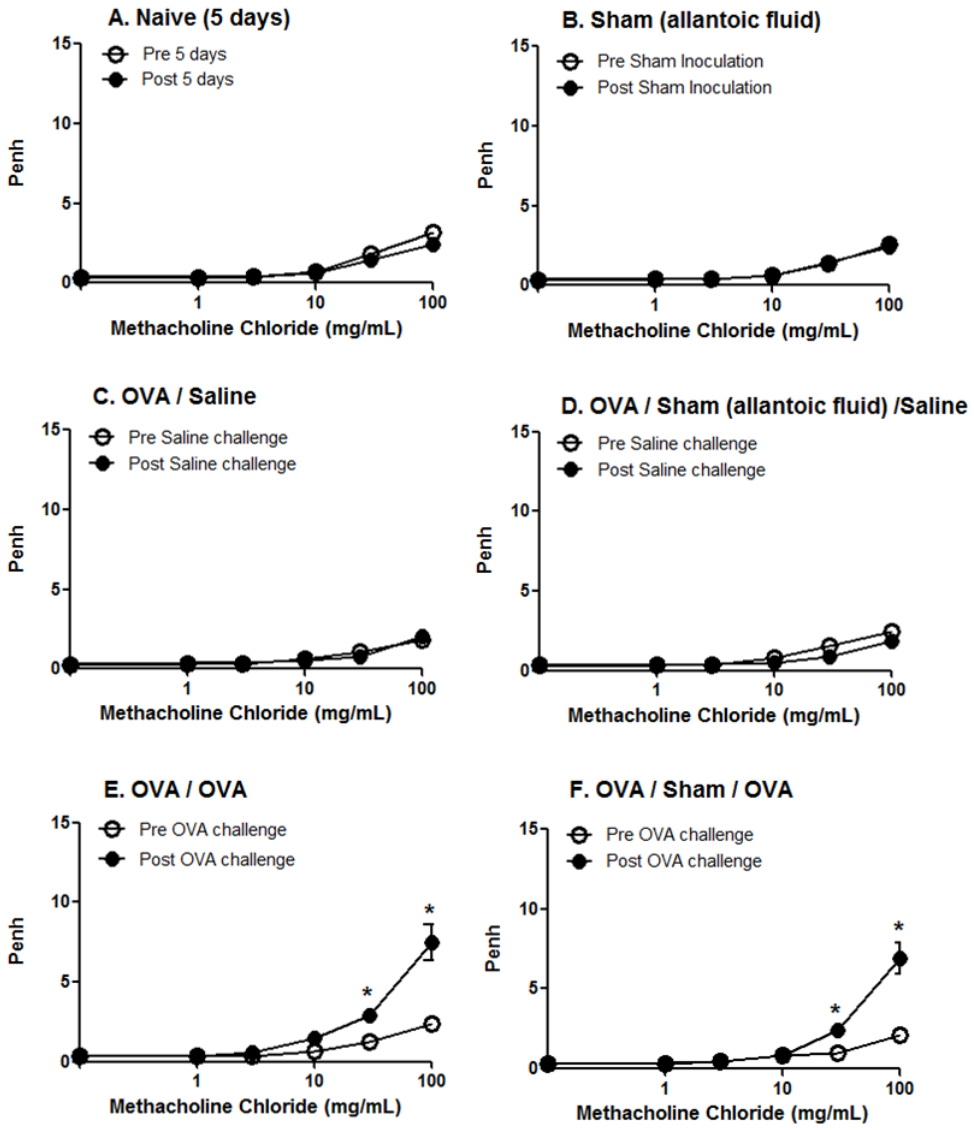


Figure 6.6. Mice were challenged with saline (C and D) or 0.5% OVA (E and F) on day 17. On day 11 and 24 h after the challenges (day 18), baseline lung function and AHR to increasing doses of methacholine were measured by whole body plethysmography. OVA challenge in sham instilled mice increased Penh levels from the Penh values before OVA exposures, while there is no significant increase of Penh on the saline challenged group similar to those of un-instilled mice. Results are expressed as mean ± SEM of absolute Penh. Data were analyzed with unpaired two tailed t-test (*P<0.05; n=6 except for sham instilled animals (B) n=4).

IV.2. THE ROLE OF INFLUENZA VIRUS INFECTION IN MURINE MODEL OF ALLERGIC AIRWAY INFLAMMATION

IV.2.1. EARLY AND LATE-PHASE REACTIONS

The previous observation showed that inoculation with influenza virus in mice enhanced pulmonary infliltration with inflammatory cells and induced airway hyperresponsiveness. The time of allergen challenge inhalation was decided based on the dose and the time point when viral infection induced apparent inflammation without severe consequences such as reduction in body weight as well as increase in baseline respiratory functions.

After being administered with OVA inhalation on day 4 after viral infection, EAR and LAR were observed (fig 6.7). Compared to sham instilled mice, the peak of EAR in influenza virus infected mice was not significantly increased.

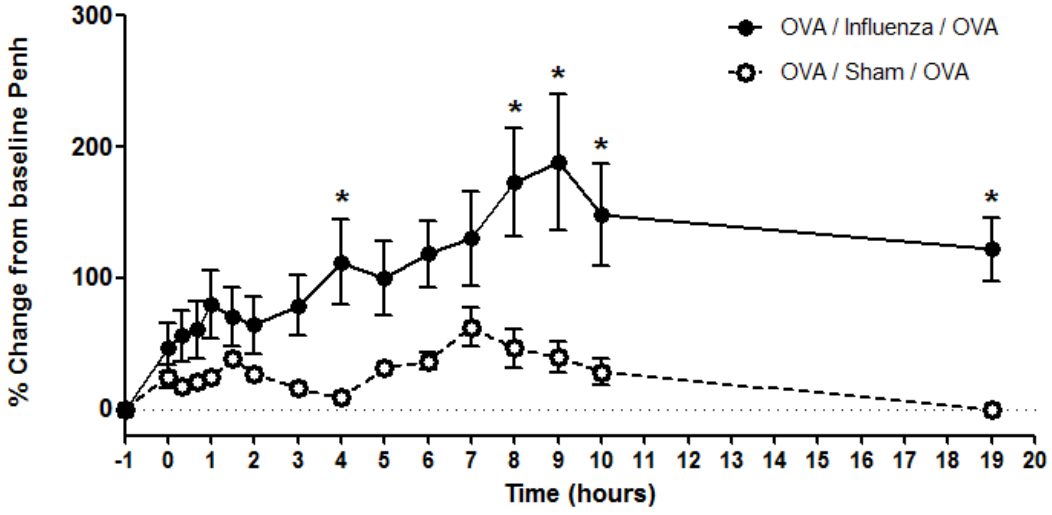


Figure 6.7. Early and late phase asthmatic bronchocostriction observed over 19 hours in sham instilled and influenza infected mice challenged with OVA 0.5%. The OVA sensitised/sham instilled/OVA challenged group and the OVA sensitised/ influenza A/OVA challenged group late asthmatic responses peaked at 7 hours and 9 hours respectively. Mean changes in Penh are expressed as mean±S.E.M. percentage change from baseline. Data were analysed with unpaired two tailed t-test (* P< 0.05; n=6).

The influenza virus infection increased the peak and prolonged the LAR compared to sham instilled mice. The peak of LAR increased from 42.30±9.7% increase from

baseline Penh in sham instilled mice to 188.3±51.9% increase from baseline Penh in influenza virus infected mice. Nineteen hours after the second OVA challenge, the respiratory functions of the infected animals did not return to normal as in the sham instilled ones as depicted in figure 6.7.

OVA sensitised-influenza infected mice but challenged with saline did not show any EAR or LAR (fig 6.8). So it can be said that the profound increase of EAR and LAR in the OVA sensitised OVA/influenza infected/OVA challenged mice was the combined effects of OVA challenge and virus infection.

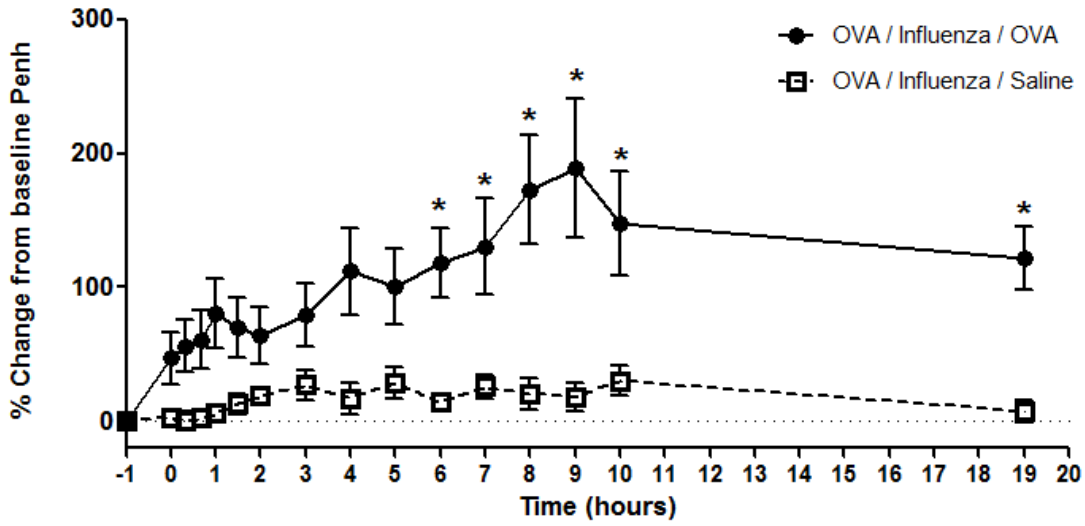


Figure 6.8. Mean time-course values of Penh in OVA sensitised and influenza A infected mice that were challenged with saline or OVA 0.5%. Mean changes in Penh are expressed as mean±S.E.M. percentage change from baseline. Data were analysed with unpaired two tailed t-test (* P< 0.05; OVA /influenza/OVA n=6, OVA/influenza/saline n=5).

IV.2.2. TOTAL AND DIFFERENTIAL CELL COUNTS

In OVA sensitised/influenza infected/OVA challenged mice, there was a significant increase in airway leucocytes counts in the BAL fluid from $25.3\pm2.7.10^5$ cells/ml and compared to OVA sensitised/sham instilled/OVA challenged mice $14.2 \pm 2.61.10^5$ cells/ml. All individual inflammatory cells were significantly elevated with neutrophils being prominent. Influenza infection increased neutrophils influx in the airways; the

neutrophil count was 0.12±0.06.10^5 cells/ml in sham instilled animals and increased to 6.2 ±1.1.10^5 cells/ml in influenza infected group (fig 6.9).

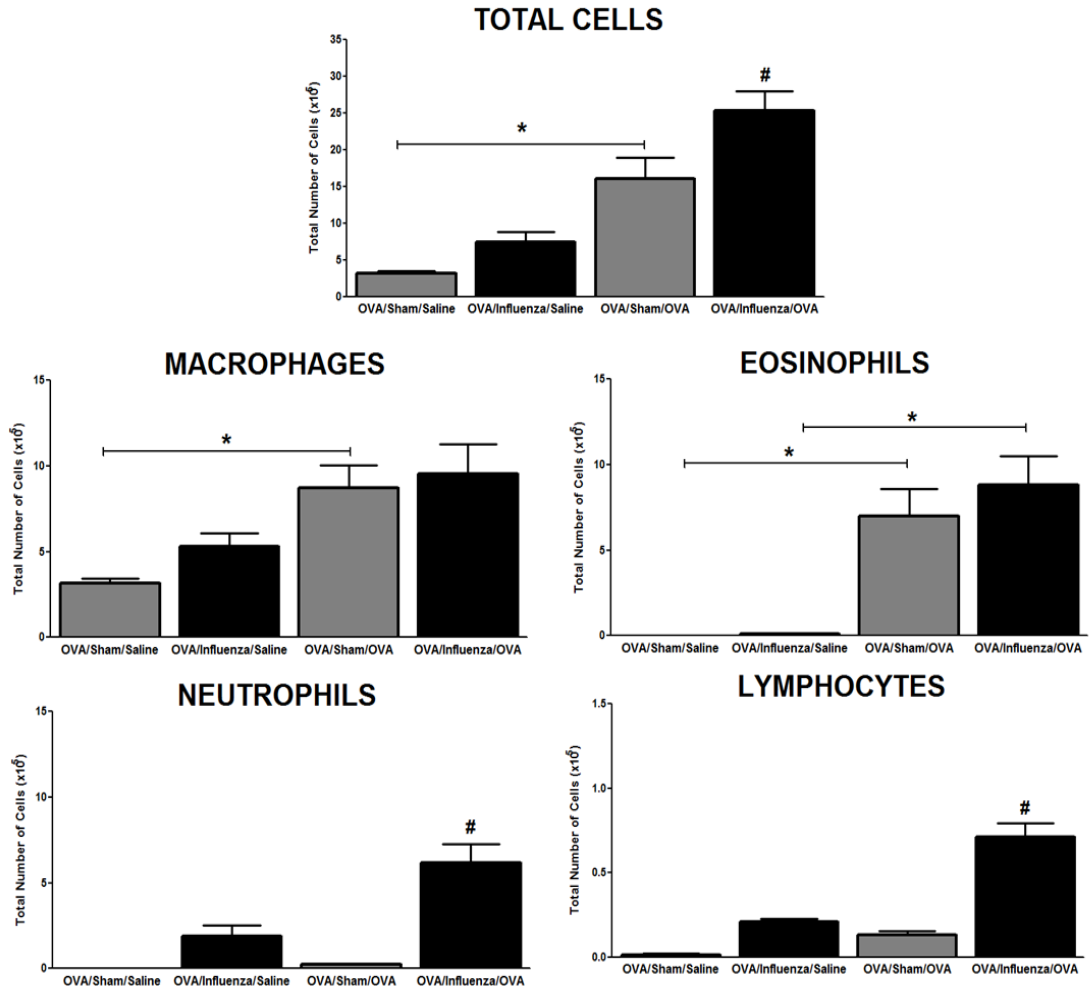


Figure 6.9. The number of total cells, macrophages, eosinophils, lymphocytes and neutrophils found in the bronchoalveolar fluid of naïve (non-sensitised), OVA sensitised, sham instilled or influenza infected, OVA or saline challenged mice. Results are expressed as mean±S.E.M. Data were analysed with One-way Analysis of Variance followed by a Bonferroni post-test. #significantly different from all other groups; *connected with corresponding bar is significantly different from each other (P<0.05; n=6 except for OVA/Influenza/Saline group n=5).

In OVA sensitised/influenza infected/saline challenged mice, the inflammatory cell count in the BAL fluid from also increased compared to OVA sensitised/sham inoculated/saline challenged mice. When the individual leukocyte counts were observed, neutrophil is being the most increased in number in influenza infected animals compared to sham infected ones. Although as not as great as the increase in neutrophil number, lymphocyte number in OVA sensitised/influenza infected/OVA

challenged mice also increased significantly compared to any other groups. There was small number of eosinophil detected in OVA sensitised/influenza infected/saline challenged group but the increase was not significant compared to sham instilled group (fig 6.9).

IV.2.3. AIRWAY RESPONSIVENESS TO METHACHOLINE

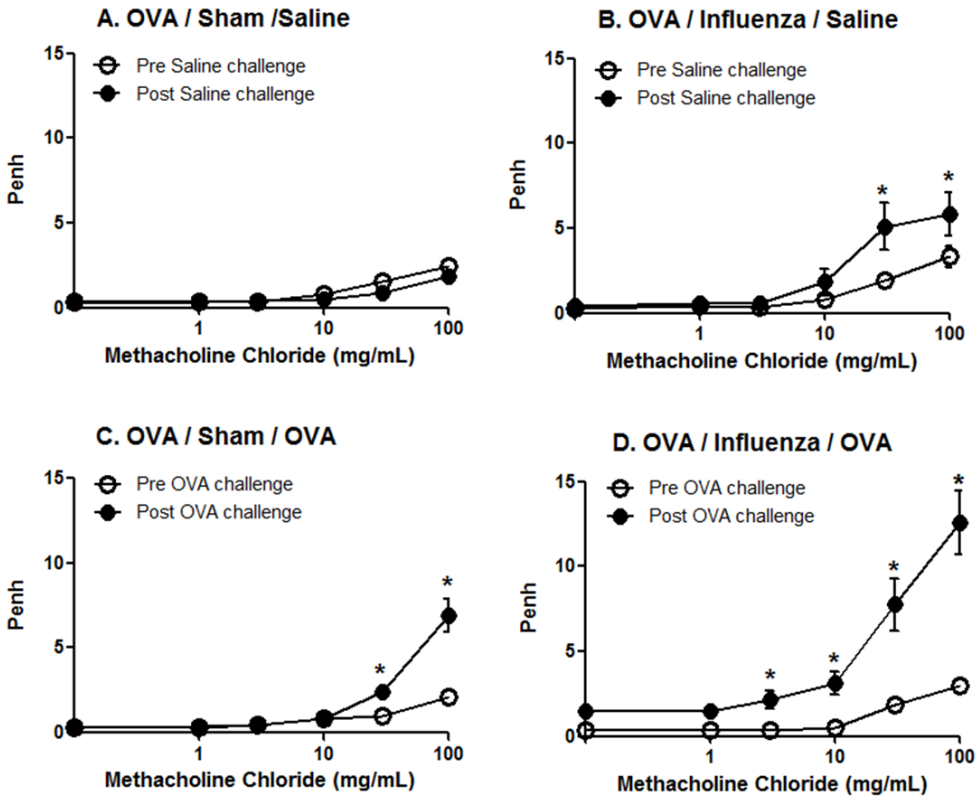


Figure 6.10. Mice were challenged with saline or 0.5% OVA on day 17. On day 10 and 24 h after the challenges (day 18), baseline lung function and AHR to increasing doses of methacholine were measured by whole body plethysmography**.** Results are expressed as mean ± SEM of absolute Penh. Data were analyzed with unpaired two tailed t-test (*P<0.05; n=6 except for except for OVA/Influenza/Saline group (B) n=5).

Airway hyperresponsiveness was induced when mice sensitised and challenged with OVA were provoked with methacholine with the maximum response pre- challenge (2.14±0.14 absolute Penh) increasing significantly to 6.9±1.1 absolute Penh post-challenge. Influenza infection on OVA induced airway allergic inflammation further increased the airway responsiveness from 2.9±0.3 absolute Penh pre-infection to 12.6±1.9 absolute Penh post-infection (fig.6.10).

In OVA sensitised/influenza infected/saline challenged mice, methacholine inhalation also significantly enhanced airway responsiveness. The airway hyperresponsiveness was not observed in OVA challenged/sham instilled/saline challenged animals (fig 6.10).

IV.2.4. PROTEIN EXUDATION IN THE AIRWAYS

Influenza when infected concurrently with allergen challenge in sensitised mice significantly increased the protein level in BAL fluid (fig 6.11).

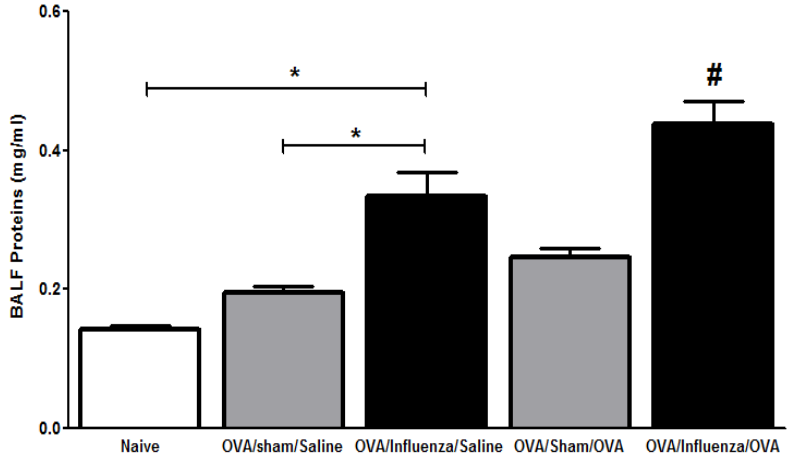


Figure 6.11. Total protein content in bronchoalveolar lavage fluid of naïve (non-sensitised), OVA sensitised, sham instilled or influenza infected, OVA and saline challenged mice. Results are expressed as mean±S.E.M. Data were analysed with one-way Analysis of Variance followed by a Bonferroni post-test. #significantly different from all other groups; *connected with corresponding bar is significantly different from each other (P<0.05; n=6 except for OVA/Influenza/Saline group n=5).

IV.2.5. INFLUENZA A (H1N1/PR8) TITRES IN THE LUNGS

Influenza infection in the lungs was confirmed with a plaque assay. The viral titre of saline challenged and OVA challenged groups were not significantly different (fig 6.12).

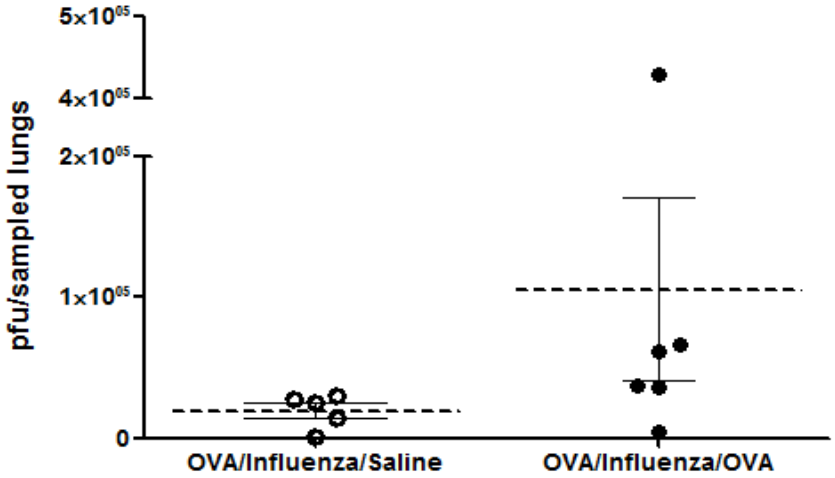


Figure 6.12. Viral titres of lungs of mice sensitised with OVA infected with influenza A 10 pfu and challenged with saline or 0.5% OVA. Virus titres were determined by plaque assay on MDCK cells. Virus titres are expressed as mean ± SEM of pfu per sampled lungs. Data were analysed with unpaired two tailed t-test (* P< 0.05 OVA/Influenza/OVA n=6, OVA/Influenza/Saline n=5).

IV.2.6. LUNG HISTOLOGY

Haematoxylin and Eosin stain was used to show general morphology of the lungs. Marked peribronchial and perivascular inflammation were observed in OVA challenged mice compared to naive or sham instilled-OVA challenged mice. There was also a noticeable change in the features of epithelial cells in OVA challenged animals. The epithelium was elongated and folded (fig 6.13 C) compared to normal intact epithelium observed in naive or sham instilled saline challenged animals (fig 6.13 B). Influenza infection caused a more severe peribronchial and perivascular inflammation (fig 6.15 D and E). In the lung of influenza infected animals, bronchial lumens with epithelial shedding or loss were also observed (fig 6.15 D and E).

OVA challenge increased the mean pathology score from naive, or saline challenged animals. However, influenza infection did not significantly change the mean pathology score compared to sham instilled animals (fig.6.14).

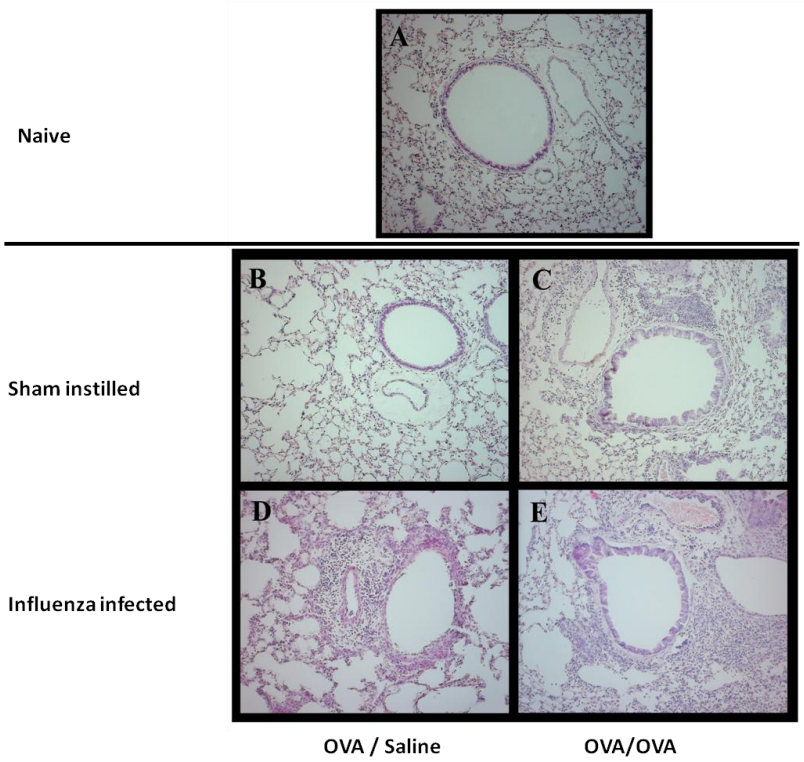


Figure 6.13. Histopathological changes of lungs of naive mice (A); or OVA sensitised mice which instilled with sham and challenged with saline (B); OVA sensitised mice which instilled with sham and challenged with OVA (C); OVA sensitised mice which infected with influenza A virus and challenged with saline (D); or OVA sensitised mice which infected with influenza virus and challenged with OVA (E). The lungs were stained with haematoxylin and eosin to display general morphology (100x magnification).

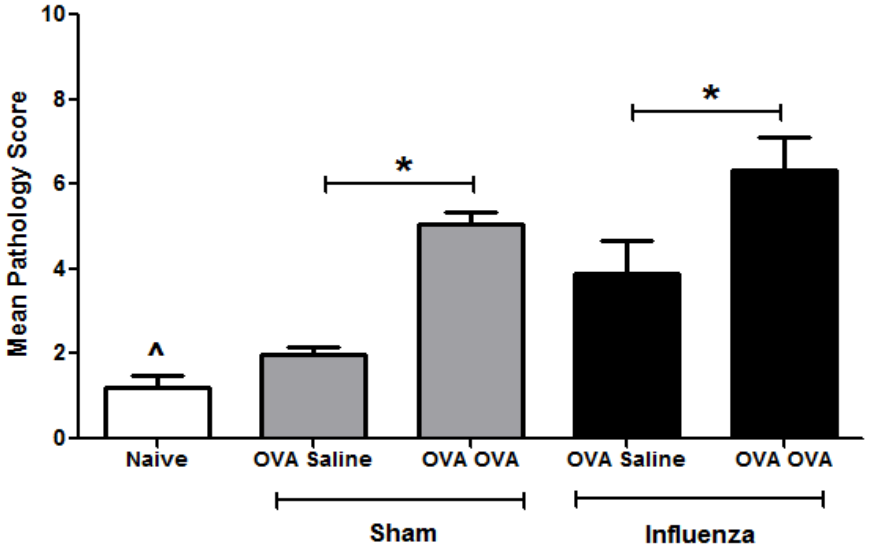


Figure 6.14. The mean lung pathology score of naïve (non-sensitised), OVA sensitised, sham instilled or influenza infected, OVA or saline challenged mice. Results are expressed as mean±S.E.M. Data were analysed using one-way ANOVA, followed by post hoc Bonferroni's test to determine the mean pathology score ^significantly different from all groups except OVA/Sham/Saline. *connected with corresponding bar is significantly different from each other ($P<0.05$; n=6, except for OVA/Influenza/Saline n=5).

