Mechanisms underlying the resolution of HDM induced allergic airways disease

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Declaration

The work presented in this thesis was conducted by me with the following exceptions: H&E and PAS immunohistochemistry was carried out by Lorraine Lawrence. Lung function machine was operated by Simone Walker. FPR2 immunohistochemistry was carried out by Lisa Gregory. Macrophage bone marrow cell cultures and ELISAs for cytokine and chemokine secretion were carried out by Dean Davda. RT-PCR for Annexin A1 and FPR2 expression was carried by Stefania Bena and Mauro Perretti at William Harvey Research Institute, Barts London. Eicosanoid analysis was carried out by Victoria Hammond, Madhav Mondhe and Val O'Donnell at Cardiff University, UK.

Abstract

Allergic asthma is a chronic inflammatory disease of the lung and deficiencies in pro-resolving mechanisms may contribute to the persistence of inflammation. The overall aim of this project was to establish a resolution model of house dust mite (HDM) induced allergic airway disease (AAD) and identify mediators of resolution.

In our model, features of disease, induced by HDM at peak disease 4 hours, airway hyper-reactivity (AHR), Th2 lymphocytes and eosinophils remained significantly elevated 7 days after last challenge, resolving to baseline by 13 days. The levels of FoxP3+ regulatory lymphocytes also follow this pattern. However, as disease waned there was an elevation in the levels of alveolar macrophages and up regulation of the homeostatic molecule CD200R up to 13 days. Exposure to a single i.n administration of HDM in the resolved airways resulted in a rapid increase in Th2 inflammation and AHR suggesting that after resolution of HDM inflammation there is altered immune homeostasis in the lung. The pro-resolving lipid Lipoxin A4 was induced in the lung by HDM exposure and remained detectable during resolution. Depletion of alveolar macrophages during the resolution phase of allergen challenge resulted in delayed clearance of Th2 lymphocytes, airway neutrophils and interstitial macrophages. Conversely, adoptive transfer of alveolar macrophages during resolution resulted in reduced numbers of lung tissue leukocytes, specifically neutrophils and interstitial macrophages. This suggests a cross talk between these macrophage subsets and a novel interaction for pulmonary homeostasis. The anti-inflammatory peptide Annexin A1 is highly expressed by alveolar macrophages and mice deficient in Annexin A1 had enhanced AHR and Th2 immunity response to HDM. Blocking the Annexin A1 receptor FPR2 enhanced AHR and lung inflammation. Conversely, therapeutic administration of an Annexin A1 mimetic improved AHR and Th2 immunity. These studies demonstrate that Annexin A1: FPR2 pathway may be important in HDM disease and that resolution of allergic airways disease is an active process resulting in altered homeostasis of the lung.

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Chapter 1. Introduction

1.1 Allergic asthma

Allergic asthma is a chronic inflammatory disease that affects approximately 300 million people worldwide, (Masoli et al 2004) and is the most common chronic disease in children (www.WHO.int.org 2008). The disease is characterised by airway hyper-reactivity (AHR), allergic inflammation, mucus production and airway remodelling. Inflammatory infiltrates in the lung are frequently composed of eosinophils and type 2 helper lymphocytes (Th2) Th2 lymphocytes. The risk factors for developing asthma include genetic predisposition or environmental influences such as childhood infections, inhalation of pollutants or allergens that induce irreversible damage to the pulmonary structure and immune environment (Moffatt, Gut et al. 2010) (von Mutius 2009). In susceptible individuals, inhalation of an innocuous protein or virus can promote an aberrant immune response resulting in the clinical symptoms of asthma (Holgate 2008) (Bousquet, Jeffery et al. 2000).