V. DISCUSSION

Influenza A virus is regarded as being among the most important viruses causing serious cases leading to morbidity and mortality in the world. How severe the consequence of influenza infection is determined by viral factor, host's genetics, and immune system, including asthma (Abe *et al.*, 2004). The influenza virus targets the epithelial cells of the respiratory system causing various symptoms in the host. Studies have shown that asthma might complicate the clinical condition as a result of influenza infection (Adkins *et al.*, 2004). The previous experiments (CHAPTER 4) have shown that mouse adapted influenza A virus can infect the airway of BALB/c mice and induce a robust airway inflammation resulting in airway hyperresponsiveness as well as influx of inflammatory cells.

V.1. THE EFFECTS OF INFLUENZA INFECTION IN ALLERGIC AIRWAY CELLULAR INFLAMMATION

The influenza virus infection concurrently with allergen challenge in allergen sensitised mice further increased the total number of leucocytes in the BAL fluid. All types of individual leukocytes (macrophages, eosinophils, lymphocytes, and neutrophils) numbers were increased. Eosinophils as a hallmark of asthma were also increased in number but the increase was not as great as the number of neutrophils which has been increased by around 30 folds compared to the non-infected group. Some studies have shown that viral associated asthma exacerbation is different from acute allergen induced asthma exacerbation by which neutrophils are more dominant while eosinophils are more prominent in the allergen induced asthma (Pizzichini *et al.*, 1998; Jarjour *et al.*, 2000; de Kluijver *et al.*, 2003).

Human and animal studies indicate that there is a synergistic effect between allergen exposure and viral infection (Teichtahl *et al.*, 1997; O'Donnell and Openshaw, 1998). In a human experimental study of rhinovirus, the concurrent administration of virus and

allergen challenge activated both eosinophil and neutrophil granulocytes (Greiff *et al.*, 2002).

In OVA sensitised, influenza infected mice challenged with saline, total leucocytes counts were also significantly greater compared to non infected animals. There are some eosinophils detected in the OVA sensitised, influenza infected, saline challenged group but no eosinophils detected in the non infected group. However, the detected eosinophil number in BAL fluid of influenza infected mice was not significantly different from any of the uninfected animals challenged with saline. This shows that in a concurrent exposure of influenza infection and antigen (OVA), OVA challenge is important to induce eosinophil influx into the airways.

Eosinophils are not the major response of non asthmatic subjects to viral infection (Barends *et al.*, 2004). Neutrophils were quite dominant in the influenza infected group of mice as the proportion was increased from being none in un-infected group to 25% of total cells in the infected group. The greatest proportion of leucocytes subtype in both groups was the macrophages which in the influenza infected groups increased by 2 folds compared to naive animals. Respiratory infection causes neutrophils and mononuclear cells influx into the airways for viral clearance (Ramshaw *et al.*, 1997).

Influenza infection has also been shown to increase the cytokines production by neutrophils and alveolar macrophages infiltration in the airways, which further leads to recruitment of additional macrophages and T lymphocytes in the pulmonary system (La Gruta *et al.*, 2007).

Lymphocytes number in influenza infected animals in both OVA and saline challenged groups was also increased compared to non infected animals in either OVA challenged or saline challenged groups. T lymphocytes have been known to be responsible for viral clearance by releasing IFN-γ and TNF-α, and the killing action of CD8+ cells

(Graham *et al.*, 1994; Ramshaw *et al.*, 1997). So their numbers are significantly increased during infection.

Influenza A infection targets epithelial cells which release chemokines and cytokines including TNF-α, IL-1, MCP-1, RANTES, MIP-1, IP-10, and IL-8 upon virus induced lysis (Julkunen *et al.*, 2001). The released chemokines enhance the recruitment of macrophages, neutrophils, and T lymphocytes to the site of infection (Ada and Jones, 1986). This could also underlie the enhanced cellular inflammation when influenza and OVA challenge were given in combination.

A recent study by Le Goffic *et al*. (2011) has shown that influenza A infection leads to the increase of IL-33 expression (Le Goffic *et al.*, 2011) as has been formerly discussed in CHAPTER 4. This cytokine could be proposed as one of the reasons to explain the augmentation of airway cellular inflammation when OVA challenge was given in the acute course of influenza infection in OVA sensitised mice. IL-33 is also a chemoattractant of Th2 cells to lymph nodes and site of inflammation. In the IL-33 environment due to influenza infection, the Th2 cells will be recruited to the airways (Komai-Koma *et al.*, 2007). In addition, dendritic cells maturation and their expression of major histocompatibility complex-II are also influenced by IL-33. The mature dendritic cells when introduced to naive CD4+ T cells induce the T cells production of IL-5 and IL-13 which both are key cytokines in asthma (Rank *et al.*, 2009). Mast cells are also responsive to IL-33 as they bear ST2, the IL-33 receptor (Neill *et al.*, 2010). The interaction of IL-33 and mast cells induce the expression of cytokines, chemokines, and inflammatory mediators including IL-8, IL-5, IL-13, IL-6, IL-1β, TNF-α, GM-CSF, and prostaglandin D2 (Allakhverdi *et al.*, 2007; Iikura *et al.*, 2007; Moulin *et al.*, 2007). Eosinophils respond to IL-33 by increasing the production of IL-8, IL-6, and eosinophil derived neurotoxin (Chow *et al.*, 2010). The interplay between IL-33, IL-5, IL-8 and other secreted chemokines would attract circulating eosinophils and neutrophils into the airways where the newly recruited cells are induced to release their

own inflammatory mediators. The cycles will have the end result of exaggerated airway inflammation.

V.2. THE EFFECTS OF INFLUENZA INFECTION ON ALLERGIC AIRWAY EARLY AND LATE PHASE BRONCHOCONSTRICTION

The infection of influenza virus concurrently with allergen challenge in mice significantly aggravated the late asthmatic reactions compared to those in mice only sensitised and challenged with OVA. The late phase reaction was magnified and prolonged. The bronchoconstrictions of non-infected mice returned to normal 19 hours after the second OVA challenge but those of influenza virus infected allergic mice were still sustained. The allergen challenge is important in the development and peak enhancement of early and late phase reaction, as OVA sensitised mice which were infected with influenza virus but only challenged with saline did not show any increase of brochoconstriction that could be regarded as early and late phase asthmatic reactions.

The potentiated late phase asthmatic reactions have also been observed in guinea pigs infected with human parainfluenza virus type 3 during OVA challenge. The early and late phase was so magnified because of the viral infection; that both phases were unified, and the bronchoconstrictions were elongated and persisted for up to 12 hours (Ford *et al.*, 2013). In humans, there is evidence of an association between acute rhinovirus infection and increased level of histamine content in the BAL fluid immediately after allergen challenge (early phase reactions) which is sustained for 48 hours after the challenge (late phase reactions). The histamine leakage was not seen in non allergic subjects (Calhoun *et al.*, 1994).

It has been suggested that early phase and late phase asthmatic reactions are driven by IgE and mast cells in atopic asthma (Fahy *et al.*, 1997). IgE production is increased in some murine models of asthma (Renz *et al.*, 1992; Hessel *et al.*, 1995; Fernandez-Rodriguez *et al.*, 2008). A human experiment shows that IgE in the BAL fluid was

significantly increased after allergen re-exposure or challenge (Wilson *et al.*, 2002). On re-exposure to allergen of sensitised animal the allergen will cross link with allergen specific IgE bound on high affinity IgE receptors on mast cells and initiates the signalling cascade which causes mast cells degranulation and release of pro-inflammatory mediators including histamine, leukotrienes, and prostaglandin, and cytokines leading to early phase reactions (Edwards *et al.*, 2009). Mast cell degranulation and the subsequent release of histamine, tryptase and IFN-γ are enhanced in acute influenza A infection (Hu *et al.*, 2012) which might contribute to the increase of early (although not statistically significant) and late phase bronchoconstriction.

The late phase reactions are the later outcome of the pro-inflammatory mediators release by degranulated mast cells. The mediators promote the recruitment of inflammatory cells particularly eosinophils, but also neutrophils, T lymphocytes, macrophages, and basophils (Bradding and Holgate, 1999). As noted all individual subtype of leukocytes in OVA sensitised/ influenza infected/OVA challenged group, were increased in number compared to non-influenza infected groups. The exaggerated accumulation of inflammatory cells in the lungs when OVA challenge was administered in the acute course of influenza infection might also play a role in the profound increase of late phase bronchoconstriction. The inflammatory eosinophil has been suggested to play a role in promoting features of allergic inflammation such as late phase asthmatic reactions and airway hyperresponsiveness (Busse and Lemanske, 2001). The significantly increased number of macrophages and neutrophils as the effector cells against viral infections leads to the increase production of prostaglandin E2, thromboxane B2, platelet activating factor, and reactive oxygen species, from macrophages (Folkerts *et al.*, 1998) as well as neutrophil elastase and protease (Liu *et al.*, 1999) which might contribute to the enhancement of bronchoconstriction.

V.3. THE EFFECTS OF INFLUENZA INFECTION ON ALLERGIC AIRWAY HYPERRESPONSIVENESS

Airway hyperresponsiveness (AHR) is one of the asthma characteristics and has been robustly simulated in the developed murine model of allergic inflammation described in CHAPTER 5. This study has shown that influenza infection superimposed on pre-existing allergic inflammation aggravated the airway hyperresponsiveness in the mouse model. The OVA sensitised, influenza infected and saline challenged animals also exhibited greater airway hyperresponsiveness compared to non-infected mice.

Viral respiratory infection targets epithelial cells which will be exfoliated and damaged following an infection. The damaged epithelial cells loose the barrier functions which would expose and increase the stimulation of sub-epithelial neural systems to allergen or pro-inflammatory cytokines. The stimulation might lead to the release of neuropeptides which will activate the parasymphatic nerve ending. The activation causes a release of acetylcholine which stimulates the M3 receptor to cause airway smooth muscle contraction. This stimulation is normally inhibited by the feedback activation of M2 receptors, but respiratory viruses are known to decrease the functions and expression of M2 receptors and therefore aggravating the bronchoconstriction (Folkerts *et al.*, 1998). This has been described in detail in CHAPTER 4.

In asthma, the development of AHR is thought to be promoted by Th2 networks, particularly IL-13. In a recent animal study, Chang *et al*. (2011) reveal that the pathogenesis of AHR in influenza A infection might differ from OVA induced airway allergic inflammation(Chang *et al.*, 2011). AHR in influenza A infection is induced independently of Th2 cells. The influenza virus triggers the AHR through a pathway requiring IL-33-NH cells- IL-13 axis as discussed in CHAPTER 4. Hence, in this model, when influenza infection is combined with allergic airway inflammation, the source of IL-13 could be from both Th2 cells and NH cells.

Two studies have examined the role of influenza infection and allergen challenge in OVA sensitised mice with quite different results (Wohlleben *et al.*, 2003; Marsland *et al.*, 2004). Wohlleben *et al*. (2003) reported that influenza (H2N3) infection 1 week before OVA challenge reduced the airway eosinophilia and the recruitment of Th2 cells into the lungs compared to non infected mice. The reduction of the eosinophilia and Th2 recruitment were associated with the increase of IFN-γ. However, AHR was still significantly more exacerbated when influenza infection was given a week before challenge (Wohlleben *et al*., 2003).

The results of this current study are more in line with Marsland *et al*'s report (2004). They developed a murine model of viral induced allergic asthma exacerbation in the previously sensitised host. The influenza A (H2N3) virus was infected before allergen challenge in OVA sensitised mice. The allergen challenge in the acute phase of infection resulted in enhancement of airway hyperresponsiveness, increase in eosinophils and OVA specific Th2 cells in the lung. The study also revealed that during acute influenza virus infection allergen (OVA) challenge induced a significant increase of dendritic cells maturation and migration into the draining lymph node. These dendritic cells significantly induced the proliferation of naive T cells specific for OVA, which shows that the dendritic cells were carrying and presenting the allergen (OVA) to the naive T cells. This might be responsible for the potentiated allergic airway inflammation during respiratory virus infection.

The difference in the timing of infection, OVA challenge, and the day of analysis could underlie the diverging results between the Wohlleben *et al.* (2003) and Marsland *et al.* (2004) studies. The OVA challenge and subsequent timing of analysis (day 7 and 13 respectively) at the point of maximum IFN-γ level might explain the suppression of Th2 responses including eosinophilia in the Wohlleben *et al.* (2003) study. In contrast, Marsland *et al.* (2004) reported that the highest inflammatory cell inflammation, including eosinophilia, which demonstrated the synergism between influenza infection

and OVA challenge occurred when OVA challenge was given at the same day of infection and analysed on day 4 after infection when the level of IFN-γ of had not risen to maximum.

The highlight of both studies with regard to the current study is that the timing of viral and allergen exposure is fundamental in defining the results, whether the Th1 driven immune responses would increase or suppress the Th2 induced immune responses and vice versa.

VI. CONCLUSION

In conclusion, when OVA challenge was administered during acute influenza A infection in OVA sensitised mice, it was able to significantly exacerbate/aggravate allergic airway inflammation. Giving mouse adapted influenza A virus (H1N1/PR8) in the established murine ovalbumin model of asthma demonstrated a prominent increased and prolonged late phase asthmatic response, enhanced airway hyperresponsiveness, and significantly increased inflammatory cells influx into the airways with marked eosinophilia as well as neutrophilia, and protein leaking into bronchoalveolar fluid. This would serve as a useful model of how virus infectionsworsens or exacerbates asthma. This developed model will be used in subsequent study to evaluate the role of anti inflammatory (corticosteroid) treatment when viral infection occurs concurrently with allergen challenge in airway allergic inflammation.

CHAPTER VII

THE EFFECTS OF CORTICOSTEROIDS IN MURINE MODEL OF ALLERGIC AIRWAYS INFLAMMATION

I. INTRODUCTION

I.1. INFLAMMATION IN ASTHMA

Asthma is a complex disease which is characterised by a fundamental pattern of airway inflammation. The airway inflammation is caused by a complex cascade involving a variety of inflammatory cell types includes mast cell degranulation, airway infiltration by eosinophils and activated lymphocytes (especially Th2 cells), and damage of the airway epithelium. This particular type of airway inflammation has been thought to lead to specific clinical features of asthma including reversible airway obstruction and airway hyperresponsiveness (Busse and Lemanske, 2001).

It is believed that the airway epithelium and vascular endothelium play a key role in the inflammatory cells recruitment by acting as a main source of inflammatory mediators in asthma. These include cytokines, chemokines, inflammatory enzymes, and growth factors (Barnes *et al.*, 1998). The increase of inflammatory mediator production in asthma is a result of increasing gene transcription which is mostly regulated by nuclear factor k-B (NF-kB) and activator protein 1 (AP-1) (Barnes and Adcock, 2003).

Corticosteroids, either inhaled or systemically delivered are the most effective therapy recommended by most if not all asthma guidelines as described in detail in CHAPTER 1, Corticosteroids have proven to be effective in the control of asthma symptoms hence improve the lung function, reduce the severity and frequency of exacerbations, as well as preventing airway remodelling which in turn improves the quality of life of the asthmatic patients (Barnes, 1998). Corticosteroids work by interfering the inflammatory gene transcription to suppress inflammation as detailed in CHAPTER 1.

I.2. MECHANISM OF CORTICOSTEROIDS IN ASTHMA CONTROL

Corticosteroids are very effective in controlling asthma because they suppress so many of the cascades involved in the inflammatory responses of asthma. The activation and recruitment of inflammatory cells, including eosinophils, T lymphocytes, dendritic cells, and macrophages are also reduced by corticosteroids. Mast cells activation is not inhibited by corticosteroid, but they reduce mast cell survival in the airways (Barnes and Adcock, 2003).

One of the remarkable actions of corticosteroids is to reduce eosinophilia presumably by suppressing the production of certain cytokines such as IL-5 and GM-CSF which are responsible for the survival of eosinophils and therefore leads to their apoptosis (Owens *et al.*, 1991).

IL-5 is produced by T-cells, mast cells, basophils, and eosinophils. Apart from being responsible for eosinophil survival, IL-5 also regulates eosinophil generation, proliferation, activation, and differentiation. Gene transcription of the central mediator in the regulation of the eosinophil in inflammation, IL-5, is suppressed by corticosteroid (Robinson *et al.*, 1993).

Corticosteroids also have been known to inhibit gene expression of IL-4 and IL-13, both responsible for the allergic reaction caused by the B cells production of IgE (Zhou *et al.*, 1997). IL-4 and IL-13 which are produced by Th2 and mast cells are thought to also increase the expression of adhesion molecule vascular cell adhesion molecule-1 (VCAM-1) which in turn attracts the inflammatory cells including eosinophils, lymphocytes, and basophils to endothelial cells (Bochner *et al.*, 1995). Apart from IL-4 and IL-13, increasing production of adhesion molecules including ICAM-1 is also regulated by IL-1 which is produced by macrophages. Corticosteroids inhibit the production of IL-1 (Yu *et al.*, 1998).

The major target of inhaled corticosteroid is probably the epithelial cell. These cells along with endothelial cells, smooth muscle cells and fibroblasts are responsible for releasing inflammatory mediators including chemokines or chemotactic cytokines which regulate the recruitment or attraction of inflammatory cell into the airways (Barnes, 1998). Chemokines particularly RANTES is an important chemoattractant for eosinophils and lymphocytes. Macropage inflammatory protein-1 (MIP-1 α) and macrophage/monocyte chemoattractant protein-3 (MCP-3) as suggested by the name are chemokines produced by macrophages. Corticosteroids inhibit the production of those mentioned chemokines (Stellato *et al.*, 1995). Macrophages and T lymphocytes produce an important cytokines in allergic inflammation, TNF-α, the production of which is downregulated by corticosteroids (Ying *et al.*, 1991). The cellular effects of corticosteroid in asthma is displayed in figure 7.1.

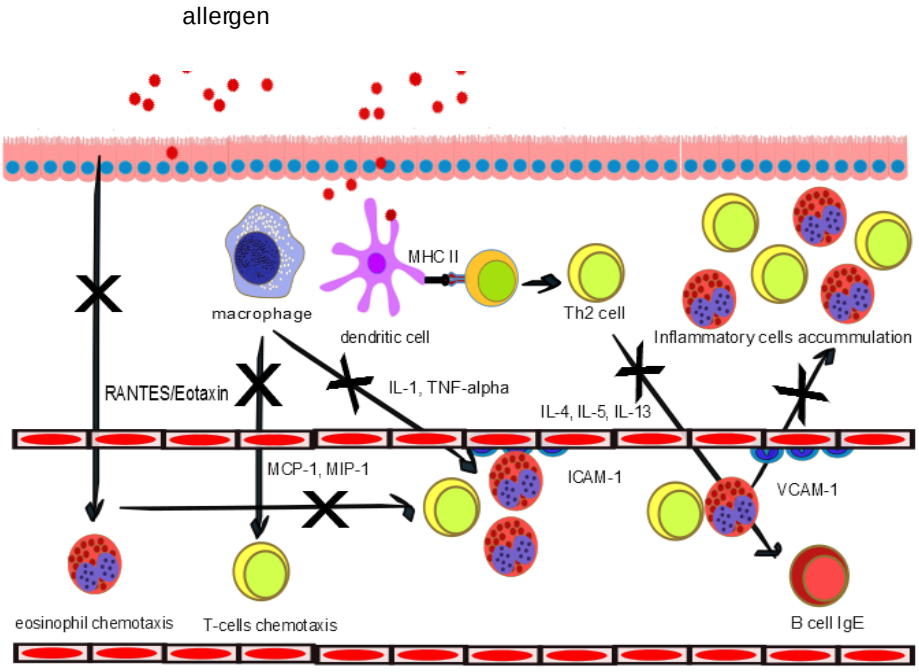


Figure 7.1. Epithelial cels produces cytokines and chemokines upon exposure to allergen. RANTES, eotaxin, MCP-1 and MIP-1 are responsible for the migration of leucocytes particularly eosinophil, T cells and macrophages. Activated macrophages also produce IL-1 and TNF-α which induce endothelial cells to upregulate ICAM-1 and VCAM-1 (adhesion molecules). T-cells also produce IL-4, IL-5, and IL-13 which upregulate the adhesion molecules and eotaxin. **X** = blocked by corticosteroids (Umland *et al.*, 2002).

The effectiveness of corticosteroids in reducing airway inflammation may lead to their ability to reduce airway hyperresponsiveness (AHR). Even though it is not firmly confirmed, there are some evidences which show that eosinophils may contribute to the development of airway hyperresponsiveness in asthma. Eosinophil infiltration into the airways leads to the release of the toxic eosinophil cationic proteins (ECP) and major basic proteins which cause dysfunction of M2 muscarinic receptors in the airways. M2 muscarinic receptors are responsible for countering the bronchoconstriction by M3 muscarinic receptor activation through a feedback mechanism. M2 receptor dysfunction is one of the mechanisms involving in the development of airway hyprerresponsiveness. Therefore, corticosteroids by inhibiting eosinophil function may reduce the airway hyperresponsiveness in asthma. Longterm treatment with corticosteroid has been proven to reduce airway responsiveness toward histamine or cholinergic agonist provocation as well as reduce the late asthmatic responses (Barnes, 1998).

II. AIMS

We have developed a murine ovalbumin model of airway allergic inflammation that shows inflammatory cell influx into the airways, AHR, as well as early and late asthmatic responses. So, it is important to determine whether corticosteroids would inhibit the allergic airway inflammatory response in the airway of allergic mouse model. Inhalation (Fluticasone Propionate) and systemically (Dexamethasone) delivered corticosteroids were evaluated in this study.

III. METHODS

The details of the methods used in the studies are described in CHAPTER 2

III.1. ANIMAL

Six Male BALB/c mice (20-25g) per group were used for all studies

III.1.1. SENSITISATION

Mice were sensitised on day 0 and 5 by intraperitoneal injections (0.25 ml) of the mixture of OVA (50 µg/mouse) and $Al(OH)_3$ (50 mg/mouse) in saline.

III.1.2. OVALBUMIN CHALLENGES

On day 17, mice were exposed to aerosolised OVA (0.5%) or saline (in a control group) inhalation challenges in a perspex box for 2 x 1 hour, with a 4 hour gap between exposures.

III.1.3. PBS INSTILLATION

Mice were anaesthetised lightly with isoflurane and then intranasally instilled with 50 µl PBS per mouse on day 13 (fig.7.2). Each mouse was given 12.5 µL of PBS in one nostril and then 12.5 µL was given to the other nostril twice to give 50 µL PBS in total. The animal was held in upright position for 2 minutes then was put back in the transfer cage. Mice were then returned to their cage once the instillation was finished.

III.1.4. CORTICOSTEROIDS INTERVENTION

Dexamethasone (DEX):

Water soluble dexamethasones, 3 or 6 mg/kg in 200 µl of PBS was administered by intraperitoneal injection once a day for 6 day starting from day 12 of the procedure. On day 17, when mice were challenged with OVA, DEX was given before the first OVA

challenge. Control mice received a 200 µl intraperitoneal injection of PBS. Details of the procedures are displayed in figure 7.2.

Fluticasone Propionate (FP):

FP 0.25 or 0.5 mg/ml was dissolved in a mixture of saline (40%), DMSO (30%) and ethanol (30%). FP inhalation for 20 minutes twice a day 6 hour apart was administered for 6 days starting from day 12 of the procedure. On the day of OVA challenge (day 17), FP was given before the first OVA challenge and 6 hours after the second OVA challenge. Control mice were given inhalation of vehicle (saline (40%), DMSO (30%) and ethanol (30%)) at the respective times. Details of the protocol are described in figure 7.2

III.1.5. EARLY PHASE AND LATE PHASE DETERMINATION

Measurement of the lung function of unrestrained mice was recorded as Penh using a Buxco system. Values of Penh after allergen challenge were recorded at 0, 20, 40, 60, 90 and 120 minutes, then hourly until 10 hours followed by a final reading at 19 hours after the second ovalbumin challenge.

III.1.6. AIRWAY RESPONSIVENESS MEASUREMENTS

Airway responsiveness in mice was measured after methacholine (acetyl-β-methylcholine chloride) provocation. Respiratory activity was recorded for 5 min, to establish baseline value for Penh. Mice were subsequently exposed to the increasing doses of aerosolized methacholine dissolved in saline (1, 3, 10, 30, 100 mg/ml). The procedures were started with preselected flow rate at 1.0 min; with 1.5 minutes of intermittent methacholine exposures (at 25% duty: 1.5 second exposure followed by 4.5 seconds without exposures on a continuous cycle), and 2 min dryer time. Plethysmographic data were recorded for another 5 minutes after aerosol administration. The Penh values measured during each 5 minutes sequence were averaged and are expressed for each methacholine concentration as absolute Penh

values. Airway responsiveness measurements were performed on day 11 and 19 hours after the final ovalbumin exposure.

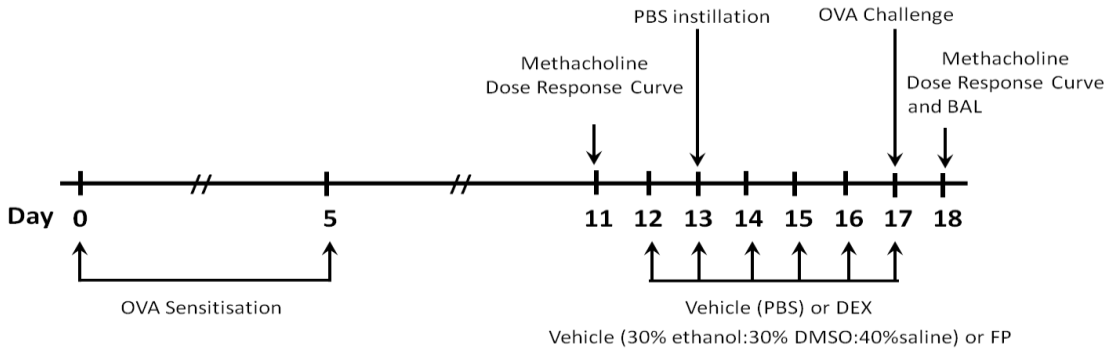


Figure 7.2. The protocol of dexamethasone (DEX) and fluticasone propionate (FP) intervention in OVA model of allergic airway inflammation. DEX or PBS was given intraperitoneally once a day while FP or vehicle FP (30% ethanol: 30% DMSO: 40% saline) were given by aerosol inhalation twice a day 6 hours apart for 6 days starting from day 12

III.2. TOTAL AND DIFFERENTIAL CELL COUNTS

After the final airway responsiveness measurements, the mice were administered a lethal dose of sodium pentobarbitone, the lungs were then removed and lavaged. Using the BAL fluid, recovered total and differential cell counts were then carried out using the methods described in CHAPTER 2.

III.3. BAL FLUID PROTEIN CONTENT

Total Protein content of BAL fluid was analysed with BCA protein assay as described in CHAPTER 2.

III.4. LUNG HISTOLOGY

Lung was stained with H and E and was analysed with histopathology scoring as described in CHAPTER 2.

IV. RESULTS

IV.1. DEXAMETHASONE (DEX)

IV.1.1. THE EFFECTS OF DEX ON EARLY AND LATE PHASE REACTIONS

In PBS treated mice, challenge with ovalbumin , early asthmatic bronchoconstrictions (EAR) were observed 2 hours after the second OVA challenge (40.3 ±12.3% increase from baseline Penh). Both doses of DEX did not affect this EAR. Mice treated with 3 or 6 mg/kg DEX also displayed the EAR at 1 hour after OVA challenge (45.7±9.7% and 33.9±7.6% respectively). Corticosteroid did not affect the EAR. Penh values in mice in PBS treated group were then gradually restored towards baseline and then increased again peaking at hour 7 (63.6±5.9%). This is regarded as late asthmatic bronchoconstriction (reactions) (LAR). Treatment with DEX 6 mg/kg significantly attenuated the LAR (22.7±6.5%), but 3 mg/kg DEX did not. Penh values in all groups returned to baseline 24 hours after the first OVA challenge or 19 hours after the second OVA challenge. The EAR and LAR of DEX and vehicle treated mice were displayed in figure 7.3.

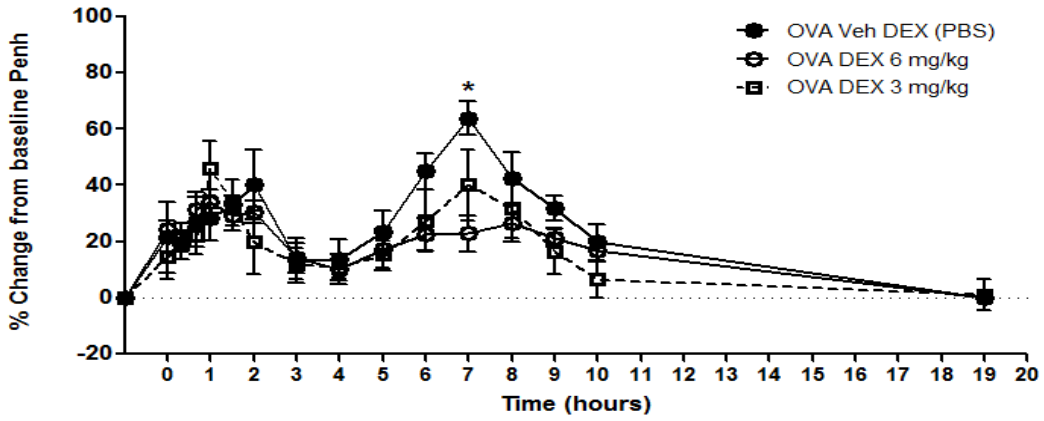


Figure 7.3. Mean time-course values of Penh in OVA sensitised and challenged mice and treated with PBS or DEX (3 or 6 mg/kg) for 6 days. Early and late phase asthmatic bronchoconstriction were observed around 0-3 hours and 3-10 hours respectively after challenge in mice which treated with PBS or DEX 3 mg/kg. Results are expressed as mean±S.E.M. percentage of Penh change from baseline. Data were analysed with unpaired two tailed t-test (* P< 0.05; n=6).

IV.1.2. THE EFFECTS OF DEX ON AIRWAY RESPONSIVENESS TO METHACHOLINE

Figure 7.4 displayed the airway responsiveness of OVA sensitised and OVA challenged mice which were treated with DEX 3 and 6 mg/kg or PBS. The PBS treated mice show a significant increase in Penh values 24 hours after OVA challenge when provoked with methacholine compared to those before OVA challenge. DEX 6 mg/kg attenuated the airway hyperresponsiveness but not DEX 3 mg/kg.

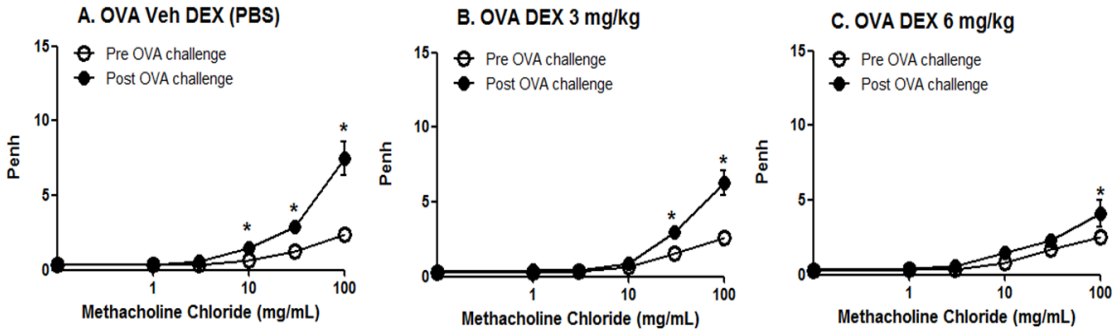


Figure 7.4. Mice were sensitised and challenged with OVA and treated with DEX (6 mg/kg or 3 mg/kg) or vehicle (PBS) for 6 days. A day before administration of DEX or vehicle, and 24 h after the challenges, baseline lung function and responses to increasing doses of methacholine was measured by whole body plethysmography**.** Results are expressed as mean ± SEM of absolute Penh. Data were analysed with unpaired two tailed t-test (* P< 0.05; n=6).

IV.1.3. THE EFFECTS OF DEX ON TOTAL AND DIFFERENTIAL CELL COUNTS

OVA sensitisation and challenge increased the total inflammatory cell numbers compared to either naive or saline challenged animals. There was a significant reduction in total inflammatory cells count in the bronchoalveolar lavage fluid (BALF) in mice treated with 3 and 6 mg/kg of DEX compared to PBS treated mice ($14.2\pm2.6.10^5$ cells/ml to $7.3\pm1.4.10^5$ cells/ml and $4.2\pm0.8.10^5$ cells/ml respectively). A significant reduction of eosinophil number was also observed in DEX treated mice compared to PBS group ($7.6\pm1.7.10^5$ cells/ml to $1.1\pm0.8.10^5$ cells/ml and $0.2\pm0.1.10^5$ cells/ml respectively) as shown in figure 7.5. No eosinophils or neutrophils detected in naive or saline challenged animals.

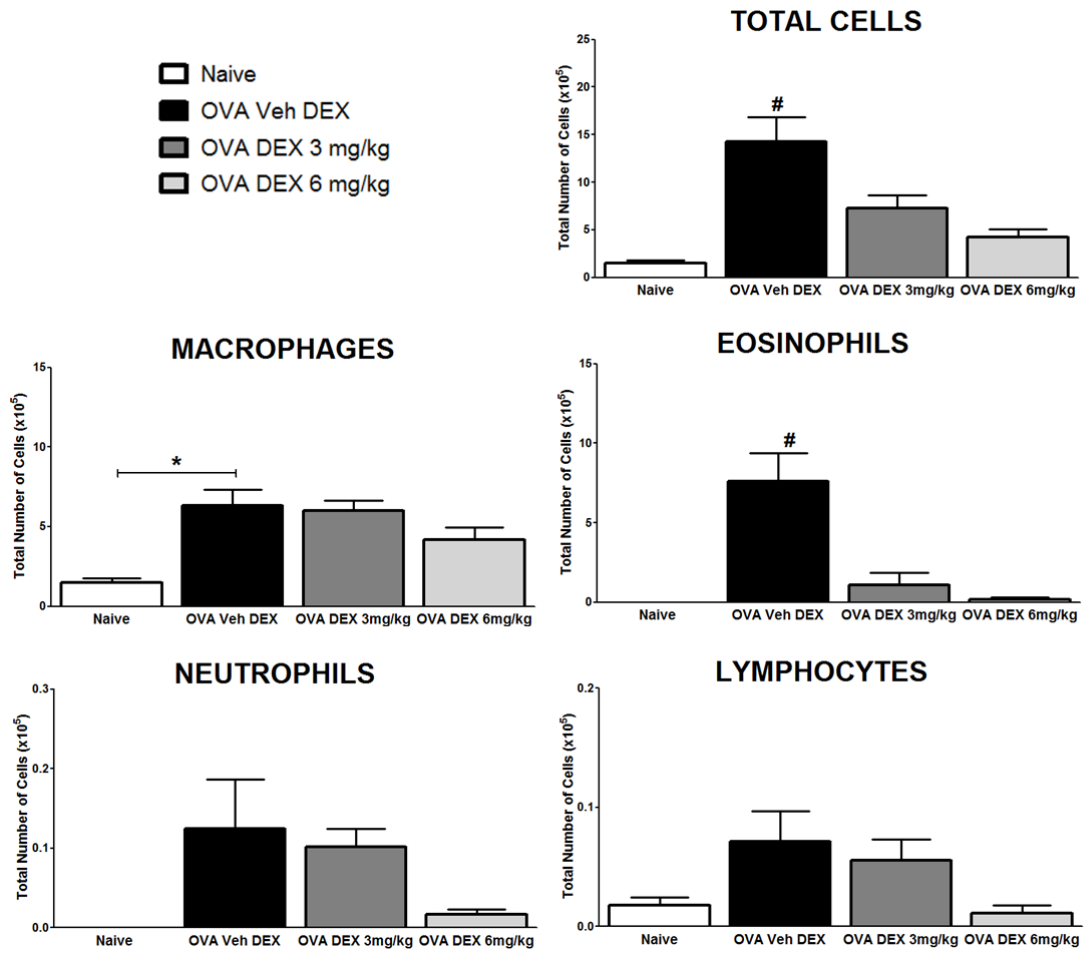


Figure 7.5. The number of total cells, macrophages, eosinophils, lymphocytes and neutrophils found in the bronchoalveolar lavage fluid of naive mice and OVA challenged treated with PBS or DEX (3 mg/kg or 6 mg/kg) mice. Results are expressed as mean±S.E.M. Data were analysed with One-way Analysis of Variance followed by a Bonferroni post-test $^{\#}$significantly different from all other groups; *connected with corresponding bar is significantly different from each other ($P<0.05$; n=6).

IV.1.4. THE EFFECTS OF DEX ON PROTEIN EXUDATION

OVA challenged and vehicle treated mice displayed significant increase in protein content in BALF compared to the level of protein content in naive mice (fig 7.6). However, the high dose of DEX (6 mg/kg) did not significantly reduce the protein concentration. Even so, the level of protein in DEX treated mice were at similar level compared to saline challenged mice (0.19±0.07 and 0.18±0.05 respectively).

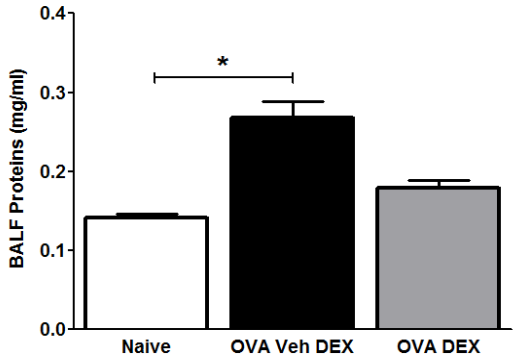


Figure 7.6. Total protein content in bronchoalveolar lavage fluid of naïve mice and mice sensitised and challenged with OVA and treated with DEX (3 or 6 mg/kg) or vehicle (PBS) for 7 days. Results are expressed as mean±S.E.M. Data were analysed with One-way Analysis of Variance followed by a Bonferroni post-test (*P<0.05; n=6).

IV.1.5. THE EFFECTS OF DEX ON LUNG HISTOLOGY

Marked peribronchial and perivascular inflammation as well as folding and elongation of epithelial cells were observed in OVA challenged/vehicle (PBS) treated mice compared to naive mice (fig 7.7.B). The mean pathological score of vehicle treated mice was also significantly increased compared to naive mice (fig 7.8). Treatment with DEX (6 mg/ml) attenuated the peribronchial and perivascular inflammation (fig 7.7.C). Although there is a trend of mean pathological score reduction in DEX (6 mg/kg) treated group, the reduction was not statistically significant compared to the vehicle treated mice.

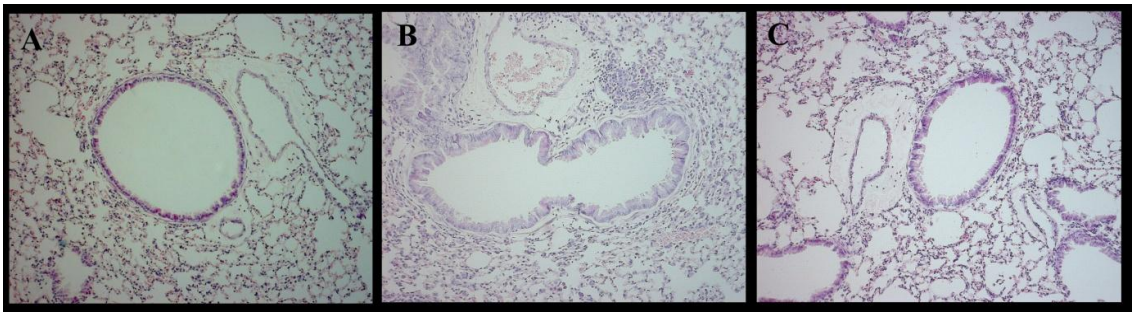


Figure 7.7. Histopathological changes of lungs of naive mice (A) or OVA sensitised and challenged mice which were treated with vehicle DEX (PBS) (B) or DEX (6 mg/kg)(C). The lungs were stained with haematoxylin and eosin to display general morphology (100x magnification).

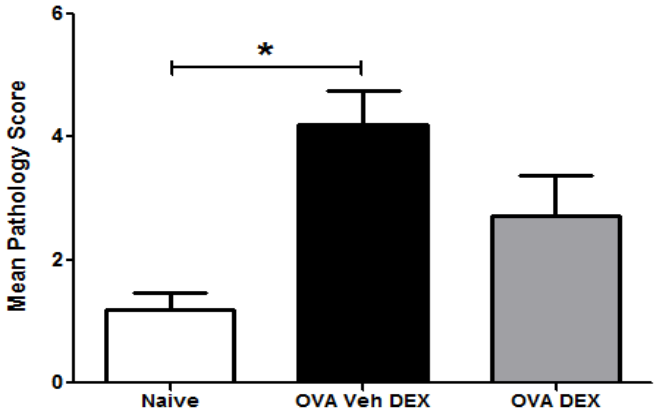


Figure 7.8. The mean lung pathology score of naïve, OVA sensitised and challenged mice which were treated with PBS (Vehicle DEX) or DEX 6 mg/kg. Results are expressed as mean±S.E.M. Data were analysed using one-way ANOVA, followed by post hoc Bonferroni's test to determine the mean pathology score (*P < 0.05; n=6).

IV.2. FLUTICASONE PROPIONATE (FP)

IV.2.1. THE EFFECTS OF FP ON EARLY AND LATE PHASE REACTIONS

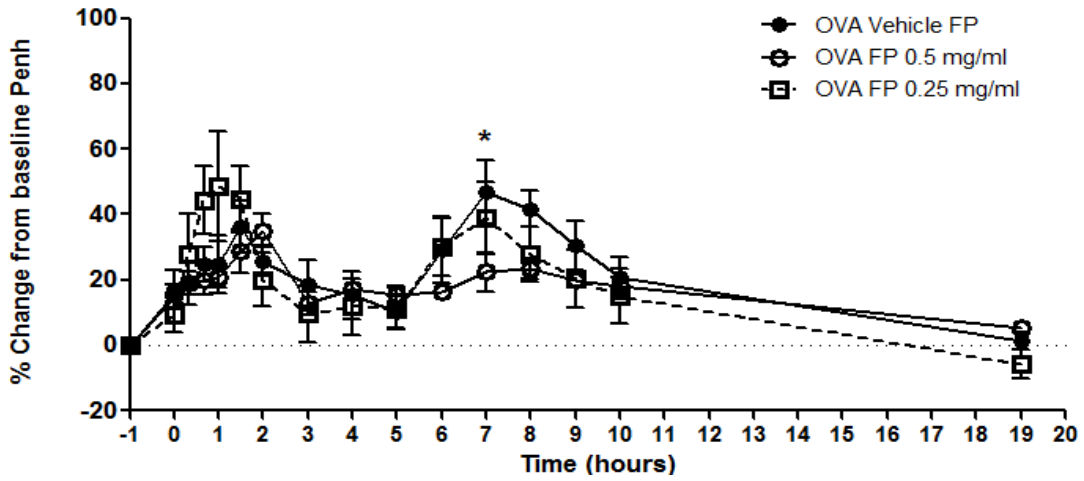


Figure 7.9. Mean time-course values of Penh in OVA sensitised and challenged mice that were treated with FP (0.25 or 0.5 mg/ml) or vehicle (saline/DMSO/ethanol (40%/30%/30)) for 6 days. Mean changes in Penh are expressed as mean±S.E.M. percentage change from baseline. Data were analysed with unpaired two tailed t-test (* P< 0.05; n=6).

The vehicle treated mice displayed a significant increase of Penh at 7 hour after OVA challenge which was regarded as the LAR (46.8 ±9.6% increase from baseline Penh). FP treatment did not significantly change EAR compared to vehicle

(saline/DMSO/ethanol (40%/30%/30%)). Treatment with 0.25 mg/ml FP did not attenuated the LAR (38.9 ±10.9% increase from baseline Penh). Therefore the higher dose (0.5 mg/ml) was delivered which was significantly reduced the LAR (22.3 ±6.0% increase from baseline Penh) (fig 7.9).

IV.2.2. THE EFFECTS OF FP ON AIRWAY RESPONSIVENESS TO METHACHOLINE

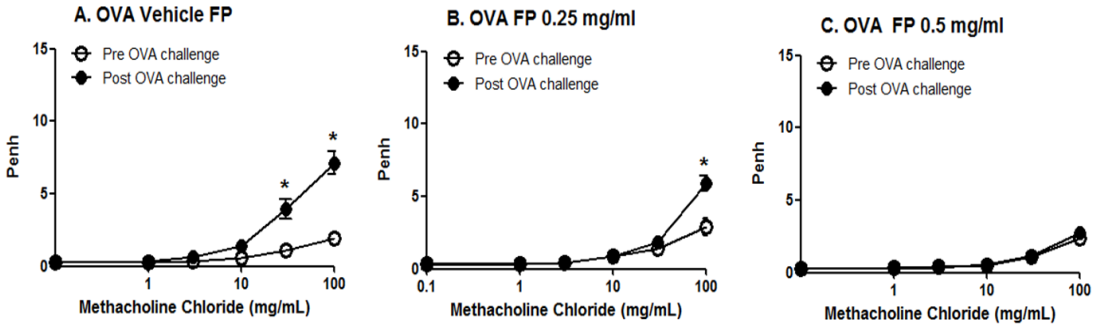


Figure 7.10. Mice were sensitised and challenged with OVA and treated with FP (0.5 mg/ml or 0.25 mg/ml) or vehicle (saline/DMSO/ethanol (40%/30%/30)) for 6 days. A day before administration of FP or vehicle, and 24 h after the challenges, baseline lung function and AHR to increasing doses of methacholine was measured by whole body plethysmography**.** Results are expressed as mean ± SEM of absolute Penh. Data were analysed with unpaired two tailed t-test (* P< 0.05; n=6).

Mice sensitised and challenged with OVA and treated with vehicle (saline/DMSO/ethanol (40%/30%/30)) showed a significant increase in airway responsiveness when provoked with methacholine (maximum response from absolute Penh 1.9±0.3 before OVA challenge to absolute Penh 7.1±0.8 after vehicle treatment and OVA challenge). The airway function responses to the highest dose of methacholine in mice treated with the highest dose of FP (0.5 mg/ml) before OVA challenge was not significantly different from those after OVA challenge. Administration of FP 0.5 mg/ml b.i.d for 7 days completely abolished the airway hyperresponsiveness induced by OVA challenge and provoked by methacholine, but not by administration of FP 0.25 mg/ml (Fig.7.10).

IV.2.3. THE EFFECTS OF FP ON TOTAL AND DIFFERENTIAL CELL COUNTS

FP 0.5 mg/ml significantly decreased BALF total inflammatory cell numbers from 15.1±1.4x10^5cells/ml in vehicle treated mice to 4.6±0.7.10^5cells/ml. Treatment with 0.5 mg/ml FP also significantly reduced eosinophil influx in the airways from 6.6±0.6x10^5cells/ml in vehicle treated group to 0.6±0.3.10^5cells/ml. FP 0.25 mg/ml was not sufficient to significantly reduce the level of inflammatory cell accumulation. The effects of FP on total and differential cells are displayed in figure 7.11.

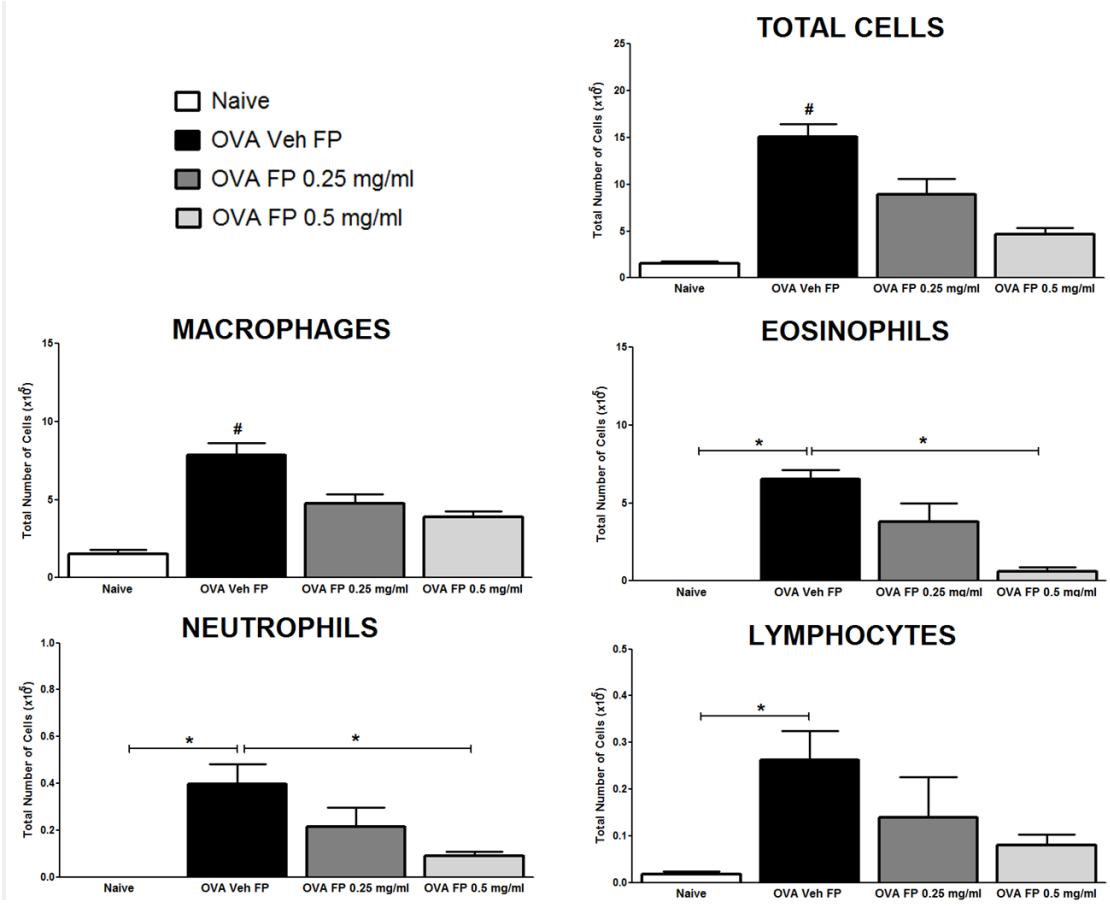


Figure 7.11. The number of total cells, macrophages, eosinophils, lymphocytes and neutrophils found in the bronchoalveolar fluid of OVA challenged mice treated with FP (0.25 and 0.5 mg/ml) or vehicle (saline/DMSO/ethanol (40%/30%/30)) for 6 days. Results are expressed as mean±S.E.M. Data were analysed with One-way Analysis of Variance followed by a Bonferroni post-test $^{\#}$significantly different from all other groups; *connected with corresponding bar is significantly different from each other (P<0.05; n=6).

IV.2.4. THE EFFECTS OF FP ON PROTEIN EXUDATION

The increase in BALF protein content after OVA sensitication and challenge in vehicle treated mice was not significant compared to naive mice. Treatment with FP 0.5 mg/ml did not significantly reduce protein content (fig.7.12).

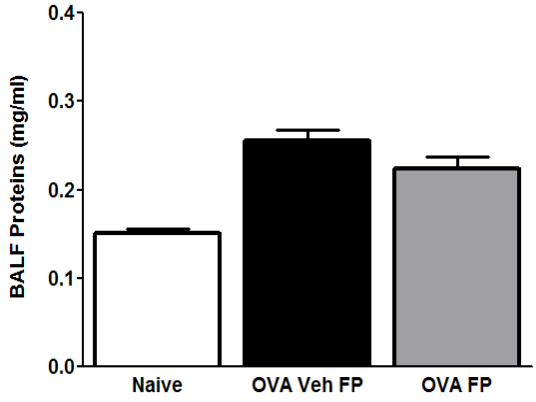


Figure 7.12. Total protein content in bronchoalveolar lavage fluid of naïve, OVA sensitised and OVA challenged mice which were treated with FP 0.5 mg/ml or vehicle (saline/DMSO/Ethanol (40%/30%/30)) for 7 days. Results are expressed as mean±S.E.M. One-way Analysis of Variance followed by a Bonferroni post-test ($P<0.05$; n=6).

IV.2.5. THE EFFECTS OF FP ON LUNG HISTOLOGY

OVA sensitisation and challenge in vehicle treated mice caused a significant peribronchial and perivascular inflammation (fig 7.13.B) compared to naive mice (fig.7.13.A). Treatment with FP 0.5 mg/ml for 7 days reduced the inflammation (fig.7.13.C). Epithelial cells were also elongated and folded in the bronchial lumen of vehicle treated mice whereas in mice treated with FP 0.5 mg/ml, epithelial cell were intact and normal in shape. The mean pathology score of OVA sensitised and challenged mice which treated with vehicle was significantly higher than naive mice. The mean pathology score of FP 0.5 mg/ml treated mice was significantly lower than that of the vehicle treated mice (fig 7.14).

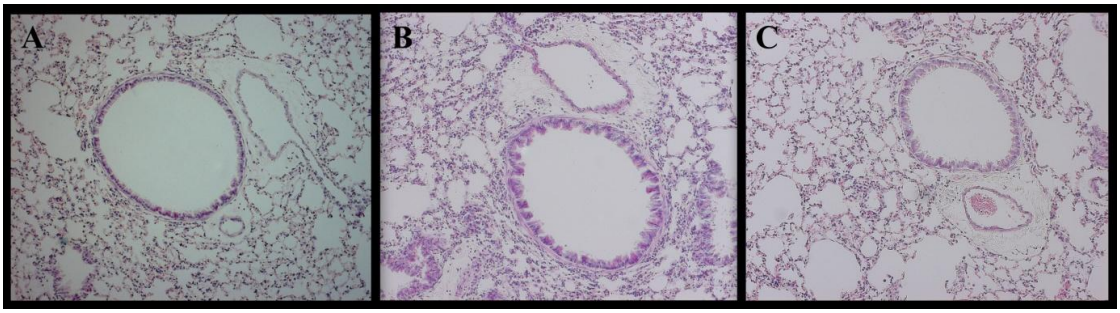


Figure 7.13. Histopathological changes of lungs of naive mice (A) or OVA sensitised and challenged mice which were treated vehicle (saline/DMSO/Ethanol (40%/30%/30)) (B) or FP (0.5 mg/ml) (C). The lungs were stained with haematoxylin and eosin to display general morphology (100x magnification).

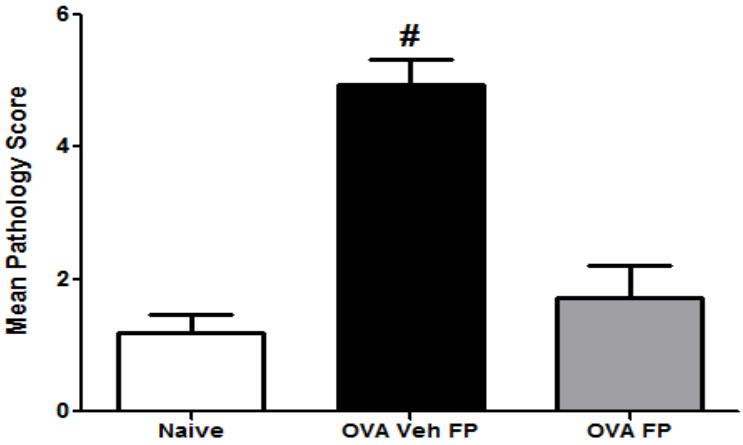


Figure 7.14. The mean lung pathology score of naïve, OVA sensitised and challenged mice which were treated with FP (0.5 mg/ml) or vehicle (saline/DMSO/Ethanol (40%/30%/30)). Results are expressed as mean±S.E.M. Data were analysed using one-way ANOVA, followed by post hoc Bonferroni's test to determine the mean pathology score. $^{\#}$Significantly different from all other groups ($P < 0.05$; n=6).

V. DISCUSSION

A murine model of airway allergic inflammation which simulates most features of human asthma including increasing airway hyperresponsiveness, infliltration of inflammatory cells into the airways which elicit Th2 skewed inflammation with eosinophilia, protein exudation and accumulation in BALF, and showing early as well as late phase asthmatic bronchoconstriction has been developed.

Most asthma guidelines recommend treatment with corticosteroid and beta agonist for asthma controller and reliever particularly for mild and moderate asthma. Inhaled corticosteroid is now the first choice for asthma management as it is convenient to use, cost effective, and has fewer side effects compared to the systemic corticosteroid (Barnes, 1998; GINA, 2012). Due to their side effects, systemic corticosteroids are now only administered for severe or difficult to control asthma (Schäcke *et al.*, 2002).

Although inhaled or systemic corticosteroids have been proven to be the most effective therapy of asthma, only a few identified studies have investigated the anti inflammatory and immunomudulation properties of corticosteroids together with their effects on functional parameters such as EAR, LAR, and AHR on animal models of airway allergic inflammation. Most studies did not measure the effect of corticosteroids in the EAR and LAR as endpoints, the studies commonly only investigating airway hyprerresponsiveness to methacholine. This reduces the value of the animal models to reflect and correlate treatment in the clinic (Stevenson and Birrell, 2011). The availability of non invasive whole body plethysmography to measure Penh as a reflection of airway function in conscious unrestrained animal has allowed the study to include the monitoring the airway function overtime to investigate the early and late phase responses to antigen challenge.

Corticosteroids, both DEX and FP, did not inhibit early asthmatic phase bronchocostriction. Early phase bronchoconstriction responses have been known to be not affected by corticosteroid. Studies in human and animal have shown this insensitivity (Evans, 2009; Ishmael, 2011). Mediators released from mast cells activation and degranulation are believed to be the main cause of the cascade which leads to early phase bronchoconstriction. Corticosteroids do not inhibit mast cells activation (Schleimer *et al.*, 1983; Liu *et al.*, 2001) hence are not effective in reducing the early phase bronchoconstriction. This is one of the reasons why corticosteroid is recommended to be coupled with β-adrenoreceptor drugs in mild and moderate persistent asthmatics (GINA Report, 2012), the bronchodilator action of the later drug effectively reducing broncoconstriction in the EAR.

The late asthmatic bronchocostriction responses in vehicle treated groups were attenuated significantly by high dose of both corticosteroids. This finding is in line with studies in human and in animal models (Parameswaran *et al.*, 2000; Evans, 2009). The nature of the inflammatory processes during late phase reactions is different from those of early phase reactions. Activation and accumulation of inflammatory cells particularly eosinophils are essential in the development of late phase reactions (Weersink *et al.*, 1994). The accumulation of eosinophils in the airways is believed to be regulated by IL-13, IL-5, and GM-CSF which are also released by mast cells. The mentioned cytokines are inhibited by corticosteroids which therefore inhibit late phase bronchoconstriction (Umland *et al.*, 2002).

Similar to previous studies in human or animals (Trifilieff *et al.*, 2000; Singer *et al.*, 2002), both corticosteroids (DEX and FP) are confirmed in this study to significantly reduce the total cell number in bronchoalveolar lavage compared to vehicle treated groups of mice in dose dependent manner. The individual numbers of eosinophils and macrophages in the lavage fluids were also reduced. Inflammatory cells including eosinophils, T lymphocytes, and dendritic cells are known to be sensitive to

corticosteroids which inhibit their migration into the airway as well as reducing their survival by inhibiting their regulators including chemokines, cytokines, and adhesion molecules (Pitzalis *et al.*, 2002).

The administration of the highest dose of both corticosteroids also significantly reduced methacholine-induced airway hyperresponsiveness. Similar findings on corticosteroids inhibiting airway hyperresponsiveness have also been reported (Gauvreau *et al.*, 2000; Inman *et al.*, 2001; Vanacker *et al.*, 2002). The exact mechanism on how corticosteroid inhibits airway hyperresponsiveness is not clear. However, there is an established relationship between airway inflammation and airway hyperresponsiveness in allergic asthma (Meurs *et al.*, 2008). Eosinophils have been thought to indirectly play a role in airway hyprresponsiveness through the production of MBP (Major Basic Proteins). MPB is toxic to the epithelial cells *in vitro* (Rothenberg, 1998). MBP may also causes dysfunction in muscarinic M2 receptors resulting in increased smooth muscle reactivity (Jacoby *et al.*, 1993).

The pathophysiology of AHR is quite complex and only partially understood. However, it is believed that there are two fundamental elements of AHR which are the variable and persistent types (Meurs *et al.*, 2008). This study only reveals variable AHR induced by acute allergen challenge as there is no noticeable structural change in the airway or airway remodelling which will cause the persistent element. Therefore the persistent element of AHR as seen in clinic is not reflected in this model.

VI. CONCLUSION

In conclusion, administration of corticosteroids (DEX and FP) inhibited airway inflammation by attenuating late asthmatic bronchocostriction, reducing the inflammatory cell accumulation in the airways, and reducing airway hyperresponsiveness in the murine model of ovalbumin induced airway inflammation. This shows that the model is sensitive to corticosteroids and closely mimics the features of human asthma. Therefore this corticosteroids intervention model would be an effective model to be further developed and used in subsequent studies of viral associated exacerbation in allergic airway inflammation.

CHAPTER VIII

EFFECTS OF CORTICOSTEROIDS ON INFLUENZA ASSOCIATED EXACERBATION OF MURINE AIRWAY ALLERGIC INFLAMMATION

I. INTRODUCTION

Classical features of allergic asthma are airway hyperreactivity and airway inflammation which are orchestrated by allergen specific Th2 cells in combination with eosinophilia as a hallmark (Robinson *et al.*, 1992). Although allergen specific Th2 cells and eosinophilia are marking the inflammation in allergic asthma, the major culprit of asthma exacerbation is actually microbial infection particularly respiratory virus infection (Traves and Proud, 2007). Respiratory viral infection is able to exacerbate asthma in almost all asthmatics. As a result, the asthma symptoms are often precipitated, becoming severe, and requiring hospitalisation. The most common cause of virus induced asthma exacerbation is rhinovirus (Message and Johnston, 2001). Influenza is also a common cause of asthma exacerbation and has been associated with significant number of morbidity and mortality particularly during influenza H1N1 pandemic in 2009 (Jain *et al.*, 2009).

Influenza on its own causes a substantial morbidity and mortality from severe pneumonia which leads to acute lung injury and acute respiratory distress syndrome (ARDS). A study showed that ARDS is the most important cause of patient death (Szretter *et al.*, 2007). One proposed mechanism for ARDS is influenza virus induced cell death in lung parenchyma and epithelial cells apoptosis (Yang *et al.*, 2011). Other important reason for the severe consequence of influenza virus infection is exaggerated immune response known as cytokine storm (Tyburski *et al.*, 2001; Li *et al.*, 2012) . In some patients the appropriate immune response will clear the virions as well as inhibit viral replication. However, the inflammation from overreacted and uncontrolled immune response may lead to an unwanted more severe disease process (Lee *et al.*, 2011).

It has been estimated that in the UK, the cost of patients with asthma exacerbation were 3.5 times higher compared to asthmatic without exacerbation (Hoskins *et al.*,

2000). Viral associated asthma exacerbations account for around 80% of asthma exacerbation cases in children and between 46% and 76% cases in adults (Johnston, 1995; Nicholson *et al.*, 1997). The exact cost of asthma exacerbation is not known. However, from the facts mentioned, the approximate figure of virus induced asthma exacerbation's cost is quite significant.

I.1. THERAPEUTIC OPTIONS

Asthma exacerbation could be life threatening and has been associated with emergency care and hospital admission, therefore any available strategies of prevention and effective treatment of virus induced asthma exacerbation is a major necessity. Several therapeutic options have the potential to prevent or to reduce the severity of viral associated asthma exacerbations. The interventions include vaccines and monoclonal antibodies for prevention, anti-inflammatory agents such as corticosteroids, and also antiviral treatments as well as interferon (Holt and Sly, 2012). In this chapter, the focus will be given to corticosteroids, the anti-inflammatory agents.

I.1.1. SYSTEMIC CORTICOSTEROIDS

Systemic corticosteroids have been known as a first choice treatment for acute exacerbation of asthma (GINA Report, 2012). Their place as a mainstay therapy for asthma have been replaced by inhaled corticosteroids (ICS) because long use of oral corticosteroid treatment can lead to serious unwanted side effects such as growth retardation, hypertension, adrenal suppression, Cushing syndrome, and osteoporosis (Covar *et al.*, 2000). Therefore, long term therapy with oral corticosteroid is not recommended unless for patients with severe disease uncontrolled with ICS and bronchodilator or patients who are not capable of using ICS (National Asthma Education and Prevention Program (2002). Short term use of oral corticosteroids is also only considered in an emergency setting requiring immediate control of asthma exacerbation (Scarfone *et al.*, 1993).

Studies have shown that the early systemic corticosteroid administration in acute exacerbation of asthma might offer some clinical advantages. In 2 randomised clinical trials, the intervention of systemic prednisolone in early acute asthma exacerbation reduced the number of hospital admission compared to placebo (Tal *et al.*, 1990; Scarfone *et al.*, 1993). Oral prednisolone given at the onset of asthma exacerbation also led to improved clinical status and respiratory functions as opposed to the deteriorating status of placebo treated group (Harris *et al.*, 1987).

Some studies showed the benefit of giving corticosteroid in respiratory viral infection associated exacerbations. The clinical conditions because of virus induced bronchiolitis also seem to be responsive to systemic corticosteroid if appropriate and sufficient dose is given (Weinberger, 2003). Oral corticosteroid has also played a role in preventing asthma exacerbation. Brunette *et al* (1988) investigated the role of oral prednisone in children with 7 times hospitalisation for acute virus infection induced exacerbations in average (Brunette *et al.*, 1988). Prednisolone treatment for one year given at the onset of viral respiratory infection has made a 90% reduction in the number of hospitalisations compared to placebo treated group. Other study by the same investigators showed that giving systemic corticosteroid in early onset of respiratory viral infection in asthmatics prevented the symptoms from progressing to severe acute exacerbation of asthma which require hospitalisation (Storr *et al.*, 1987). The studies define the role of early systemic corticosteroid intervention in acute asthma which leads to shorter hospital stay, reducing the probability of emergency hospital care, and preventing the worsening of exacerbations for patients requiring emergency care. At present, the asthma treatment guidelines agree on using systemic corticosteroid along with β_2-agonists, anticholinergic drugs, and adequate oxygenation as the main intervention for acute asthma in emergency departments (GINA Report, 2012; National Asthma Education and Prevention Program, 2002; NHLBI/WHO, 2002).

I.1.2. INHALED CORTICOSTEROID

Consideration of the adverse effects of systemic corticosteroids has led to the development of inhaled corticosteroids (ICS). The inhaled administration means the direct delivery of the corticosteroid into the inflamed lungs. The direct administration has also made the lower dose and therefore less systemic adverse effects possible. Currently ICS has been regarded as first line therapy for all level of persistent asthma with the addition of bronchodilator in patient with moderate and severe diseases (National Asthma Education and Prevention Program, 2002).

Some studies have reported the role of ICS in reducing the risk of asthma exacerbation (Juniper *et al.*, 1990; Dahl *et al.*, 1993). In one study, asthmatics were treated with high, moderate, and low dose budesonide. The rate of asthma exacerbation was reduced by 50% in the group treated with high dose of budesonide. The benefit of budesonide treatment was in a dose response relationship (Tattersfield *et al.*, 1999). Another study reported that treatment with budesonide in newly diagnosed mild persistent asthmatics was able to reduce the number of exacerbations by 25% compared to placebo treated patients (Pauwels *et al.*, 2003). The OPTIMA study also showed a greater benefit of ICS in reducing the risk of asthma exacerbation by reducing the risk of annual exacerbation from 0.77 per patient to 0.29 per patient when given to ICS naive patients (O'Byrne *et al.*, 2001).

The role of ICS in exacerbation of asthma is still debated. However, a meta analysis which was conducted to compare the use of ICS to systemic corticosteroid or combination of ICS and systemic corticosteroid to combination of placebo and systemic corticosteroid found that ICS has additional benefit to systemic corticosteroid in the acute asthma exacerbation in the emergency setting (Rodrigo, 2006). The combination of ICS and bronchodilator (salbutamol) in the same setting also showed a greater level of bronchodilation compared to salbutamol alone (Rodrigo, 2005). Based on the

mentioned meta analyses, some guidelines including Global Initiative for Asthma (2012) suggested that the use of ICS in acute exacerbation of asthma could be an effective part of acute asthma exacerbation therapy. However, other guidelines such as the National Heart, Lung, and Blood Institute (NHLBI) do not list ICS as part of acute asthma exacerbation therapy (NHLBI, 2007).

I.2. THE NATURE OF INFLAMMATION IN VIRUS-INDUCED ASTHMA EXACERBATION AND THE ROLE OF CORTICOSTEROIDS

Despite some evidence that corticosteroids have benefit in exacerbation of asthma as well as being listed as part of therapy in acute exacerbation of asthma, some might argue that there is a reduced efficacy of corticosteroid in virus-induced exacerbation of asthma. In a human experimental Rhinovirus infection, treatment with prednisolone or beclomethasone before challenge as prophylactic were effective in reducing nasal obstruction, nasal mucus, and level of intranasal kinin, but the effects ceased when the treatments were stopped (Farr *et al.*, 1990). In other human experimental RV infection, the administration of inhaled budesonide in mild asthmatic during the infection was able to improve lung functions and reduced the number of eosinophil but not the total inflammatory cells in the airways (Grunberg *et al.*, 2001). In children with asthma, the administration of oral corticosteroid in an early course of acute viral respiratory infection reduced the duration and the level of severity of asthma exacerbation, but did not alleviate the cold symptoms (Brunette *et al.*, 1988).

The use of corticosteroid in the case of ARDS because of influenza infection is still in debate. There is evidence that giving corticosteroid in H1N1 induced ARDS improve the oxygenation and reduce the mortality rate (Confalonieri *et al.*, 2010; Quispe-Laime *et al.*, 2010). The administration of corticosteroid in H1N1 induced ARDS has justified reasons as the ARDS in pandemic H1N1 2009 was largely due to uncontrolled lung and systemic inflammation rather than excessive viral infection (Annane, 2011). Hence

the use of anti inflammatory corticosteroids in this case is rational. However, WHO does not recommend the use high dose corticosteroids in severely ill H1N1 infected patient considering the unwanted serious outcome that might come from opportunistic infection, enhancement of viral replication, and inhibition of viral clearance (WHO, 2009).

There are differences between allergen and virus induced exacerbation of asthma. One distinct different between the two is the effectiveness of corticosteroids, as they are less effective in the virus-induced exacerbations (Farr *et al.*, 1990; Grunberg *et al.*, 2001; FitzGerald *et al.*, 2004; Harrison *et al.*, 2004). It has been mentioned that viral infection may act synergistically with allergic airway inflammation worsening the symptoms, the outcomes and hence the severity of asthma exacerbation (Contoli *et al.*, 2005). One of the proposed mechanisms of the enhancement of the severity is that the immune system responds to viral infection by attracting neutrophils. The neutrophilia in combination with allergen induced eosinophilia increases the severity of the inflammation (Liu *et al.*, 1999). While eosinophil number is also significantly increased in asthma exacerbations, neutrophils seem to a play more prominent role in virus-induced asthma exacerbation (Pizzichini *et al.*, 1998; de Kluijver *et al.*, 2003; Wark, 2005)

One of the well known clinical benefits of corticosteroids to reduce the allergic airway inflammation appears to be through reducing the number and activity of eosinophils (Gibson *et al.*, 2000). However, corticosteroids do not seem to have effect in reducing the number of neutrophils and their survival (Cox, 1995). Therefore, some might say, corticosteroids, the potent anti-inflammatory agents do not fully ameliorate inflammation and seems to only have a partial protection in virus induced asthma exacerbation.

II. AIMS

The studies aim to determine the role of systemic and inhaled corticosteroids in the established murine model of allergic airway inflammation when challenged with the allergen (ovalbumin) during acute influenza infection. The endpoints include airway cell inflammation, early and late phase asthmatic reactions, and airway hyperresponsiveness. The studies would investigate whether the effective dose of corticosteroids in murine model of ovalbumin induced allergic airway inflammation were still able to reduce the inflammation during viral exacerbations.

III. METHODS

The methods used in the studies are described in more detail in CHAPTER 2

III.1. ANIMAL

Six Male BALB/c mice (20-25g) per group were used for all studies

III.1.1. SENSITISATION

Mice were sensitised by intraperitoneal injections (0.25 ml) of the mixture of OVA (50 µg/mouse) and $Al(OH)_3$ (50 mg/mouse) in saline or saline on day 0 and 5.

III.1.2. INFLUENZA VIRUS INFECTION

Mice were anaesthetised lightly with isoflurane and then intranasally infected with 10 pfu in 50 µl PBS per mouse on day 13 (fig.8.1). Each mouse was given 12.5 µL of virus stock in one nostril and then 12.5 µL was given to the other nostril twice to give 10 pfu/50 µL virus stock in total. The animal was held in upright position for 2 minutes then was put back in the transfer cage in prone position to facilitate the virus to spread evenly to the right and the left lungs. Control mice were treated the same and subjected to intranasal administration of sham. Sham is allantoic fluid from PBS

instilled embryonated egg treated similarly with influenza virus infected embryonated egg. All procedures were conducted in class II biosafety hood/cabinet. Mice were then returned to their cage in the mouse isolator once the infection was finished.

III.1.3. CORTICOSTEROIDS INTERVENTION

Dexamethasone (DEX):

Water soluble dexamethasone, 6 mg/kg in 200 µl of PBS was administered by intraperitoneal injection once a day for 6 days starting from day 12 of the procedure. On day 17, when mice were challenged with OVA, DEX was given before the first OVA challenge. Control mice received a 200 µl intraperitoneal injection of PBS. Details of the procedures are displayed in figure 8.1.

Fluticasone Propionate (FP):

FP 0.5 mg/ml was dissolved in a mixture of saline (40%), DMSO (30%) and ethanol (30%). FP inhalation for 20 minutes twice a day 6 hour apart was administered for 6 days starting from day 12 of the procedure. On the day of OVA challenge (day 17), FP was given before the first OVA challenge and 6 hours after the second OVA challenge. Control mice were given inhalation of vehicle (saline (40%), DMSO (30%) and ethanol (30%)) at the respective times. Details of the protocol are described in figure 8.1.

III.1.4. OVALBUMIN CHALLENGES

Mice were challenged with aerosolised OVA (0.5%) or saline (in a control group) inhalation challenges in a perspex box for 2 x 1 hour, with a 4 hour gap between exposures.

III.1.5. EARLY PHASE AND LATE PHASE DETERMINATION

The lung function of unrestrained mice was measured as Penh using a whole body plethysmography system from Buxco. Values of Penh after allergen challenge were measured at 0, 20, 40, 60, 90 and 120 minutes, then hourly until 10 hours with a final reading at 19 hours after the second ovalbumin challenge.

III.1.6. AIRWAY RESPONSIVENESS MEASUREMENTS

Airway responsiveness in mice was measured after methacholine (acetyl-β-methylcholine chloride) provocation. Respiratory activity was recorded for 5 min, to establish baseline value for Penh. Mice were subsequently exposed to the increasing doses of aerosolized methacholine dissolved in saline (1, 3, 10, 30, 100 mg/ml). The procedures were started with preselected flow rate at 1.0 min; with 1.5 minutes of intermittent methacholine exposures (at 25% duty: 1.5 second exposure followed by 4.5 seconds without exposures on a continuous cycle), and 2 min dryer time. Plethysmographic data were recorded for another 5 minutes after aerosol administration. The Penh values measured during each 5 minutes sequence were averaged and are expressed for each methacholine concentration as absolute Penh values. Airway responsiveness measurements were performed on day 11 and 19 hours after the final ovalbumin exposure.

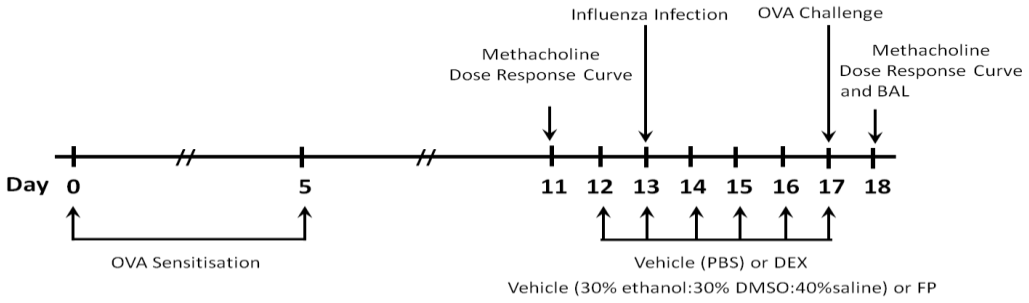


Figure 8.1. The protocol of DEX or FP interventions given in the acute course of influenza A infection in OVA model of allergic airway inflammation. DEX or PBS was given intraperitoneally once a day while FP or vehicle FP (30% ethanol: 30% DMSO: 40% saline) were given by aerosol inhalation twice a day 6 hours apart for 6 days starting from day 12

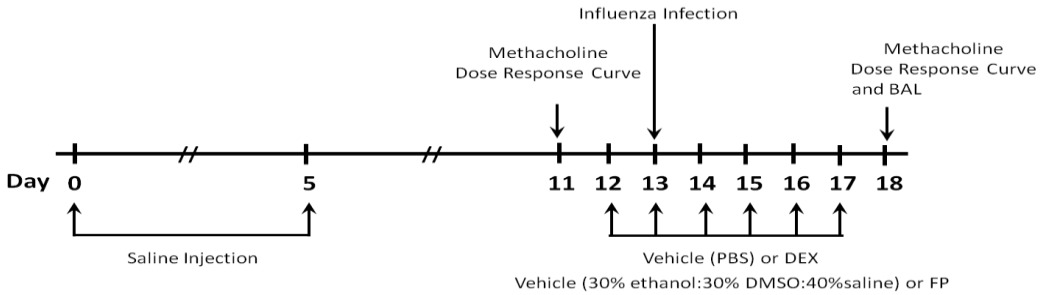


Figure 8.2. The protocol of DEX or FP interventions given in the acute course of influenza A infection. DEX or PBS was given intraperitoneally once a day while FP or vehicle FP (30% ethanol: 30% DMSO: 40% saline) were given by aerosol inhalation twice a day 6 hours apart for 6 days starting from day 12

III.2. TOTAL AND DIFFERENTIAL CELL COUNTS

After the final airway responsiveness measurements, the mice were administered a lethal dose of sodium pentobarbitone, the lungs were then removed and lavaged. Using the BAL fluid, recovered total and differential cell counts were then carried out using the methods described in CHAPTER 2.

III.3. BAL FLUID PROTEIN CONTENT

Total Protein content of BAL fluid was analysed with BCA protein assay as described in CHAPTER 2.

III.4. VIRUS TITRE DETERMINATION

The virus titre was quantitated by plaque assay with AEC immunocytochemistry as described previously in CHAPTER 4.

III.5. LUNG HISTOLOGY

Lung was stained with H and E and was analysed with histopathology scoring as described in CHAPTER 2.

IV. RESULTS

IV.1. THE EFFECTS OF SYSTEMIC CORTICOSTEROID (DEX) ON INFLUENZA ASSOCIATED EXACERBATION OF MURINE AIRWAY ALLERGIC INFLAMMATION

IV.1.1. THE EFFECTS OF DEX ON EARLY AND LATE PHASE REACTIONS

OVA challenge induced early phase reactions in both vehicle (PBS) and DEX treated group. The EAR for both vehicle treated and DEX treated groups peaked on 2 hours after the second OVA challenge (61.9 ±20.9% and 78.7 ±20.5% increase from baseline Penh respectively). Late phase responses were clearly demonstrated in vehicle treated group and peaked (194.6 ±36.01% increases from baseline Penh) on the 8^{th} hour after the second OVA challenge. The bronchoconstrictions were sustained and did not return to normal baseline 19 hours after OVA challenge.

DEX reduced the peak of the LAR (46.5 ±19.02% increase from baseline Penh) on 8 hours after OVA challenge. However, after the greatest reduction of the change from baseline Penh on 7 hours after OVA challenge, the level of bronchocostriction was gradually increased up to the last observation time point i.e. 10 hours after the second OVA challenge. Ten hours after the last OVA challenge, the change from baseline Penh of vehicle and DEX treated animals were not significantly different. Nineteen hours after the second OVA challenge Penh did not return to baseline and the increase in baseline Penh was greater (although not statistically significant) compared to those on hour 10 when the mice were last observed as depicted on figure 8.3. Change from baseline Penh at 19 hour were no significantly different between vehicle treated and DEX treated group.

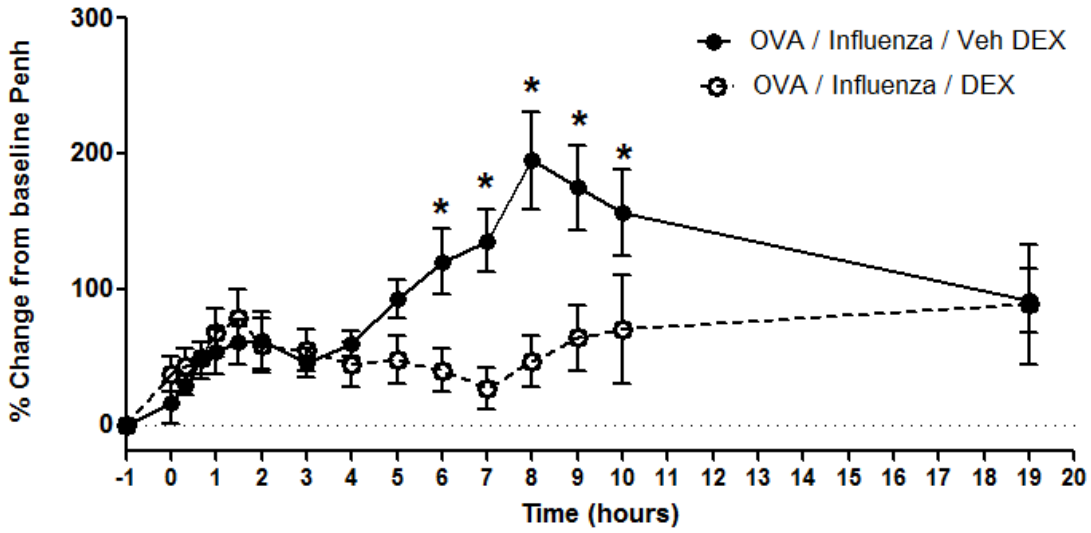


Figure 8.3. Mean time-course values of Penh in OVA sensitised, influenza infected, and OVA challenged mice and treated with PBS or DEX (6 mg/kg) for 6 days. Mean changes in Penh are expressed as mean±S.E.M. percentage change from baseline. Data were analysed with unpaired two tailed t-test (* P< 0.05; n=6).

IV.1.2. THE EFFECTS OF DEX ON AIRWAY RESPONSIVENESS

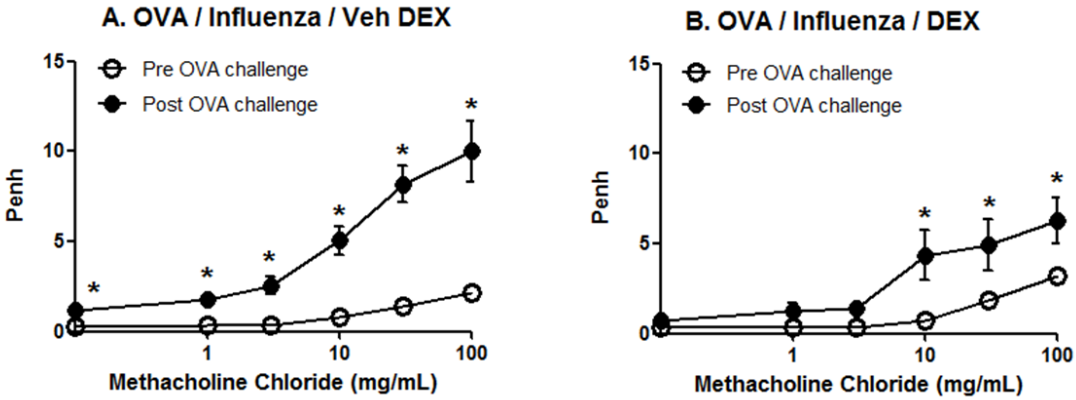


Figure 8.4. Mice were OVA sensitised, influenza infected, challenged with OVA and treated with DEX (6 mg/kg) or vehicle (PBS) for 6 days. A day before administration of DEX or vehicle, and 24 h after the challenges, baseline lung function and AHR to increasing doses of methacholine was measured by whole body plethysmography**.** Results are expressed as mean ± SEM of absolute Penh. Data were analyzed with unpaired two tailed t-test (*P<0.05; n=6).

The OVA challenge in the acute course of influenza infection induced airway hyperresponsiveness. The absolute Penh by methacholine provocation was enhaced

significantly compared to those read before the influenza infection and OVA challenge. There was still a significantly increased airway responsiveness to methacholine after DEX (Fig. 8.4).

IV.1.3. THE EFFECTS OF DEX ON TOTAL AND DIFFERENTIAL CELL COUNTS

Influenza virus infection and OVA challenge increased the total cell counts significantly (Fig. 8.5) in vehicle treated animals compared to naive animal. Treatment with DEX significantly reduced the influx of inflammatory cells recovered in bronchoalveolar fluid from $28.6\pm3.6.10^5$ cells/ml in vehicle treated group to $14.3=5\pm2.7.10^5$ cells/ml in DEX treated group. The macrophages and neutrophil counts were not affected by DEX. However, eosinophil and lymphocyte numbers were significantly attenuated by DEX. Lymphocyte number was reduced from $1.5\pm0.2.10^5$ cells/ml in vehicle treated animal to $0.3\pm0.1.10^5$ cells/ml in DEX treated animal, while the eosinophil number was reduced from $9.3\pm1.6.10^5$ cells/ml to $0.06\pm0.02.10^5$ cells/ml respectively.

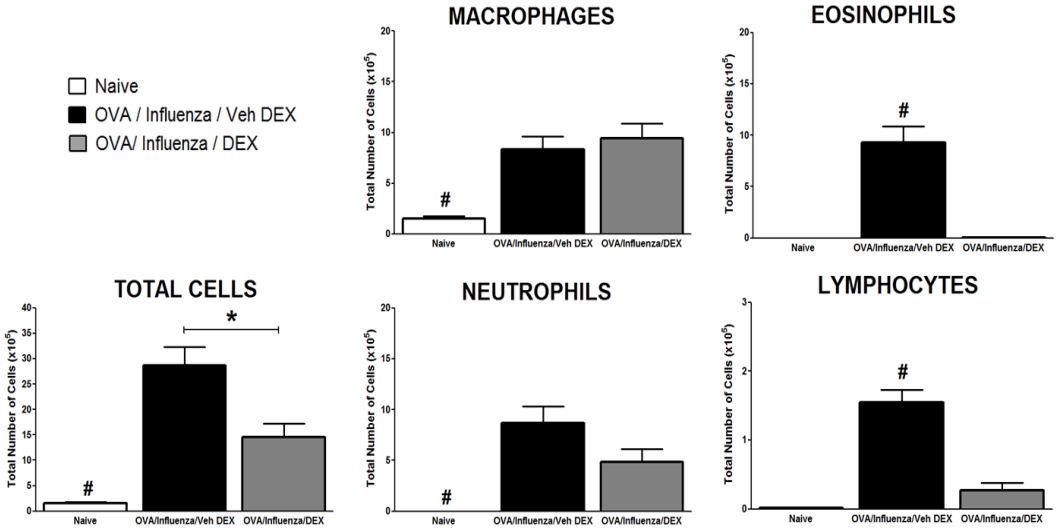


Figure 8.5. Broncholaveolar lavage total and differential cells number (macrophages, eosinophils, lymphocytes and neutrophils) of naïve (non-sensitised) and OVA sensitised, influenza infected, OVA challenged mice which were treated with PBS or DEX (6 mg/kg) for 6 days. Values represent mean±S.E.M. Data were analysed with One-way Analysis of Variance followed by a Bonferroni post-test. #significantly different from all other groups; *connected with corresponding bar is significantly different from each other ($P<0.05$; n=6)

The percentage of each subtype of leucocytes in the total inflammatory cells recovered in BALF was also observed. The DEX treatment significantly altered the proportion of macrophages, eosinophil, and lymphocyte. However, it did not change the proportion of neutrophils from the total leucocytes influx (Fig 8.6).

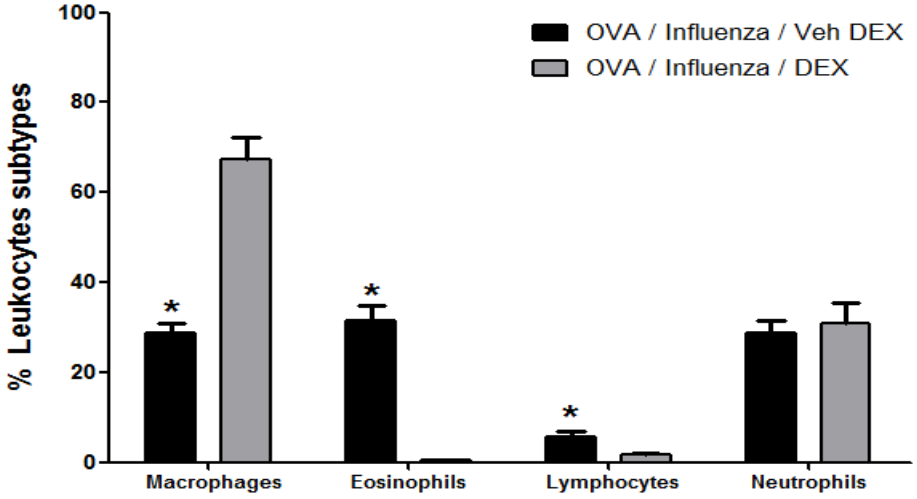


Figure 8.6. The percentage of macrophages, eosinophils, lymphocytes and neutrophils found in the bronchoalveolar fluid of naïve AND OVA sensitised, influenza infected, OVA challenged mice which were treated with PBS or DEX for 6 days. Results are expressed as mean±S.E.M. Data were analysed with One-way Analysis of Variance followed by a Bonferroni post-test (*P<0.05; n=6).

IV.2. THE EFFECTS OF SYSTEMIC CORTICOSTEROID (DEX) ON THE ACUTE COURSE OF INFLUENZA INFECTION IN SALINE SENSITISED AND NON-CHALLENGED MICE

The reading of Penh (airway resistance) in these groups of mice was not conducted as saline challenged mice did not show any significant change in Penh to be regarded as EAR and LAR.

IV.2.1. THE EFFECTS OF DEX ON AIRWAY RESPONSIVENESS

The maximum airway responsiveness was increased significantly from 3.5±0.9 absolute Penh before mice were infected with influenza virus to 8.7±0.7 absolute Penh after infection. DEX treatment did not reduce the increasing AHR (fig 8.7).

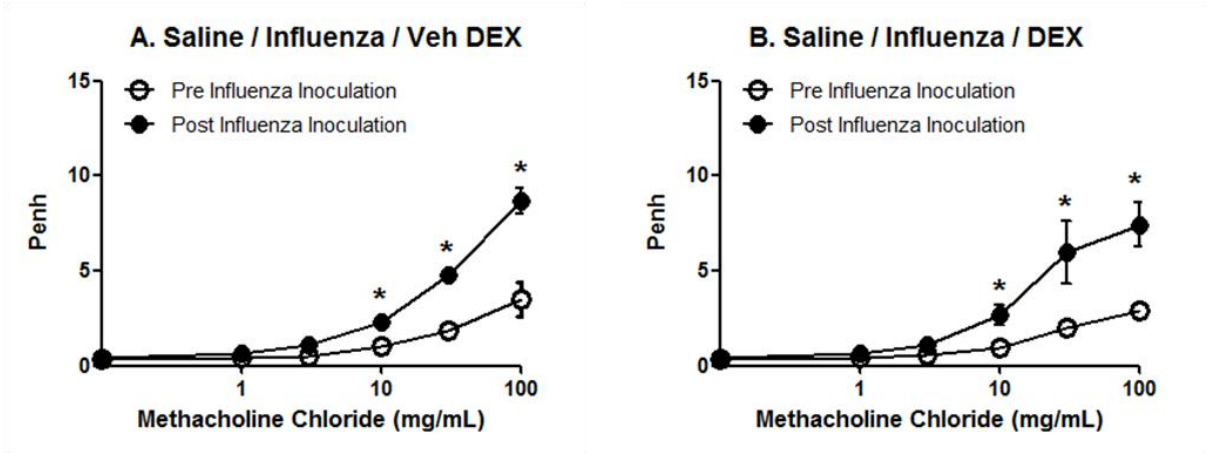


Figure 8.7. Mice were saline sensitised, influenza infected, and treated with DEX (6 mg/kg) or vehicle (PBS) for 6 days. A day before administration of DEX or vehicle, and 24 h after the challenges, baseline lung function and AHR to increasing doses of methacholine was measured by whole body plethysmography**.** Results are expressed as mean ± SEM of absolute Penh. Data were analyzed with unpaired two tailed t-test (*P<0.05; n=6).

IV.2.2. THE EFFECTS OF DEX ON TOTAL AND DIFFERENTIAL CELL COUNTS

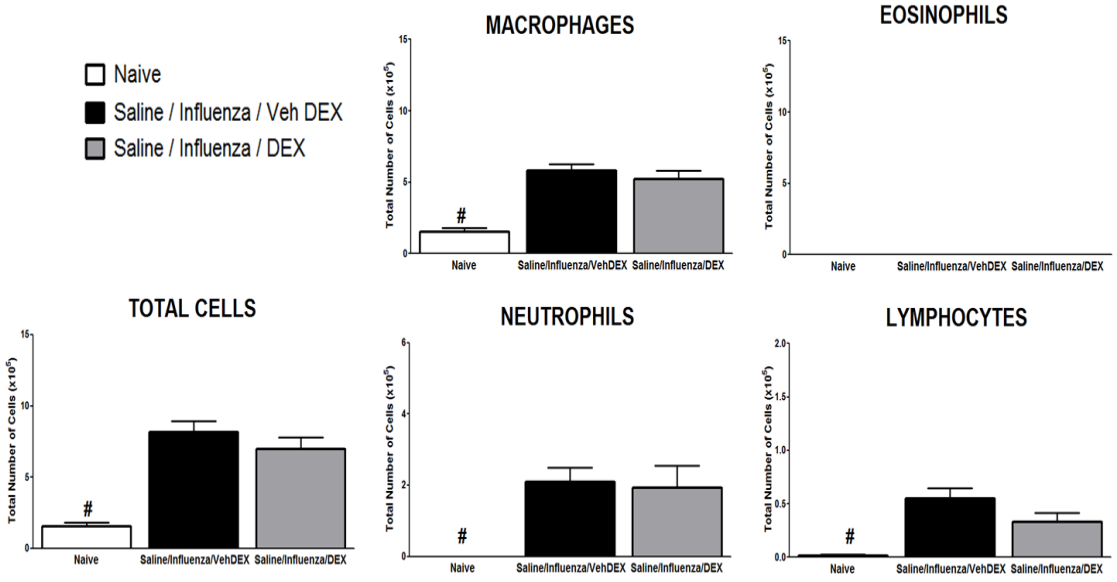


Figure 8.8. Broncholaveolar lavage total and differential cells number (macrophages, eosinophils, lymphocytes and neutrophils) of naïve and saline sensitised, influenza infected, and treated with PBS or DEX for 6 days. Results are expressed as mean±S.E.M. Data were analysed with One-way Analysis of Variance followed by a Bonferroni post-test. #significantly different from all other groups (P<0.05; n=6).

Influenza Infection increased the total inflammatory cells influx into the airway. The number of macrophage, lymphocyte, and neutrophil were significantly greater than those in naive animals. The increase in the total cell count, macrophage count, lymphocyte count, and neutrophil count were not affected by DEX (fig 8.8).

IV.2.3. THE EFFECTS OF DEX ON PLASMA EXUDATION/PROTEIN LEAKAGE

OVA sensitisation, OVA challenge, influenza infection, and DEX treatment, when all given concurrently enhanced protein content in BALF. However, influenza infection per se did not increase the protein level significantly compared to naive mice (Fig 8.9).

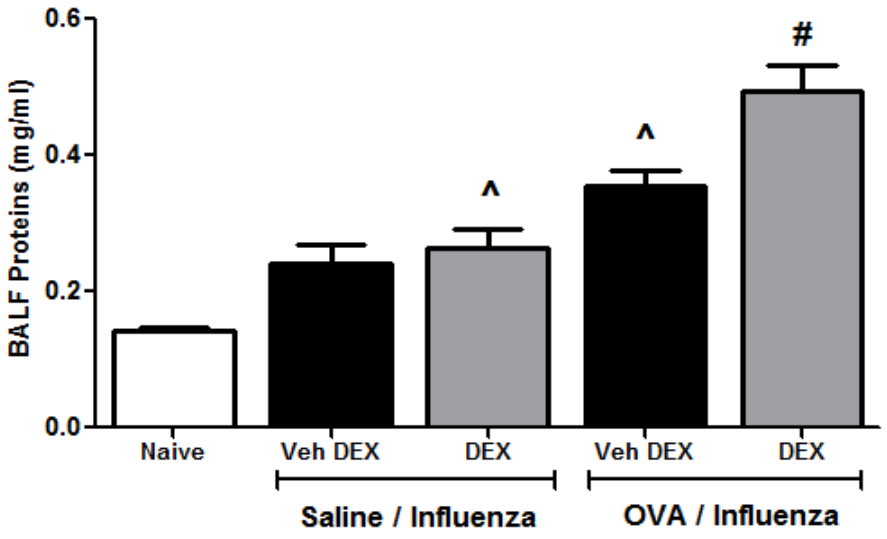


Figure 8.9. Total protein content in bronchoalveolar lavage fluid of naïve, naïve (non-sensitised) and OVA sensitised, influenza infected, OVA challenged mice which were treated with PBS or DEX (6 mg/kg) for 6 days. Results are expressed as mean±S.E.M. Data were analysed with One-way Analysis of Variance followed by a Bonferroni post-test. $^{\#}$significantly different from all other groups, ^significantly different from naive mice ($P<0.05$; n=6).

IV.2.4. THE EFFECTS OF DEX ON VIRAL TITRES

OVA sensitisation and challenge did not significantly increase the viral titre. Treatment with DEX did not affect viral titre in either saline sensitised or OVA sensitised and challenged animals (fig. 8.10).

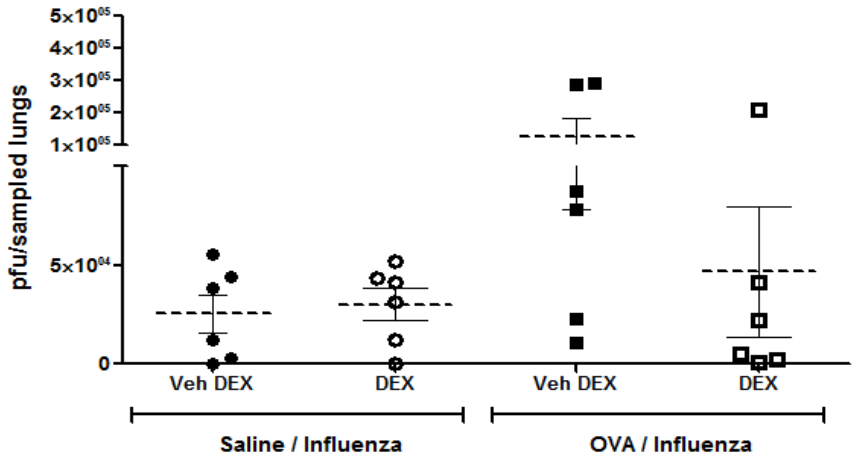


Figure 8.10. Viral titres of lungs of mice sensitised with OVA infected with influenza A, challenged with OVA and treated with DEX (6mg/kg) or PBS for 6 days. Virus titres were determined by plaque assay on MDCK cells. Virus titres are expressed as mean ± SEM of pfu per sampled lungs. Data were analysed with one-way Analysis of Variance, followed by post hoc Bonferroni's test to determine the difference in viral titres.

IV.2.5. THE EFFECTS OF DEX LUNG HISTOLOGY

Figure 8.11 displays change in lung histological features of mice when they infected with influenza virus and challenged with OVA compared to naive mice. The lung from mice which were infected with influenza virus alone and treated with vehicle DEX (PBS) showed a distinct peribronchial and perivascular inflammation as well as epithelial loss or shedding (fig 8.11.B). Treatment with DEX did not significantly change the lung histological features (fig.8.11.C). Lungs of OVA sensitised, influenza infected and OVA challenged mice display a more severe perivascular and peribronchial inflammation (fig 8.11.D) compared to the non challenged mice (fig 8.11.B and C). DEX slightly reduced the density of inflammatory cells around bronchioles and lung blood vessel (fig 8.11 E).

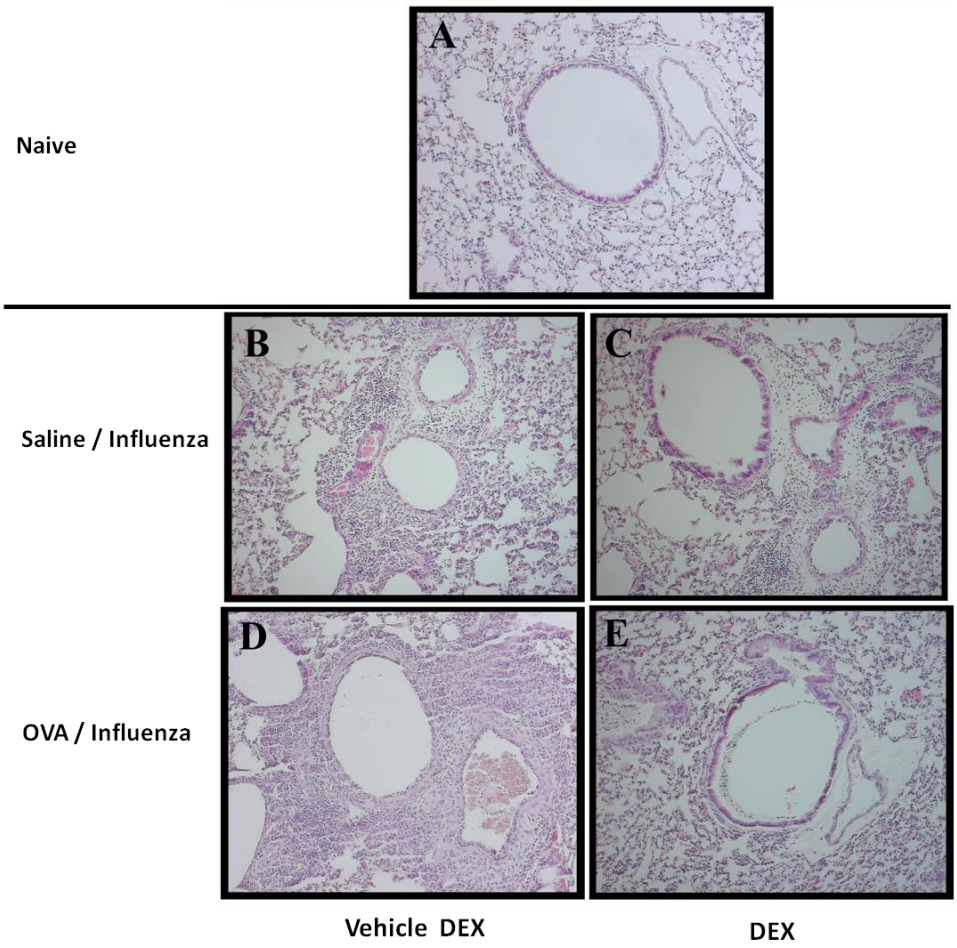


Figure 8.11. Histopathological changes of lungs of naive mice (A); or saline sensitised/influenza infected mice which were treated with PBS (vehicle DEX) (B); Saline sensitised/influenza infected mice which were treated with DEX (C); OVA sensitised/influenza infected mice which were treated with PBS (D); or OVA sensitised/influenza infected mice which were treated with DEX (E). The lungs were stained with haematoxylin and eosin to display general morphology (100x magnification).

Influenza infection increased the mean pathological scores compared to naive animals. However, DEX treatment or OVA sensitisation and challenge did not significantly alter the mean pathology scores compared any other groups (fig. 8.12).

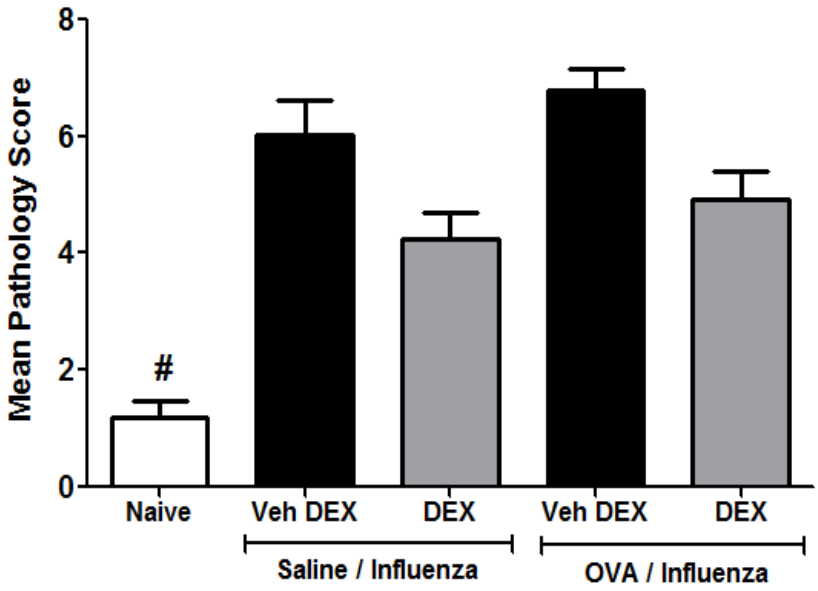


Figure 8.12. The mean lung pathology score of naïve or saline sensitised mice which were treated with PBS (Vehicle DEX) or DEX 6 mg/kg or OVA sensitised and challenged mice which were treated with PBS or DEX 6 mg/kg. Results are expressed as mean±S.E.M. Data were analysed using one-way ANOVA, followed by post hoc Bonferroni's test to determine the mean pathology score. *significantly different from all other groups ($P<0.05$; $n=6$).

IV.3. THE EFFECTS OF INHALED CORTICOSTEROID (FP) ON INFLUENZA ASSOCIATED EXACERBATION OF MURINE AIRWAY ALLERGIC INFLAMMATION

IV.3.1. THE EFFECTS OF FP ON EARLY AND LATE PHASE REACTIONS

When the OVA sensitised and influenza infected animals were challenged with OVA, the EAR was observed on both vehicle treated and FP treated mice. The EAR of the vehicle treated mice peaked 1.5 hour after the last OVA challenge (107.6 ±18.9% increase from baseline Penh), while the EAR of FP treated animals peaked 2 hours after the challenge (59.7±33.6% increase from baseline Penh) (fig 8.13).

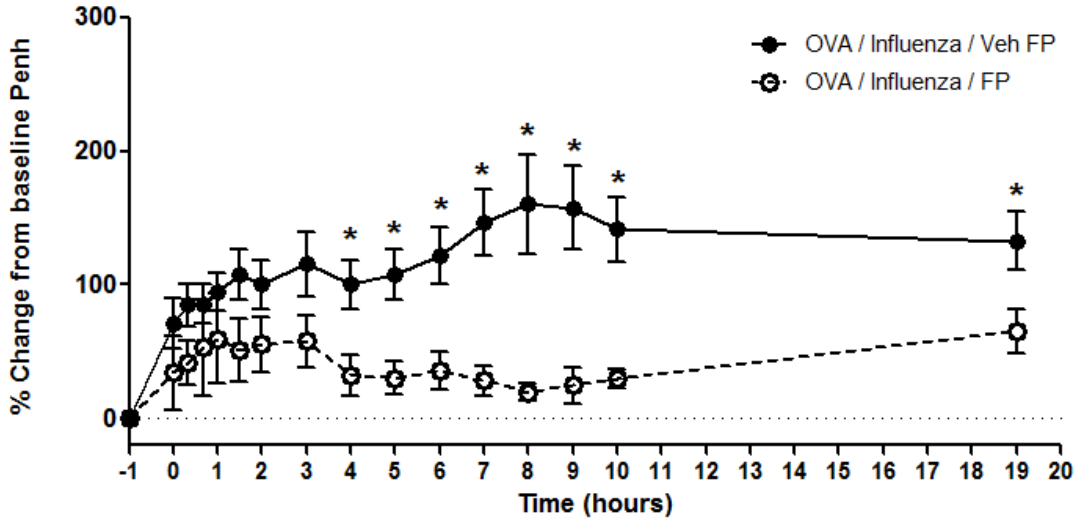


Figure 8.13 Mean time-course values of Penh in OVA sensitised, influenza infected, and OVA challenged mice and treated with vehicle (saline/DMSO/Ethanol (40%/30%/30)) or FP (0.5 mg/ml) for 6 days. Mean changes in Penh are expressed as mean±S.E.M. percentage change from baseline. Data were analysed with unpaired two tailed t-test (* P< 0.05; n=6).

The peak of LAR in vehicle treated mice was detected 8 hours after the last OVA challenge in vehicle treated mice (160.3±36.9% increase from baseline Penh). There was no LAR peak detected in mice treated with FP, hence FP attenuated the LAR. Nineteen hours after the last OVA challenge, the baseline airway functions did not return to baseline on either vehicle treated or FP treated mice. However, the baseline

airway functions of FP treated mice were significantly lower compared to vehicle treated mice (fig 8.13).

IV.3.2. THE EFFECTS OF FP ON AIRWAY RESPONSIVENESS

There was a marked increase in airway responsiveness when OVA challenge and influenza infection were given simultaneously in OVA sensitised animals. FP did not significantly reduce the airway responsiveness induced by viral infection and allergen challenge at 24 hours (fig 8.14).

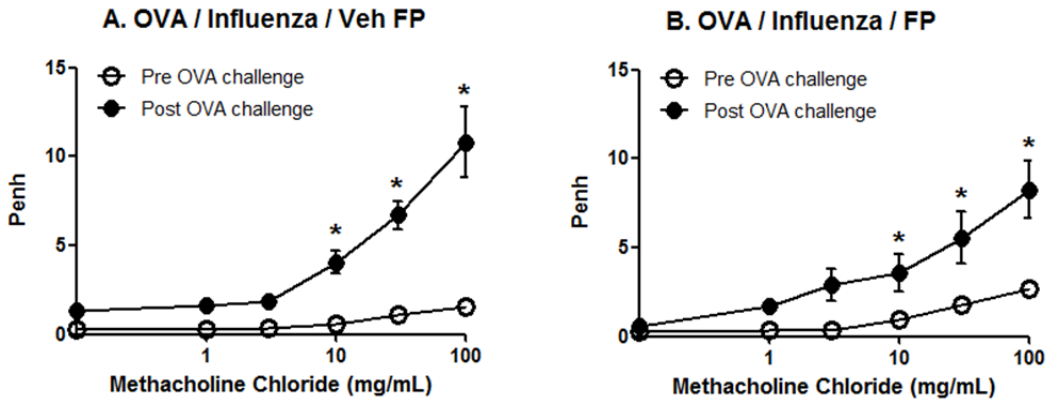


Figure 8.14. Mice were OVA sensitised, influenza infected, challenged with OVA and treated with FP (0.5 mg/ml)or vehicle (saline/DMSO/ethanol (40%/30%/30)) for 6 days. A day before administration of FP or vehicle, and 24 h after the challenges, baseline lung function and AHR to increasing doses of methacholine was measured by whole body plethysmography**.** Results are expressed as mean±S.E.M of absolute Penh. Data were analyzed with unpaired two tailed t-test (*P<0.05; n=6).

IV.3.3. THE EFFECTS OF FP ON TOTAL AND DIFFERENTIAL CELL COUNTS

Influenza infection and OVA challenge in OVA sensitised mice increased the total inflammatory cells recovered in BAL fluid compared to naive animal. The macrophage, eosinophil, lymphocyte, and neutrophil number were also significantly greater than naive. FP treatment significantly reduced the total leukocytes count. The numbers of all individual cells were also attenuated by FP (Fig 8.15).

FP also significantly affected the proportion of individual leukocyte subtypes particularly macrophages and eosinophils in total influx of inflammatory cells in BAL fluid. The proportion of lymphocytes and neutrophils were not altered by FP (fig 8.16).

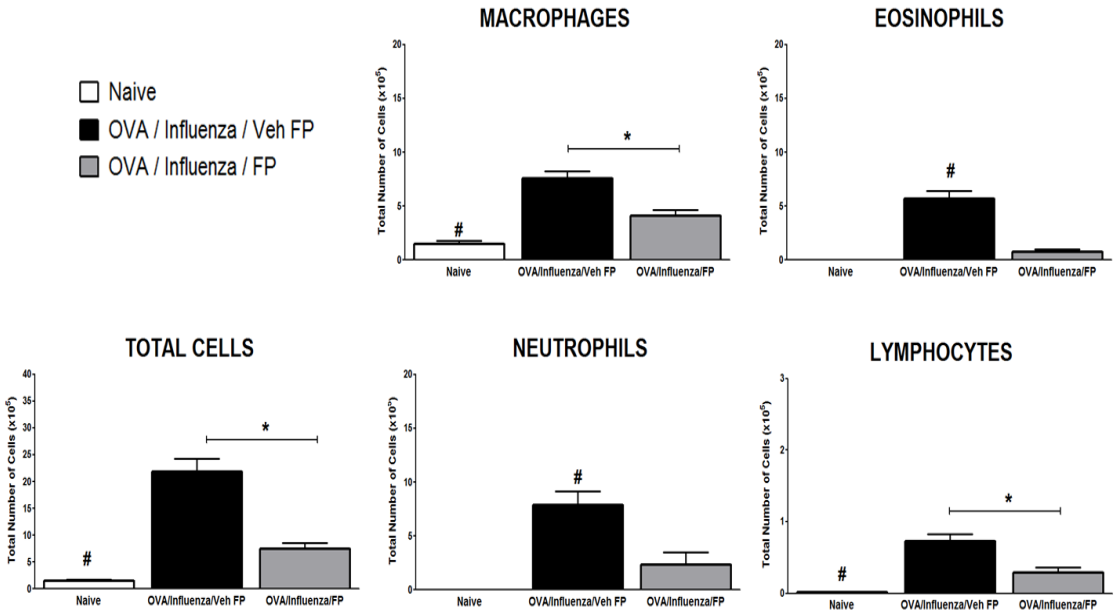


Figure 8.15. Broncholaveolar lavage total and differential cells number (macrophages, eosinophils, lymphocytes and neutrophils) of naïve (non-sensitised) and OVA sensitised, influenza infected, OVA challenged and treated with Vehicle (saline/DMSO/ethanol (40%/30%/30) or FP (0.5 mg/ml) for 6 days. Values represent mean±S.E.M. Data were analysed with One-way Analysis of Variance followed by a Bonferroni post-test. #significantly different from all other groups; *connected with corresponding bar is significantly different from each other (P<0.05; n=6)

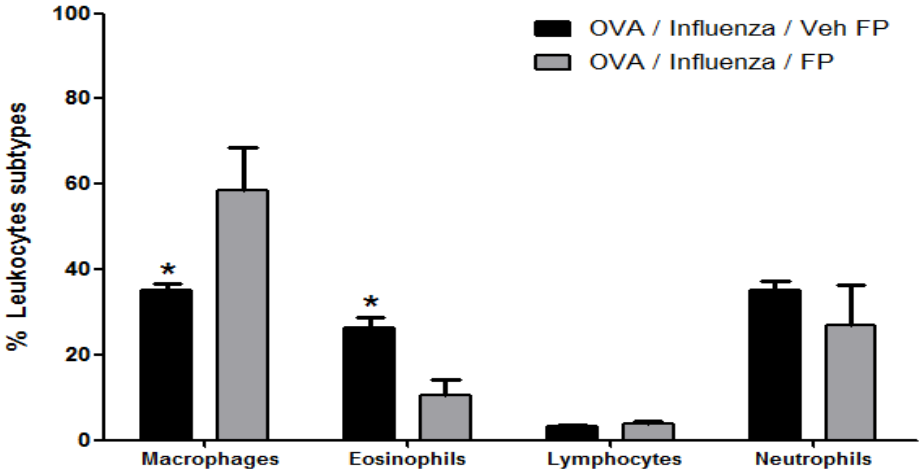


Figure 8.16. The percentage of macrophages, eosinophils, lymphocytes and neutrophils found in the bronchoalveolar fluid of naïve AND OVA sensitised, influenza infected, OVA challenged and treated with vehicle (saline/DMSO/ethanol (40%/30%/30)) or FP (0.5 mg/ml) for 6 days mice. Results are expressed as mean±S.E.M. Data were analysed with One-way Analysis of Variance followed by a Bonferroni post-test (*P<0.05; n=6).

IV.4. THE EFFECTS OF INHALED CORTICOSTEROID (FP) ON THE ACUTE COURSE OF INFLUENZA INFECTION IN SALINE SENSITISED AND UNCHALLENGED MICE

Observation of the airway functions (Penh) overtime to determine AER and LAR was also not performed in these groups of animal for the same reasons as per the DEX treated mice.

IV.4.1. THE EFFECTS OF FP ON AIRWAY RESPONSIVENESS

The airway responsiveness increased by influenza virus infection was not reduced by FP as described on figure 8.17.

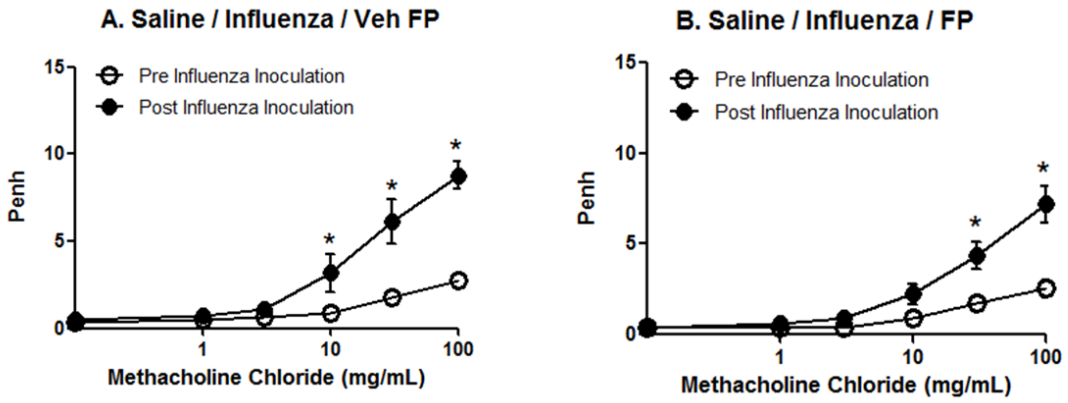


Figure 8.17. Mice were saline sensitised, influenza infected, and treated with FP (0.5 mg/ml) or vehicle (saline/DMSO/ethanol (40%/30%/30)) for 6 days. A day before administration of FP or vehicle, and 24 h after the challenges, baseline lung function and AHR to increasing doses of methacholine was measured by whole body plethysmography**.** Results are expressed as mean±S.E.M of absolute Penh. Data were analyzed with unpaired two tailed t-test (*P<0.05; n=6). .

IV.4.2. THE EFFECTS OF FP ON TOTAL AND DIFFERENTIAL CELL COUNTS

FP treatment did not significantly reduce the increasing total cell counts induced by influenza virus infection (fig 8.18). The individual inflammatory cells subtype number was also not affected by FP treatment except for lymphocytes which reduced from $0.3\pm0.1.10^5$ cells/ml in vehicle treated mice to $0.99\pm0.07.10^5$ cells/ml in FP treated mice.

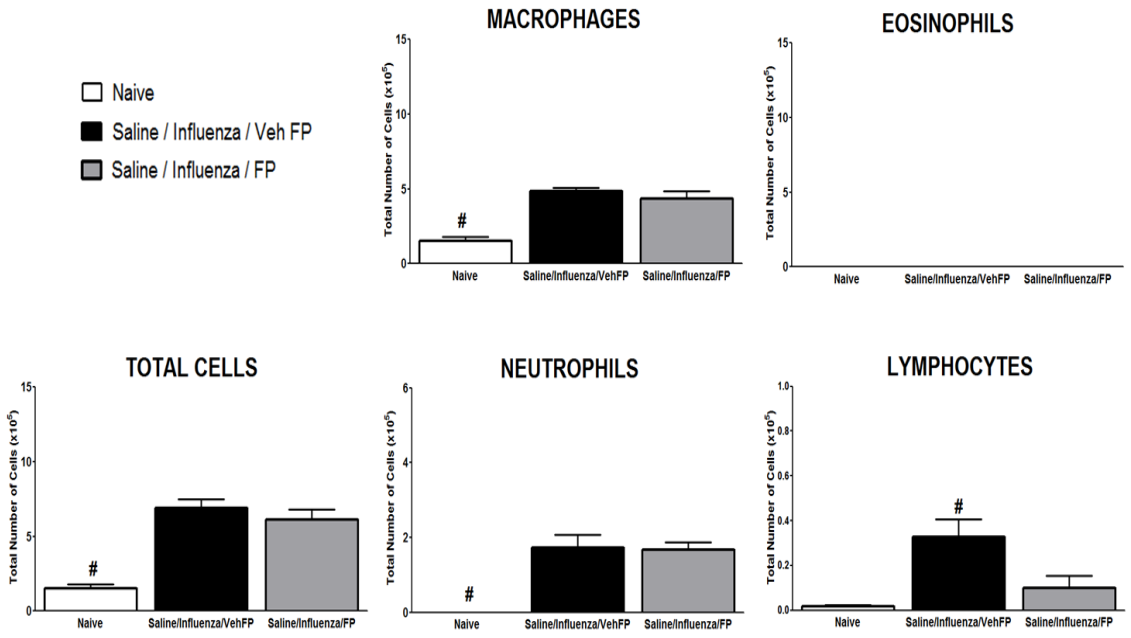


Figure 8.18. Broncholaveolar lavage total and differential cells number (macrophages, eosinophils, lymphocytes and neutrophils) of naïve and saline sensitised, influenza infected, and treated with vehicle (saline/DMSO/ethanol (40%/30%/30)) or FP (0.5 mg/ml) for 6 days. Results are expressed as mean±S.E.M. Data were analysed with One-way Analysis of Variance followed by a Bonferroni post-test. #significantly different from all other groups (P<0.05; n=6).

IV.4.3. THE EFFECTS OF FP ON PLASMA EXUDATION/PROTEIN LEAKAGE

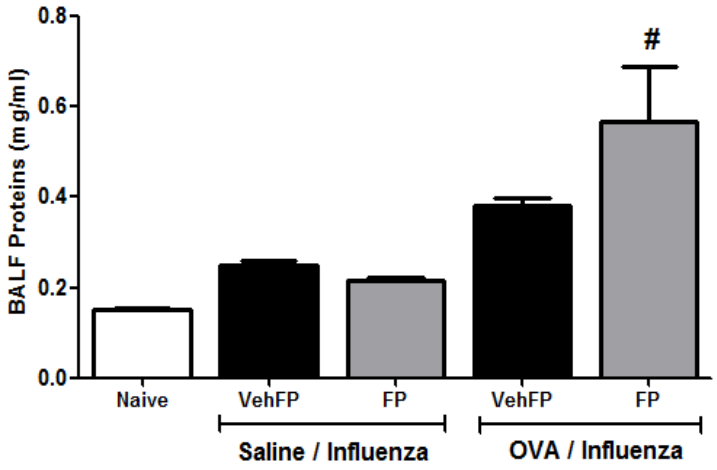


Figure 8.19. Total protein content in bronchoalveolar lavage fluid of naïve (non-sensitised) and OVA sensitised, influenza infected, OVA challenged mice which were treated with vehicle (saline/DMSO/ethanol (40%/30%/30)) or FP (0.5 mg/ml) for 6 days. Results are expressed as mean±S.E.M. Data were analysed with One-way Analysis of Variance followed by a Bonferroni post-test. #significantly different from all other groups (P<0.05; n=6).

Protein level in the BAL fluid from OVA sensitised, OVA challenged, influenza infected, and FP treated animals was significantly higher than any other groups (fig 8.19).

IV.4.4. THE EFFECTS OF FP ON VIRAL TITRES

OVA sensitisation and challenge did not significantly change viral titre compared to saline sensitised and non challenged animal. Treatment with FP did not have any significant effect on influenza virus titre (Fig 8.20).

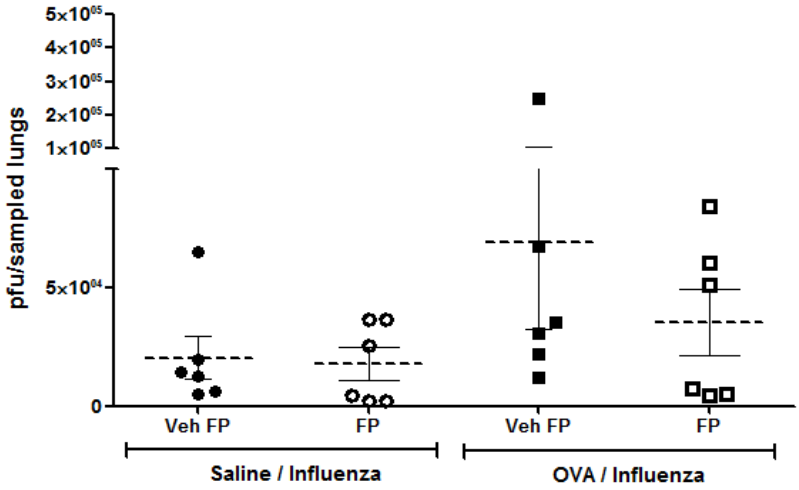


Figure 8.20. Viral titres of lungs of mice sensitised with OVA infected with influenza A, challenged with OVA and treated with vehicle (saline/DMSO/ethanol (40%/30%/30)) or FP (0.5 mg/ml) for 6 days. Virus titres were determined by plaque assay on MDCK cells. Virus titres are expressed as mean ± SEM of pfu per sampled lungs. Data analysed for each differential cell type and total count using one-way ANOVA, followed by post hoc Bonferroni's test to determine the difference in viral titres.

IV.4.5. THE EFFECTS OF FP LUNG HISTOLOGY

Marked peribronchial and perivascular inflammation were observed in influenza infected mice (fig 8.21.B, C, D, and E) compared to naive animals. In the mice which OVA sensitised, influenza virus infected, and challenged with OVA there is an evidence of epithelial cells elongation and folding (fig 8.21.D and E). OVA sensitisation and challenge caused denser accumulation of cells in peribronchial and perivascular area (fig 8.21.E). Treatment with FP reduce the density of the inflammatory cells accumulation in OVA sensitised and OVA challenged group (fig 8.21.E) but did not

significantly change the general histological appearance of the lungs from influenza infected mice which only sensitised with saline (fig 8.21.C).

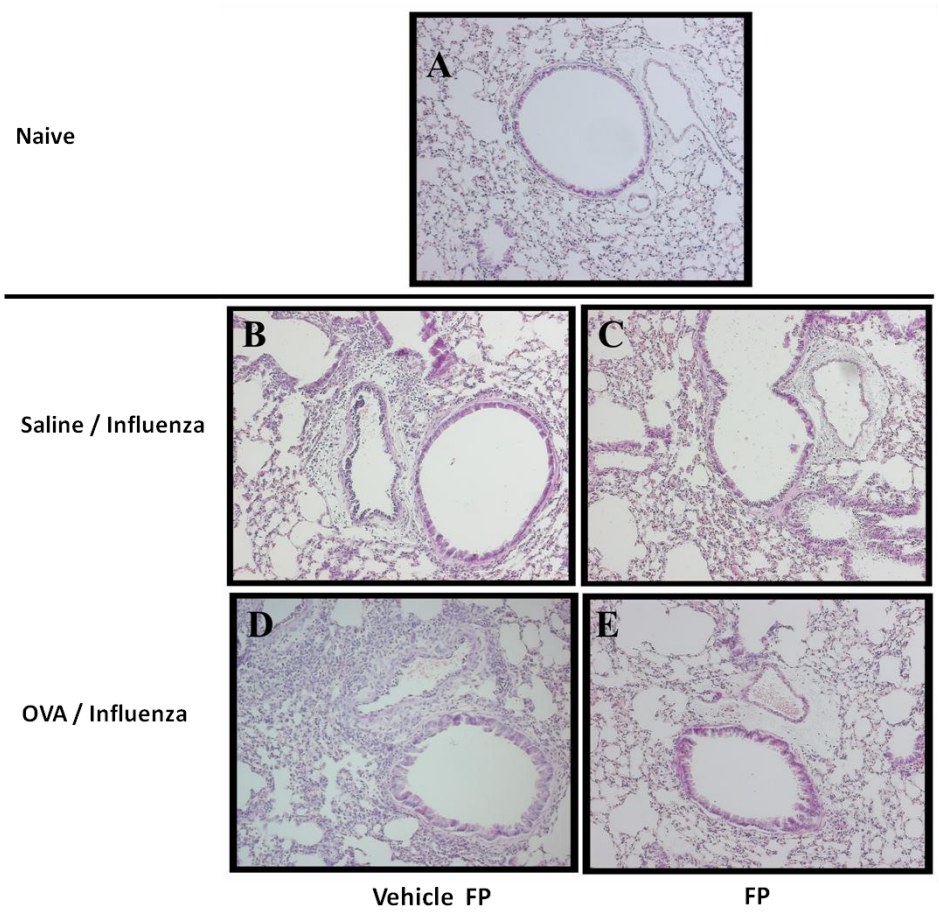


Figure 8.21. Histopathological changes of lungs of naive mice (A); or saline sensitised/influenza infected mice which were treated with vehicle FP (B); Saline sensitised/influenza infected mice which were treated with FP (C); OVA sensitised/influenza infected mice which were treated with vehicle FP (D); or OVA sensitised/influenza infected mice which were treated with FP (E). The lungs were stained with haematoxylin and eosin to display general morphology (100x magnification).

Influenza infection increased the mean pathology score compared to naive mice. Treatment with FP 0.5 mg/ml significantly reduced the mean pathology score in OVA challenge, influenza infected, and OVA challenged animals but did not change the score of the animals which were only sensitised with saline (fig 8.22).

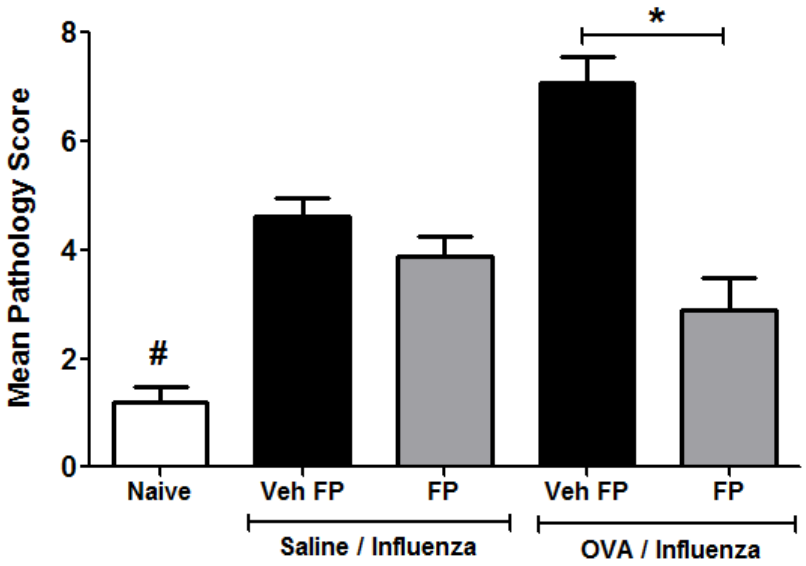


Figure 8.22. The mean lung pathology score of naïve or saline sensitised mice which were treated with vehicle (saline/DMSO/ethanol (40%/30%/30)) or FP (0.5 mg/ml) or OVA sensitised and challenged mice which were treated with vehicle (saline/DMSO/Ethanol (40%/30%/30)) or FP (0.5 mg/ml) for 6 days. Results are expressed as mean±S.E.M. Data were analysed using one-way ANOVA, followed by post hoc Bonferroni's test to determine the mean pathology score. #significantly different from all other groups; *connected with corresponding bar is significantly different from each other ($P<0.05$; n=6).

V. DISCUSSION

Currently, corticosteroids either inhaled or systemically administered are the mainstay of asthma therapy. Corticosteroid is a potent anti inflammatory agent which can attenuate the inflammation in asthmatics via their genomic or non-genomic actions. In the case of asthma exacerbation, particularly viral associated asthma exacerbation, theoretically corticosteroids should be effective in reducing the inflammation from the interplay between allergic inflammation and respiratory viral infection. Therefore, corticosteroids have been commonly prescribed in the case of viral induced asthma exacerbation both in general practice or emergency setting. However, in reality, the benefit of corticosteroid in the case of asthma exacerbations induced by respiratory viral infection is still controversial as described in the introduction section.

In this series of experiments, the role of both systemic (DEX) and inhaled (FP) corticosteroid in the course of acute influenza infection of mice with allergic airway inflammation when they are challenged with allergen were determined.

V.1. THE EFFECTS OF CORTICOSTEROIDS ON THE INFLAMMATORY CELLS INFLUX INTO THE AIRWAYS

OVA challenge in acute influenza infection on OVA sensitised mice in which they were treated with the vehicle of corticosteroid led to a very significant inflammatory cells influx into the airways. This is consistent with our results in CHAPTER 6. DEX significantly reduced the total inflammatory cell. DEX reduced the individual number of lymphocytes and eosinophils. Eosinophil number was almost completely abrogated by DEX. The number of macrophages and neutrophils though were not affected by DEX.

Influenza infection alone in saline sensitised and non-challenged mice also increased the total inflammatory cells counts. The number of macrophages, lymphocytes, and neutrophils were higher than those numbers in naive animals. The treatment of DEX

did not reduce the total inflammatory cells count or the individual count of inflammatory cells subtype i.e. macrophages, neutrophils, and lymphocytes.

Influenza infection targets the epithelial cells which line the airways. In the course of acute influenza infection, the infected epithelial cells become necrotic, lysed, and release a range of cytokines and chemokines such as TNF-α, IL-1, MCP-1, RANTES, MIP-1, IP-10, and IL-8 (Julkunen *et al.*, 2001). The release of the inflammatory mediators by necrotic epithelial cells promotes the extravasation of macrophages, neutrophils, and T lymphocytes from peripheral blood trough the endothelial-epithelial barrier to the infected tissue in the airways (Ada and Jones, 1986). Macrophages have an essential role as both killing the virus and secreting inflammatory mediators which further recruit inflammatory cells into the lungs. Macrophages and neutrophils also act as phagocytes to the virus infected apoptotic cells (Hashimoto *et al.*, 2007). Hence, along with neutrophils, macrophages have a role in viral clearance (Watanabe *et al.*, 2005).

In acute viral-infected allergic mice, allergen challenge exaggerates the migration of inflammatory cells into the airways as there is a synergism between the two as discussed in CHAPTER 6. The administration of DEX reduced the number of eosinophils in BAL fluid. Eosinophilia is the hallmark of asthma. Eosinophils are known to be sensitive to corticosteroid. The pharmacologic characteristic of corticosteroid in the treatment of asthma includes the action of the anti-inflammatory agent in the inhibition of eosinophil recruitment, reduction of the number of eosinophil including their secretary products (Birrell *et al.*, 2003) and induction of eosinophil apoptosis (Anderson, 1996).

Schleimer (2004) in his review argues that corticosteroids, while effective in reducing inflammation as a result of activated adaptive immune responses, they have a minimum effect on innate immune responses which usually triggered by infection.

Macrophages and neutrophils are essential effector cells in the innate immune responses. The function of macrophages as phagocytes is increased by corticosteroid (Heasman *et al.*, 2003). Systemic corticosteroids are also known to significantly increase the number neutrophils and increase their survival (Schleimer, 2004). The action of sparing innate immune response by corticosteroids might be the reasons why DEX had a minimal effect in the reduction of macrophages and neutrophils in either OVA sensitised/influenza infected/OVA challenged mice or saline sensitised/influenza infected/non-challenged mice.

Similar to DEX, in acute influenza infection of saline sensitised and un-challenged mice, FP treatment did not reduce the total cell count. However, different from DEX, FP reduced the number of lymphocytes significantly compared to vehicle treated animals.

Treatment of FP in OVA sensitised/influenza infected/OVA challenged mice also reduced the total inflammatory cells count significantly compared to the cells count in vehicle treated animals. Different from DEX though, the number of all individual subtypes of leukocytes was significantly lower in FP treated group than those in vehicle treated group. However, when compared the effects of the DEX and FP treatments on the proportion of individual subtypes of leukocytes recovered in BAL fluid of OVA sensitised, influenza infected, and OVA challenged groups were quite similar with the increased proportion of macrophages and the reduced proportion of eosinophils compared to vehicle treated groups.

Corticosteroids have been proposed to largely act on reducing the migration and accumulation of inflammatory cells in the airway by reducing the production of chemokines/chemoattractant and inhibiting the upregulation of some particular adhesion molecules (Schleimer, 2004). Therefore, Schleimer (2004) also propose that corticosteroids primarily affect the production of chemoattractants and not the leukocytes responses to their chemoattractants. And in this study, FP effects on the

reduction of the inflammatory cells chemoattractants might be larger than those of DEX.

Corticosteroids, even though almost similar in their molecular structures have a varied rank of potency. FP has a relatively higher affinity for the glucocorticoid receptor (GR) compared to DEX (Johnson, 1998). The rate of dissociation of the FP-GR complex is also slower than DEX. The GR-FP profile is associated with a relatively high anti-inflammatory activity. The FP-GR interacts with glucocorticoid response element on target genes at significantly lower concentration than DEX (EC_{50} FP = range 0.01 to 0.1 nmol/L and EC_{50} DEX = 3 nmol/L respectively) (Adcock *et al.*, 1999). To the best of my knowledge, there are no study to find the direct comparison between FP and DEX effect on every chemoattractant for the individual leucocytes, but there are some reference that showed FP is more potent compared to DEX in inhibiting human T-lymphocytes migration and proliferation. FP is also more potent in inhibiting the production of IL-5 and IL-4 from Th2 lymphocytes (Johnson, 1998). So, FP may be more potent in reducing the number of inflammatory cells in the lungs compared to DEX in OVA sensitised/influenza infected/OVA challenged animals. However, FP was not effective in attenuating the inflammatory cell influx in the lungs of the animals which only infected with influenza virus.

V.2. THE EFFECTS OF CORTICOSTEROIDS ON EARLY AND LATE PHASE ASTHMATIC RESPONSES

The allergic cascade initiated by re-exposure of allergen has been known to cause early and late phase bronchoconstriction. Previous series of experiments (CHAPTER 6) highlighted interesting findings demonstrating that influenza virus infection when occurs concomitantly with allergen exposure/challenge in allergen sensitised mice profoundly accentuate the brochoconstriction particularly late phase asthmatic response. Late phase asthmatic reaction was also prolonged and the lung functions did not return to baseline 19 hours after the second (24 hours after the first) allergen

challenge. Mice treated with vehicle corticosteroids also had a significant increase in late phase asthmatic bronchoconstriction. The bronchoconstriction still persisted 19 hours after the second OVA challenge. This was a measure of exacerbation. Neither DEX nor FP reduced early phase asthmatic reactions.

The number of mast cells in airway smooth muscle (Brightling *et al.*, 2002) and within the epithelial layer (Balzar *et al.*, 2011) of asthmatics is higher than that in their healthy counterpart. After allergen challenge, mast cells in the allergic airways are rich in allergen specific IgE. They degranulate and release inflammatory mediators such as prostaglandin, leukotrienes, and histamine which contribute to early phase brochoconstriction (Edwards *et al.*, 2009). Influenza A infection has also been noted to induce mast cells proliferation and activation. In the acute phase of influenza infection, mast cells when degranulated have been suspected to have a significant contribution on the lung injury by releasing histamine, tryptase and IFN-γ ((Hu *et al.*, 2012).

Inhaled corticosteroid (Budesonide) has been known to selectively reduce the number of mast cells in the smooth muscle and epithelium of asthmatic's airways but not sub-mucosal mast cells (James *et al.*, 2012). FP was also able to reduce the number of mast cell in COPD patients after 3 months treatment (Gizycki *et al.*, 2002). Nonetheless, corticosteroid has failed to inhibit the activation and release of histamine from degranulated mast cells (Liu *et al.*, 2001). Therefore, this could explain why corticosteroid has a minimum effect in inhibiting early phase bronchoconstrictions in this study.

Late phase reaction was sensitive to FP. Mice treated with FP had the late phase bronchoconstriction completely abrogated. Mice treated with FP also had significantly lower baseline lung functions 19 hours after the second OVA challenge compared to vehicle treated mice.

The effects of DEX on the late phase bronchoconstrictions were slightly different from FP. DEX was able to reduce the peak of late phase bronchoconstriction. However, starting from hour 7 after the second OVA challenge, the mice beginning to have ongoing increase of bronchoconstriction up to hour 10 when the observation was paused. The next day (19 hours after the second OVA challenge) there was no significant reduction of bronchoconstriction and the increase of baseline Penh was not significantly different from vehicle treated mice. It is interesting because, anything could happen in 10-19 hour when the Penh reading was paused. The late phase bronchoconstriction might just have been delayed and not completely attenuated by DEX.

Late phase bronchoconstriction has been known as the continuing consequences of mast cells degranulation. Mast cells generate a variety of chemokines which attracts Th1 and Th2 lymphocytes, eosinophils and neutrophils into the airway. The migrated Th2 lymphocytes also produce Th2 specific cytokines including IL-5 which is important for eosinophil differentiation, proliferation, activation, and survival (Renauld, 2001). Mast cells also produce a range of Th2 cytokines including IL-4 and IL-13 which contribute to the increasing production of chemokines from epithelial and airway smooth muscle cells. IL-13 is recognized to highly contribute to the increase of airway hyperresponsiveness in asthma (Leigh *et al.*, 2004; Yang *et al.*, 2004). The inflammatory cells influx into the airway, particularly the Th2 lymphocytes has been thought to have an important role in the development of the late asthmatic responses.

The difference of the relative potency between DEX and FP in the inhibition of T lymphocytes proliferation and migration as well as IL-4 and IL-5 secretion might contribute in their differences in the abolishment of late phase brochoconstriction in this study. This seems to be associated with the effectiveness of DEX and FP in reducing the influx of inflammatory cells into the airways.

As noted, the levels of circulating alveolar macrophages and neutrophils as part of the innate immune response effector cells toward respiratory viral infection seem to be spared by corticosteroid (Schleimer, 2004). The accumulated macrophages can also be a source of inflammatory cytokines by their ability to secrete IL-1, TNF-α, and IL-8 which lead to the amplifications of inflammation in the airways by triggering the activation and further recruitment of inflammatory cells into the infected sites (Julkunen *et al.*, 2000). Macrophages infected with respiratory virus *in vitro* were capable of producing prostaglandine E2, thromboxane B2, platelet activating factor, and reactive oxygen species, which might contribute to the enhancement of bronchoconstriction (Folkerts *et al.*, 1998). Neutrophils were also capable of secreting inflammatory mediators, elastase, and protease (Liu *et al.*, 1999). Protease produced by neutrophils can activate eosinophils (Liu *et al.,* 1999). Eosinophils were sensitive to corticosteroid treatment. However, there is a study which reported that neutrophils take up and store the eosinophil cationic protein released by eosinophils (OrdoÑEz *et al.*, 2000).

DEX seems to have a minimal effect to reduce the increased number macrophages and neutrophils induced by influenza infection. This might lead to the persistent airway bronchoconstriction, 19 hours after OVA challenge even after DEX treatment. FP treatment though was capable of reducing the number of macrophages and neutrophis in mice, which might lead to the significant reduction of airway bronchoconstrictions observed 19 hours after OVA challenge.

V.3. THE EFFECTS OF CORTICOSTEROIDS ON AIRWAY HYPERRESPONSIVENESS

Similar with the result of the study to develop a model of virus infection-induced asthma exacerbation described in CHAPTER 6; OVA sensitised, influenza infected, and OVA challenged mice had a very significant increase in airway hyperresponsiveness after they were treated with the vehicle of corticosteroids, compared to the airway

responsiveness read before any of the interventions. DEX did not significantly reduce the increase in airway hyperresponsiveness and neither did FP.

The augmentation of airway hyperresponsiveness after influenza infection was also observed in mice which were only saline sensitised and not given any challenge. Neither DEX nor FP significantly reduced the increase of airway hyperresponsiveness. The hypothesis of possible causes of the enhancement of airway responsiveness in influenza infection alone; OVA allergic airway inflammation model; and OVA challenge in influenza infected in OVA sensitised mice have been discussed CHAPTER 4, 5, and 6. In the OVA allergic inflammation model, Th-2 cytokine, IL-13 has been thought to play a significant role in the development of airway hyperresponsiveness (Bousquet *et al.*, 2000). The airway hyperresponsiveness induced by influenza infection is shown to have a different mechanism from OVA induced airway hyperresponsiveness as it requires IL-33-IL-13 axis to develop as described in CHAPTER 4 and 6.

IL-33 expression in the lung is profoundly augmented by influenza A infection (Le Goffic *et al.*, 2011). The cells which are responsible for the up regulation of IL-33 include epithelial cells, macrophages, and airway smooth muscle cells. IL-33 is an 'alarmin' or a danger signal released when cells undergoing apoptosis or necrosis (Borish and Steinke, 2011). In the case of influenza virus infection, IL-33 could be released by the infected epithelial cells which undergo apoptosis or necrosis.

Recently, it has been noted that there is a new comer in innate immune response, the natural helper (NH) cells which bear ST2 receptor and is responsive to IL-33. In response to IL-33, NH cells are capable of producing IL-5 and large amounts of IL-13. IL-33 also plays a role in the activation and proliferation of NH cells (Oboki *et al.*, 2011). DEX has been reported to significantly reduce the IL-33 induced NH cell accumulation in the lung (Kabata *et al.*, 2013). However, in the same study, it was reported that administration of IL-33 in OVA induced allergic airway inflammation in mice significantly

reduced the sensitivity of NH cells to DEX. Thus, the production of IL-5 and IL-13 by NH cells was also resistant to DEX. The important player in the induction of corticosteroid resistance is thymic stromal lymphopoietin (TSLP) (Kabata *et al.,* 2013) which has been noted to be upregulated in asthma (Borish and Steinke, 2011). In asthma, TSLP plays an important role in the Th2 differentiation (Oboki *et al.,* 2011). Airway smooth muscle cells are also known to release TSLP both *in vivo* and *in vitro* (Redhu *et al.*, 2011).

To the best of my knowledge, the study of NH cells sensitivity to FP has not been reported. Nevertheless, NH cells insensitivity to corticosteroid in the presence of TSLP might contribute to the insensitivity of the increased airway hyperresponsiveness to either DEX or FP in this model.

The enhancement of AHR induced by influenza infection alone without OVA sensitisation or challenge was also refractory to the treatment of DEX and FP. TNF-α is also worth to be considered as a reason which underlie the mechanism of the AHR insensitivity to corticosteroid. TNF-α is one of significantly identified cytokines in allergic airway inflammation as an inducer of AHR (Thomas and Heywood, 2002). TNF-α is also found in BAL fluid during acute viral respiratory infection (Folkerts *et al.*, 1998). IL-33 in the airways has been up-regulated by TNF-α in a dose dependent manner. IFN-γ which is abundantly released in the course of viral infection, synergistically work with TNF-α to increase the level of IL-33. The TNF-α stimulated IL-33 was resistant to DEX (Prefontaine *et al.*, 2009).

Apart from the IL-33 - IL-13 - NH cells axis previously discussed, a physical mechanism might also underlie the corticosteroid insensitivity. Respiratory tract epithelial cells are the first target of influenza virus infection. In addition, influenza infection leads to epithelial cells lysis, necrosis and apoptosis (Hashimoto *et al.*, 2007). The epithelial shedding might alter the permeability and the barrier function of epithelial cells which

increases the access of methacholine to the smooth muscle cells and increase the AHR. If the infection persists and the repair mechanism's rate is slower than the cell lysis, the integrity of airway epithelial cells would be further destroyed. Corticosteroid has been shown to suppress the early state of epithelial cells migration and proliferation in the repair process (Dorscheid *et al.*, 2006) which might contribute to the insensitivity of the AHR to corticosteroid treatments.

V.4. THE EFFECTS OF CORTICOSTEROIDS ON AIRWAY PROTEIN EXUDATION

Influenza virus infection when given simultaneously with OVA challenge in OVA sensitised mice increased the protein content recovered in BAL fluid even though not statistically significant. Interestingly, treatment with DEX or FP which was expected to reduce the protein level, in fact significantly increased the level of protein in BAL fluid compared to vehicle treated mice.

Asthma associated protein leakage has been thought to originate from capillary leakage because of the loose intracellular gaps (McDonald, 1994). In influenza infection, this condition is worsened by the epithelial shedding due to viral infection which also alters the permeability of epithelial-capillary barrier and allows plasma extravasation (Bozanich *et al.*, 2008).

Corticosteroids are capable of reducing the increased capillary permeability and plasma leakage into the respiratory lumen in asthma (Nocker *et al.*, 1999). In rat sensitised with OVA, DEX reduced the protein leakage (Damazo *et al.*, 2001). In previous studies discussed in CHAPTER 7, DEX and FP also slightly reduced the enhanced protein content recovered in BAL fluid even though not statistically significant. However, when viral infection which given concurrently with antigen challenge is treated with corticosteroid, the protein originating from cell lysis, necrosis,

and apoptosis might becoming more accumulated as a result of viral infection and corticosteroid induced apoptosis.

The proteins can also originate from the increased release of inflammatory proteins. The airway lining fluid is known to contain some major antimicrobial component to protect it from infection (Travis *et al.*, 1999). Influenza infection activates the innate immune system such as collectins and defensin to restrict the spread of the virus. Collectins including mannose binding lectins (MBL), surfactant protein D (SP-D), and surfactant protein A (SP-A) which are present in the lung and play an essential role in inhibiting influenza virus replication (Whitsett, 2005; White *et al.*, 2008)

In this study, influenza infection, OVA challenge, and corticosteroid when given concurrently potentiated the protein content in the alveolar compartments. Corticosteroids have been reported to upregulate SP-D and SP-A *in vivo* and *in vitro*. DEX doubled total SP-A level in the rat lungs (Floros *et al.*, 1989). The expression of SP-D mRNA is also upregulated by corticosteroids *in vivo* and *in vitro* (Deterding *et al.*, 1994). Corticosteroids also do not inhibit the expression of defensin (van Wetering *et al.*, 2002).

As previously discussed, Schleimer (2004) argued that corticosteroid spare innate immune responses if not enhance their expression in response to infection. This could also explain why the protein content in BAL fluid from mice sensitised with OVA, infected with influenza, challenged with OVA, and treated with corticosteroids were significantly higher than any other groups.

V.5. THE EFFECTS OF CORTICOSTEROIDS ON VIRAL TITRE

Corticosteroid intervention in the course of acute infection has been debated as there is a possibility that the immunosuppressive properties of corticosteroids might facilitate viral replication and increase viral titre which could harm the patient. In this study, treatment with corticosteroids did not significantly alter the viral titre in either OVA sensitised/OVA challenged mice or saline sensitised/non-challenged mice.

VI. CONCLUSION

The series of studies demonstrated that systemically delivered DEX was able to reduce the influx of inflammatory cells in the airways when influenza infection was given concurrently with OVA challenge in OVA sensitised mice. The numbers of eosinophil and lymphocyte was reduced by DEX, but not the number of macrophages and neutrophils. Despite being able to reduce the augmented late phase responses, DEX did not reduce the baseline respiratory functions 19 hours after the OVA challenge. Airway hyperresponsiveness was also insensitive to DEX treatment. In mice sensitised with saline, infected with influenza but not receiving any allergen challenge, DEX did not significantly alter the number of total inflammatory cell recovered in BAL fluid as well as the airway hyperresposiveness.

FP on other hand significantly reduced the number of inflammatory cells influx in the respiratory system with all individual subset of leucocytes' numbers also significantly reduced. FP also reduced the late phase responses. Furthermore, the suppression of late phase reaction or the low bronchoconstriction was preserved 19 hours after the second OVA challenge when the last Penh reading was taken. Airway hyperresponsiveness though, was not significantly altered by FP. The FP effects in saline sensitised, influenza infected, unchallenged mice were quite similar to those of DEX. FP did not significantly alter both leukocytes influx in the airways and airway hyperresponsiveness.

The treatment of DEX and FP significantly increased the level of protein in BALF but did not significantly change viral titre in the lungs. Therefore, taken together, the findings suggest that some features of asthma portrayed in this virus associated exacerbation model of airway allergic inflammation such as the airway hyperresponsiveness are selectively resistant to corticosteroid treatment.

CHAPTER IX

GENERAL DISCUSSION

I. THESIS AIMS

Asthma is a major health problem affecting almost 300 million people worldwide. It is characterised by airway inflammation with inflammatory cells particularly eosinophils, T lymphocytes, and mast cells largely present in the airway and involved in the process of inflammation (Barnes, 1996; Tattersfield *et al.*, 2002).

Airway hyperresponsiveness or exaggerating airway response to stimuli that would not cause any response to people without asthma is also one of the important asthma features (Grootendorst and Rabe, 2004). In addition, airway remodelling which is defined as airway structural change due to sub-epithelial fibrosis, goblet cell hyperplasia, angiogenesis, and smooth muscle thickening is also observed in chronic asthma (Fish and Peters, 1999; Bousquet *et al.*, 2000).

In allergic asthma, the most common type of asthma, which affect two third of the asthmatic population (Temelkovski *et al.*, 1998) the progression of the disease is started when the person is sensitised with allergen. Dendritic cells detect the allergen which is presented to T and B lymphocytes in the lymph nodes. This process initiates the production of allergen specific IgE by B cells. The allergen specific IgE would then bind to the high affinity receptor (FCεRI) on the surface of mast cells. Future allergen exposure will cause a cross linking with this IgE- FCεRI on the surface of mast cells and lead to mast cells degranulation with subsequent release of inflammatory mediators. The release of the mediators results in early asthmatic responses (EAR) with features including bronchoconstriction which starts immediately after allergen challenge. Some asthmatics are dual responders which mean they also experience late asthmatic responses (LAR) after the EAR which commonly resolves in 24 hours (Picado, 1992). This cascade is thought to contribute to the influx of inflammatory leukocytes, development of airway hyperresponsiveness, and further airway remodelling.

Asthma is generally present as a chronic inflammation with periodic exacerbation at some points of the stable disease. Virus infection is the most common cause of acute asthma exacerbations. Rhinovirus, respiratory syncytial virus, and parainfluenza virus are the most frequently identified virus in the case of virus associated asthma exacerbation. Influenza is also a significant cause of asthma exacerbation particularly during the H1N1 2009 pandemic (Jain *et al.*, 2009).

With regard to treatment, corticosteroid based therapy remains the most effective treatment for asthma at the moment. However, the use of corticosteroid in virus associated asthma exacerbation is still controversial. In theory, corticosteroid should be able to effectively reduce the inflammation due to viral infection, but some evidence shows that the administration of corticosteroid alone is only partially effective in virus associated asthma exacerbation (Tattersfield *et al.*, 1999; FitzGerald *et al.*, 2004; Harrison *et al.*, 2004).

This thesis sought to establish the role of acute viral infection when combined with allergen exposure in allergen-sensitised host. The thesis also determined if there is any alteration of corticosteroid effectiveness in the exacerbations or worsening of allergic airway inflammation associated with viral respiratory infection. The study was carried out using a mouse ovalbumin (OVA) model of allergic airway inflammation. The murine ovalbumin model was subsequently developed by combining allergen (OVA) and virus infection to further evaluate the interaction between the two. The developed models were then used to study the role of either systemically delivered (dexamethasone/DEX) or topically delivered (fluticasone propionate/FP) corticosteroid.

The endpoints evaluated and assessed were inflammatory cells influx, early and late phase asthmatic responses (EAR and LAR), airway hyperresponsiveness (AHR), bronchoalveolar lavage protein content, viral titre in the lungs, and histopahological features.

Most asthma studies using murine models do not include the important endpoints such as EAR and LAR as part of their assessments. AHR is probably the most measured physiological function by investigators in murine models of allergic inflammation studies. However, measuring AHR only would not completely portray what really happens in the clinic, whereas the animal model should be developed as closely as possible to resemble the disease (in human). The physiological endpoints such as EAR and LAR are more important to be a part of assessments when studying the efficacy of a treatment or the role of viral infection in the murine model of allergic inflammation to get a more complete reflection of the clinical implications resulting from the interventions.

II. OUTCOMES

II.1. *IN VITRO* ANTIVIRAL STUDIES

The series of studies was started with *in vitro* assays to assess whether corticosteroids (budesonide, dexamethasone, fluticasone propionate, and prednisolone) that would be used in the studies had any inhibitory effects on the viral targets (parainfluenza virus type 3 and influenza A /H1N1/PR8). A cell based system using the viral plaque reduction assay and RT-PCR assay was used to assess the antiviral activity of corticosteroids against parainfluenza virus type 3. Ribavirin was used as a positive control. DEX had an inhibitory effect against the virus, but not the other corticosteroids. Gao *et al*'s study (2000) showed that HPIV3 infection upregulate the ICAM-1 expression in epithelial cells which has been thought to facilitate viral entry into the cell target (Gao *et al.*, 2000). Studies to evaluate the effect of corticosteroids on ICAM-1 expression were carried out but the inhibitory effect of corticosteroids on HPIV3 infection had no correlation with the level of ICAM-1 expression. For this reason, DEX might not inhibit HPIV3 replication via ICAM-1 pathway.

The antiviral activity of corticosteroids against influenza infection was examined using a cell based plaque reduction assay with zanamivir as a positive control. Again, DEX was the only corticosteroid to have the inhibitory effect against virus infection among all corticosteroids tested.

The antiviral effects might be mediated by the NF-kB pathways, as corticosteroids reduce or inhibit the NF-kB activity (Barnes and Adcock, 2003) and some influenza virus is known to upregulate the NF-kB (Flory *et al.*, 2000; Hiscott *et al.*, 2006; Kumar *et al.*, 2008) for its own benefits. However, reducing the NF-kB activity is not the only mechanism that might underlie the antiviral properties of DEX as other corticosteroids tested too are known to inhibit NF-kB pathway. Solubility in media might also influence the higher antiviral activity of the DEX compared to other corticosteroids tested as DEX is the only water soluble corticosteroid among others which are quite liphophilic.

II.2. *IN VIVO* INFECTION STUDIES

Respiratory viral infection is the major culprit to cause asthma exacerbations. The following studies to be conducted after *in vitro* assay were *in vivo* screening of respiratory infective agents. The first candidate was human parainfluenza virus type 3. HPIV3 has been proven to cause a profound allergic airway inflammation exacerbation in guinea pigs in our laboratory (Ford *et al.*, 2013). However, despite being given in either anaesthetised or un-anaesthetised mice, HPIV3 did not seem to infect mice as there was no virus detected in the lungs of HPIV3 infected mice when the lung samples were analysed with either cell plaque assay or RT-PCR.

The unsuccessful infection by HPIV3 in mice led to the screening of another candidate which is poly (I:C), a synthetic dsRNA and TLR3 ligand. The study was successful, a high dose (100µg/50µl) of poly (I:C) instillation in mice was able to induce lung inflammation with neutrophilia accompanied by significant AHR.

Considering that poly (I:C) is a synthetic agent which therefore will not activate the murine immune system as complete as the real virus would, the screening for a respiratory infective agent was continued and led to the pilot *in vivo* infection of mouse adapted influenza A/H1N1/PR8. The influenza virus infected the mice effectively in 10 pfu/mouse dose. The infection successfully caused cellular airway inflammation with neutrophilia, lymphopenia, and marked airway hyperresponsiveness. The dose given was relatively mild in order to more reflect the common influenza virus infection and not severe pneumonia.

The influenza A /H1N1/PR8 is known to induce marked lung cell inflammation and AHR (Brown *et al*., 2004). The development of AHR in influenza infection is thought to involve the IL-33 – NH cells - IL-13 axis (Oboki *et al.*, 2011). IL-33 level in the lung is significantly increased during influenza infection (Le Goffic *et al.*, 2011). The source of IL-33 upregulation which peaked on day 5 post infection is largely originated from macrophages but could be from infected epithelial cells. The IL-33 subsequently induces NH cells to produce IL-13 which play an important role in AHR (Oboki *et al.*, 2011). A defect of M2 receptors is also known to contribute to the increase of AHR in influenza infection (Folkerts *et al.*, 1998).

This influenza A virus was chosen to be used in subsequent study to evaluate the interaction between viral infection and allergen challenge in murine ovalbumin model of allergic airway inflammation. Four days after infection was chosen as the time point for OVA challenge, and five days after infection as the analysis day.

II.3. STUDIES OF THE INTERACTION BETWEEN INFLUENZA VIRUS INFECTION AND ALLERGEN CHALLENGE IN MURINE MODEL OF OVALBUMIN INDUCED ALLERGIC AIRWAY INFLAMMATION

The ovalbumin model of allergic airway inflammation was established by sensitising the mice twice intraperitoneally with OVA and giving them the OVA challenge twice 4 hours apart. The murine model showed the important hallmarks of asthma including eosinophilic airway inflammation, biphasic broncoconstrictions responses (LAR and EAR), and airway hyperresponsiveness (Fernandez-Rodriguez *et al.*, 2008).

The intervention of influenza infection was introduced in the developed OVA murine model 4 days before the OVA challenge. The last procedure and analysis were conducted on day 5 after influenza infection. This is coinciding with the maximum inflammatory responses to influenza alone in the previous studies. Therefore, in the chosen time points, it was expected that the dynamic inflammatory responses would be best reflected in the model.

Allergen challenge in the acute course of influenza infection in OVA sensitised mice exaggerated the inflammatory response demonstrated by a significant increase of inflammatory cells influx in the airways, the profoundly augmented and prolonged the LAR bronchoconstriction, and significant increase in the airway hyperresponsiveness compared to the negative control group which were sham (allantoic fluid) instilled mice.

In contrast to OVA sensitised and OVA challenged mice; OVA sensitised, influenza infected, and saline challenged mice did not develop airway eosinophilia which indicates that OVA challenge is more important than influenza infection to induce eosinophilia. OVA sensitised, influenza infected, and saline challenged group also had significant increase in AHR which was largely due to influenza infection.

The results are in agreement with Marsland *et al* (2004) studies, where influenza infection was given concomitantly with OVA challenge in OVA sensitised mice, the

maximum eosinophilic response was observed when the OVA challenge was given on day 4 after influenza infection. They also observed a significant increase of AHR due to viral infection. Marsland *et al* (2004) conclude that the increasing number of allergen carrying dendritic cells in the lung draining lymph nodes and the migration of allergen specific T lymphocytes underlie the exaggerated immune responses (Marsland *et al.*, 2004).

Kabata *et al.* (2013) who studied the addition of IL-33 in the OVA murine model of allergic airway inflammation also found a marked increase in AHR and airway eosinophilia compared to the group of mice which only being given IL-33 (Kabata *et al.*, 2013). This might explain the exaggerated inflammation demonstrated in the present model.

II.4. STUDIES ON CORTICOSTEROIDS

A good animal model of asthma has to best reflect the features of human asthma. The animal model has also to be sensitive to the mainstay treatment of human asthma, corticosteroid. Dexamethasone (DEX) which is commonly systemically delivered and fluticasone propionate (FP) which is topically (inhaled) delivered in human asthma were used to examine the corticosteroid sensitivity of developed murine model of OVA induced allergic airway inflammation.

Corticosteroids are well documented to effectively reduce the airway cellular inflammation, particularly eosinophilic inflammation in human and animals (Birrell *et al.*, 2003). T lymphocytes play an important role in the airway eosinophilic inflammation by releasing Th2 cytokines including IL-4, IL-5, and IL-13 and some chemokines (Giembycz and Lindsay, 1999). The presence of eosinophil and the Th2 cytokines lead to the development of late asthmatic responses (LAR) and airway hyperresponsiveness (AHR). Corticosteroid acts to reduce the upregulation of the Th2 cytokines and chemokines, as well as induce eosinophil apoptosis, and increase the

clearance of eosinophil into the airway lumen (Belvisi, 2004) which in turn also reduces the LAR and AHR.

The therapeutic benefit of corticosteroids in virus-associated exacerbation of asthma remains controversial. Furthermore there is some evidence showing the lack of efficacy of corticosteroid in exacerbations (Tattersfield *et al.*, 1999; FitzGerald *et al.*, 2004; Harrison *et al.*, 2004).

This thesis aimed to investigate the role of corticosteroid in the developed model of virus associated asthma exacerbation model; whether there is an alteration of corticosteroid efficacy/sensitivity when influenza infection was introduced in allergic airway inflammation.

II.4.1. DEXAMETHASONE

The model was confirmed to be sensitive to DEX which attenuated airway cellular inflammation, reduced eosinophilic inflammation, abrogated late phase bronchoconstriction, and reduced airway hyperresponsiveness in a dose dependent manner. The experiments confirmed the effective dose of DEX which attenuated the airway allergic inflammatory responses i.e. 6 mg/kg DEX.

The augmentation of cellular airway inflammation when influenza infection was combined with OVA (sensitisation and challenge) was sensitive to DEX. In both non infected and infected OVA allergic airway inflammation models, DEX effectively reduced the numbers of eosinophil and lymphocytes. Even though when influenza virus was given superimposed on the OVA induced lung inflammation model, the macrophages and neutrophils numbers which in the non-infected OVA model were sensitive to DEX (fig 7.5), becoming resistant (fig 8.5).

DEX treatment in mice with influenza infection alone did not affect the increasing number of inflammatory cells influx due to influenza infection. The number of macrophages, neutrophils, and lymphocytes were also unchanged by DEX.

The late phase bronchoconstriction in both uninfected and influenza infected OVA models was also responsive to DEX. In the OVA only group, DEX returned the airway functions to baseline 19 hours after the second OVA challenge (24 hours after the first OVA challenge). When influenza was given concurrently with OVA though, DEX was failed to restore the airway function to a baseline level (fig 8.3).

There was an obvious change of DEX sensitivity in the increase of airway hyperresponsiveness when influenza was superimposed in the OVA model. Airway hyperresponsiveness in all influenza infected groups were refractory to DEX, contrasting with the effectiveness of DEX in reducing the airway hyperresponsiveness in OVA only groups.

II.4.2. FLUTICASONE PROPIONATE

The OVA induced allergic lung inflammation was also sensitive to inhaled FP in a dose related manner. The higher dose of FP (0.5 mg/ml) reduced the inflammation features of asthma reflected in the OVA model of mice and was therefore chosen to be subsequently evaluated in the combined influenza and OVA model.

In both OVA allergic lung inflammation groups with and without influenza infection, FP effectively reduced total inflammatory cells recovered in BALF. Regarding the individual subsets of leukocyte numbers, different from DEX, all individual cell type i.e. macrophages, eosinophils, lymphocytes, and neutrophils were sensitive to the treatment of FP in both virus infected and non infected OVA groups.

FP similar to DEX did not change the airway cells inflammation when the mice were given influenza infection alone. The number of lymphocytes though was sensitive to FP.

The effectiveness of FP in attenuating the late phase bronchoconstriction was also unchanged by influenza infection. On either virus infected or uninfected OVA sensitised and OVA challenged animals, FP significantly reduced the late phase bronchoconstrictions, and the respiratory baseline functions at 19 hours after the second OVA challenge (fig 8.13).

Influenza infection however changed the efficacy of FP in reducing the airway hyperresponsiveness when influenza infection was given simultaneously with OVA challenge. FP reduced the increased airway hyperresponsiveness in OVA induced allergic airway inflammation but the airway hyperresponsiveness became refractory to FP when influenza infection was given to the animals.

DEX and FP did not change the increase in protein content in BAL fluid in OVA only group, but significantly increased the level of protein in BALF in mice infected with influenza and challenged with OVA. The corticosteroids also did not significantly change the virus titre in the lungs.

The measurement of Th1 and Th2 cytokines and chemokines could possibly help explain the alteration in corticosteroid efficacy even further. However, some suggestions can be proposed. Firstly, corticosteroids while effective by suppressing the adaptive immune response seem to spare or enhace the innate immune response to the viral infection (Schleimer, 2004). In the case of influenza infection, macrophages and and neutrophils are effector cells of the innate immune response which are responsible for viral elimination (Watanabe *et al.*, 2005) hence both types of leukocytes are significantly upregulated in the influenza infection groups. Corticosteroid increases the activation of both macrophages and neutrophils, induce the survival of neutrophils

but increase the apoptosis of dendritic cells (Schleimer, 2004). The induction of dendritic cells apoptosis by corticosteroid might counteract the increasing activity and number of dendritic cells when influenza infection is given concurrently in with OVA challenge in OVA sensitised mice (Marsland *et al.*, 2004) and further contribute to the reduction of exaggerated inflammatory cells accumulation in the airways, including reducing the number of lymphocytes and eosinophils which regulated by the cytokines released from the activated lymphocytes. The number of macrophages and neutrophils though are preserved in the course of corticosteroid treatment.

Both DEX and FP have a quite similar pattern in altering the proportion of leucocytes subtype recruitments into the airway in the influenza combined with OVA groups i.e. the portion of macrophages was significantly increased; the percentage of eosinophils was decreased; and the neutrophils portion was unchanged from the uninfected OVA allergic lung inflammation groups. The difference in DEX and FP's potencies to downregulate chemoattractants and adhesion molecules which are responsible for the accumulation of the inflammatory cells in the airways could be suggested to underlie their distinct effect in reducing total and individual inflammatory cells when influenza infection was given to the OVA model. FP seems to be more potent than DEX in inhibiting the chemokines and adhesion molecules.

The corticosteroid resistant accumulating macrophages and neutrophils might be involved in the development of persistent airway bronchoconstriction as they are capable of releasing the inflammatory mediators involve in bronchoconstrictions (Folkerts *et al.*, 1998; Liu *et al.*, 1999) which might explain why unlike FP, DEX failed to significantly reduce the respiratory function 19 hours after OVA challenge.

The second proposed mechanism is based on IL-33 – NH cells – IL-13 axis. In the presence of TSLP (thymic stromal lymphopoietin), which upregulated in asthma, NH cells which release IL-5 and large proportion of IL-13 in response to IL-33 (which

increased upon influenza infection) are refractory to corticosteroid. The release of IL-13 which is uninhibited by corticosteroid contributes to the refractory airway hyperresponsiveness (Kabata *et al.*, 2013).

The third proposed mechanism is the increase of nervous sensitivity to methacholine due to epithelial shedding. The loss of barrier function of the shed epithelium leads to the increasing exposure of smooth muscle cells and nervous system to methacholine chloride provocation causing an elevation in airway hyperresponsiveness.

III. EXPERIMENTAL LIMITATIONS

The mouse model of asthma is not the most reliable animal model to predict human asthma (Zosky and Sly, 2007). Mouse furthermore does not spontaneously develop asthma. So it has to be induced, generally by sensitisation with allergen and subsequent challenge with the same allergen to obtain the allergic response. The use of ovalbumin (OVA) as specific protein antigen to induce asthma in mice has also been criticised to have a lack of relationship with common allergen causing human asthma, such as house dust mites.

Nevertheless the OVA model of asthma is still popular for modelling allergic response as BALB/c mice genetically have a Th2 biased immune response. The OVA model displays most essential features of human asthma such as upregulation of OVA specific IgE expression, eosinophilic airway inflammation, EAR and LAR, and AHR as well as being sensitive to the basic therapies of asthma (Stevenson and Birrell, 2011).

The acute OVA model used in these studies might also raise another caveat of the thesis. Asthma is a chronic disease which is characterised by airway remodelling. The current acute OVA model lacks this chronic feature. Nonetheless, this model does resemble most of the essential acute allergic airway inflammatory features in asthma.

The term exacerbation in this current study of a murine model of allergic airway inflammation might also raise a concern as the virus infection was given in sensitised mice before antigen challenge hence the infection is administered before the allergic response. However, the term exacerbation in this model is defined as the exaggerated airway allergic inflammatory responses and the worsening of respiratory functions as seen in the exacerbation of asthma in atopic individuals (Busse and Lemanske Jr., 2007). As in any other animal model the mouse asthma or asthma exacerbation model does not fully replicate human asthma. Several models and design of studies using different species of animals have been applied to model human asthma which demonstrated that there is no generally validated model of human asthma (INTRODUCTION to CHAPTER 5). Nonetheless, the model of virus-associated exacerbation of allergic airway inflammation developed in this thesis demonstrated characteristics of human asthma exacerbation i.e worsening symptoms of human asthma. These includes exaggerated cellular inflammation with neutrophilia, significant peribronchial inflammation as shown in histopathological analysis, worsening of respiratory functions, significant increase in airway hyperresponsiveness, and plasma leakage in broncho-alveolar lavage fluid (CHAPTER 6). The aggravated airway inflammation and respiratory functions were significantly worse compared to those in animals without respiratory viral infection which is also seen in human asthma (Message *et al*., 2008).

Airway function was determined using non invasive whole-body unrestrained plethysmography. Penh (enhanced pause) is a dimensionless index based on airflow patterns of expiration and inspiration as the animal breathes which changes the box signal. The bronchoconstriction will also alter the enhanced pause. Penh has been criticised to have a limited physiological outcome and does not actually determine airway mechanics/resistance (Lunblad *et al.*, 2002). However, Lomask (2006) has argued and showed that Penh has the effect of resistance in flow whole-body

plethysmoghraphy waveform from which Penh is mathematically derived (Lomask, 2006). Furthermore, Hamelmann *et al.* (1997) have experimentally proven that Penh in methacholine dose response curve measurements observed in BALB/c OVA allergic airway inflammation model is correlated to airway resistance (Hamelmann *et al.*, 1997). Whole-body plethysmography also offers some advantage over the more invasive methods as the airway function observation can be repeated and conducted overtime allowing the long period measurement such as the assessment of EAR/LAR and the evaluation of methacholine provoked airway responsiveness before and after treatment.

The findings in these current studies might not be applicable to be generalised to other respiratory infection. The difference in the nature of inflammation of respiratory synctial virus (RSV) and influenza A infection superimposed on house dust mite (HDM) model has been noted in a recent study. The same study also revealed that the sensitivity of corticosteroid (Prednisolone) was also different between the RSV/HDM and influenza/HDM. Influenza/HDM features was more sensitive to prednisolone than RSV/HDM (Mori *et al.*, 2013).

Nevertheless an animal model of asthma is only a tool to effectively investigate the pathophysiological mechanism of the disease and needs a very judicious approach to extrapolate to human asthma as it only portrays a snapshot of a very complex and heterogeneous nature of the disease.

IV. FUTURE WORKS

There are several studies that can be addressed in the future to complete the current work in the thesis. Firstly, the investigation of several key cytokines (IL-4, IL-5, IL-9, IL-13, IL-33, IFN-γ, TNF-α, and TSLP) and chemokines (IL-8, or KC in mouse) and inflammatory mediators produced by inflammatory cells would be useful to further characterise the number of mechanism/pathways involved in the alteration of corticosteroid efficacy when influenza infection is given superimposed on the OVA induced allergic lung inflammation. The study of NH cells sensitivity to corticosteroid in the current model using fluorescence-activated cell sorting (FACS) would also be interesting to be conducted.

Second proposed further works is to develop a chronic murine OVA allergic lung inflammation model to study the interaction between influenza infection and repeated allergen challenge. The model can then be used to study the role of corticosteroid which will be more closely resembles the human asthma.

V. GENERAL CONCLUSION

In this series of experiments, a murine model of allergic airway inflammation exacerbation using respiratory virus infection has been developed. Influenza infection acted additively with OVA challenge in OVA-sensitised mice resulting in profoundly augmented inflammatory cell influx in the airways, late phase bronchocnstriction, and airway hyperresponsiveness. Mouse-adapted influenza A/H1N1/PR8 acutely induced airway inflammation with profound AHR. The low dose infection (10 pfu/mice) was able to provoke sufficient inflammation without pneumonia which was suitable to be incorporated with the established OVA induced allergic airway inflammation in mice.

The effectiveness of corticosteroids (DEX or FP) in these models has also been established. The OVA lung allergic inflammation model reflected most of the essential features of human asthma including airway cellular inflammation with eosinophilia, early and late phase bronchoconstrictions, and airway hyperresponsiveness which all were sensitive to corticosteroid treatment except the early phase bronchoconstriction. Unlike the OVA only model, the influenza-OVA model was only partially responsive to corticosteroid treatment which suggests there are some components in the model which are refractory or not regulated by corticosteroid.

These models offer a way to understand the immunopathological mechanisms underlying the alteration of corticosteroid effectiveness in viral-associated asthma exacerbation in man and also will meet the need in the process of novel drug discovery, development and evaluation. This animal model of viral-associated exacerbation of allergic airway inflammation and evaluation of the roles of corticosteroid sensitivity in this model will provide invaluable insight to address unresolved questions regarding the interplay of respiratory virus infections, allergen, asthma, and corticosteroids.

CHAPTER X

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APPENDICES

APPENDIX 1

1. EQUIPMENT

Amicon Ultracel® 100K tube – Merck Millipore, USA

Blotting cassettes – Fisher Scientific, UK

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.

CO_2 Incubator – Hera Cells, Thermo Scientific, UK

Coverslip - Fisher Scientific UK, Loughborough, UK.

Digital Camera (Leica DC500) - Leica Microsystems, Germany

Egg Incubator – Maino, Italy

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.

Mouse Isolator – Harlan, UK

Neubauer Haemocytometer - Superior, 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.

Well plate (6, 24, 96) - Fisher Scientific UK, Loughborough, UK.

Western Blot system – GE Healthcare Lifesciences

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

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

2. MATERIALS

Acetic acid - Sigma, Poole, UK.

Acrylamide - Sigma, Poole, UK.

ACTB antibody – Abcam, UK

Aluminium hydroxide - Sigma, Poole, UK.

Antimouse IgG HRP antibody – Vector Laboratories, USA

Antirabbit IgG HRP antibody– Vector Laboratories, USA

Aprotinin - Sigma, Poole, UK.

Aqueous acetic acid - Sigma, Poole, UK.

Avicel – FMC Biopolymer, USA

Avidin-Biotin-Peroxidase Complex – Vector Laboratories, USA

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

Beta-Mercaptoethanol – Invitrogen, USA

Bovine Serum Albumins - Sigma, Poole, UK

Bromophenol blue - Sigma, Poole UK

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

Budesonide – Sigma, Poole UK

cDNA primers - Primer design Ltd

Chloroform - Sigma, Poole, UK.

Chloral hydrate - Fisher Scientific UK, Loughborough, UK.

Cloning supplement – Invitrogen, USA

Crystal Violet - Sigma, Poole, UK.

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

Dimethyl sulfoxide - Sigma, Poole, UK.

DMEM - Invitrogen, USA

DPBS – Sigma, Gibco, UK

ECL developing media – Amersham, UK

EGTA disodium salt - Sigma, Poole, UK.

Embryonated chicken egg (Dekalb white) – Henry Stewart Co., UK

Eosin - Surgipath Europe Ltd, Peterborough, UK.

Ethanol - Sigma, Poole, UK.

Fluticasone propionate - Sigma, Poole, UK.

Foetal bovine serum - Sigma, Poole, UK.

Formaldehyde - Fisher Scientific UK, Loughborough, UK.

GAPDH primary antibody – Abcam, UK

Glutamine - Sigma, Poole, UK.

Haematoxylin - Fisher scientific, UK

Histoclear - Fisher scientific, UK

Histomount - Fisher scientific, UK

Human Parainfluenza Virus Type 3 - National Collection of Pathogenic Viruses, UK

Hydrochloric acid - Sigma, Poole, UK.

Hybridoma cells - Given by Dr. Awen Gallimore and Dr. Sarah Lauder (Cardiff University)

Hyperfilm – Amersham Biosciences, UK

IL-6 – Peprotech, UK

IMDM – Invitrogen, USA

Influenza A (H1N1/PR8) - Given by Dr. Awen Gallimore and Dr. Sarah Lauder (Cardiff University)

Insulin transferrine selenium – Invitrogen, USA

Leishman's Powder - Sigma, Poole, UK.

Leupeptin - Sigma, Poole, UK.

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

MAb Trap Kit™ - GE Healthcare Lifesciences, USA

Magnesium sulphate - Fisher Scientific UK, Loughborough, UK.

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

MDCK cells – Given by Dr. Awen Gallimore and Dr. Sarah Lauder (Cardiff University)

Methacholine Chloride - Sigma, Poole, UK.

Methanol - Fisher Scientific UK, Loughborough, UK.

Mice (BALB/c) - Harlan, UK

Milk (Powdered, non-fat) – Shop bought.

Monoclonal Mouse HPIV3 antibody – AbD Serotec, UK

Monkey ICAM-1 monoclonal antibody – Abcam, UK

MTS cell proliferation assay reagent – Promega, UK

Nitrocellulose membrane – Amersham Biosciences, UK

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

Ovalbumin - VWR International Ltd., Leicestershire, UK.

Paraffin wax - Surgipath Europe Ltd, Peterborough, UK.

Penicillin/streptomycin – Invitrogen, USA

Peptone primatone – Sigma, Poole UK

Periodic acid - Surgipath Europe Ltd, Peterborough, UK.

PMSF - Sigma, Poole, UK.

Phenylarsine oxide - Sigma, Poole, UK.

Picric acid - Sigma, Poole, UK.

Poly (I:C) – Invitrogen, USA

Potassium alum - Fisher Scientific UK, Loughborough, UK.

Prednisolone – Sigma, Poole UK

Ribavirin – Sigma, Poole UK

Saline tablets - Fisher Scientific UK, Loughborough, UK.

Sodium bicarbonate - Fisher Scientific UK, Loughborough, UK.

Sodium chloride - Sigma, Poole, UK.

Sodium dodecyl sulfate- 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

NaVO4 - Sigma, Poole, UK.

Sulphuric acid - Sigma, Poole, UK.

Superscript first-strand synthesis system kit - Invitrogen, USA

TEMED (N,N,N′,N′-Tetramethylethylenediamine) - Sigma, Poole, UK

Triton X - Sigma, Poole, UK.

Trizma base - Sigma, Poole, UK.

Trypsin-EDTA – Invitrogen USA

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).

Zanamivir – Given by GSK, Stevenage UK

3. COMPUTER PROGRAMS

Data acquisition - Fine point software, Buxco

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

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

Leica QWin software - Leica Microsystems, Germany

APPENDIX 2

Figure A. displays a typical waveform of a flow whole body plethysmography. The index assessed is Penh (enhanced pause) which correlate to the airway resistance. In a single breath in the case of bronchoconstriction (following administration of bronchoconstritor) the slowing of late expiration correlates to Penh. PIP is peak inspiratory pressure, while PEP is peak expiratory pressure. Ti defined as the inspiratory time from the start to the end of inspiration, Te is expiration time from the end of inspiration to the end of expiration, and Tr is an early relaxation phase of expiration when pressure decays to 36% from the total expiratory pressure signal (Lomask, 2006). Therefore Pause is

Pause = (Te-Tr)/Tr

And Penh is calculated as follow

P_{enh} (Enhanced pause) = (PEP/PIP) x Pause

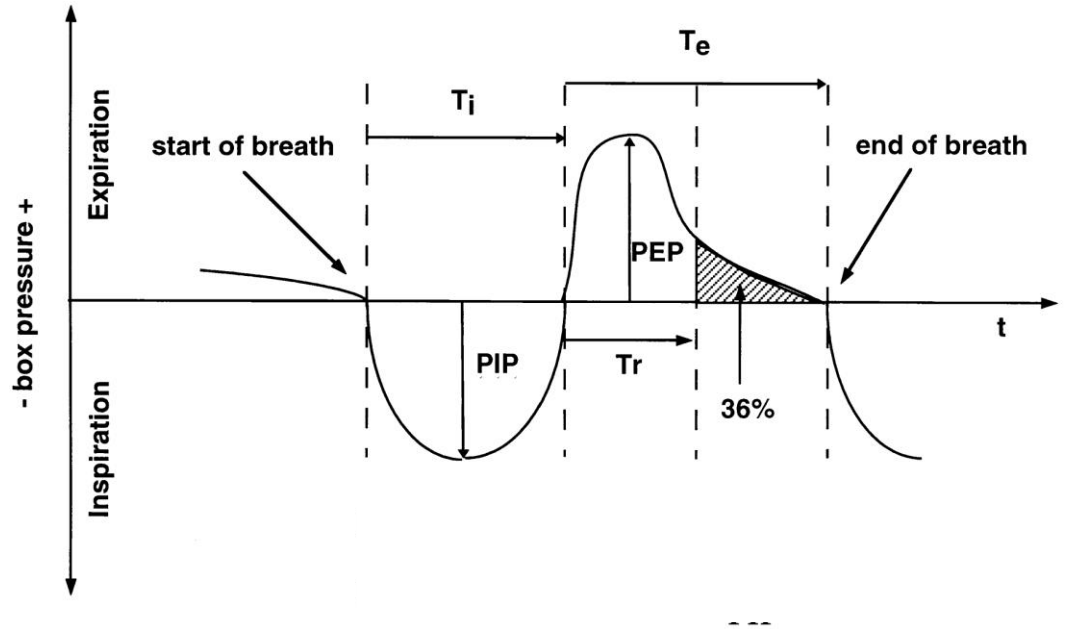

Figure A: A diagram with some parameters which Penh is derived (Hamelmann *et al.,* 1997).