Current therapies consist of leukotriene receptor antagonists and corticosteroids to dampen inflammation, along with long acting β-agonists to relax the airway smooth muscle and restore airflow (Holgate 2013). However, none of these treatment regimens “cures” asthma. The majority of allergic asthma research has focused on finding more selective inhibitors of inflammation specifically targeting either the initiation of the inflammatory response or adaptive immune mechanisms involved in pro-inflammatory pathways of Th2 mediated allergic inflammation. Trials with biologics to target Th2 cytokines revealed subpopulations of responders, however the overall outcome is of small clinical impact and therapies have not advanced beyond inhaled steroids and bronchodilators (Holgate and Davies 2009) (Holgate, Arshad et al. 2010(Wenzel 2012, Holgate 2013). It is becoming apparent from these trials that asthma is a heterogeneous disease with phenotypes and aetiology of disease inception likely to be different between these groups. A cluster analysis of a severe asthma resistant patient dataset in the US grouped the range of recorded phenotypic characterisations, that included, lung function, age of onset, symptoms, medication use, into five distinct phenotypes which supports clinical heterogeneity in asthma and the need for new

approaches for the classification of disease severity in asthma. (Moore, Meyers et al. 2009). Very recently, a study in paediatric severe therapy resistant patients showed that these children exhibited symptoms of asthma and reduced lung function without significant elevation of Th2 cytokines (Bossley, Fleming et al. 2012). Ultimately the goal of asthma treatment will be to stratify adult and paediatric patients based on biomarkers and predict the best therapeutic options on an individual basis. However, this task requires further understanding of the mechanisms that may contribute to disease (Holgate 2012, Wenzel 2012, Holgate 2013).

An emerging concept in understanding disease pathogenesis is that deficiencies in pro-resolution pathways may contribute to chronic inflammation (Haworth and Levy 2007). The resolution of acute inflammation is required for continuing health. It is driven by temporal and spatial upregulation of mediators that promote a return to tissue homeostasis. (Serhan, Brain et al. 2007, Planaguma and Levy 2008). In asthma, resolution lipids Lipoxin A4 derived from archidonic acid, and Protectin D1 synthesised from omega 3 acid, have been identified in the lung and peripheral blood of cohorts of patients. (Levy, Kohli et al. 2007, Planaguma, Kazani et al. 2008). However, the specific mechanisms involved in the resolution of allergic airway inflammation are not fully understood and will be the focus of this thesis.

1.2 Allergic inflammation

Allergic inflammation is a characteristic feature of allergic asthma (Bousquet, Jeffery et al. 2000) (Barnes Immunol Rev 2011). There is a complex interplay between cells including mast cells, eosinophils, dendritic cells (DC) and CD4 lymphocytes and their soluble mediators. Inflammatory cells produce an array of cytokines, chemokines and lipids that drive Th2 inflammation and are associated with the features of asthma disease such as mucus production, airway hyper-reactivity, collagen deposition and reticular basement membrane thickening. The classic paradigm for the pathophysiology of allergic inflammation has traditionally been attributed to elevated levels of Immunoglobulin E (IgE)

that recognises allergen, the subsequent binding of the high affinity FCεR1, and rapid degranulation of mast cells. The contents of mast cell granules can simultaneously promote AHR directly and indirectly via initiation of the adaptive Th2 inflammatory cascade (Bradding, Walls et al. 2006). Initiation of CD4 Th2 inflammation follows antigen presentation by dendritic cells and is characterised by the ensuing production of type 2 cytokines, IL-4, IL-5, IL-9 and IL-13. However, it has become apparent that this disease model is too simplistic and there has been a shift in the thinking behind the asthma paradigm (Holgate 2011, Wenzel 2012). The discovery of a new family of leukocytes, the innate lymphocyte cells (ILCs) has exposed new avenues for investigation (Spits, Artis et al. 2013). A subset of these, ILC2, have been shown to be a major source of "adaptive" Th2 cytokines particularly IL-13 and these cells may play a crucial role in asthma (Scanlon and McKenzie 2012). ILCs are distinct from adaptive T-helper lymphocytes as they do not express a T cell receptor, are lineage negative lacking CD3 and CD4, and have a different lineage to CD4 lymphocytes (Spits, Artis et al. 2013). Importantly, these innate cells reside in the airway mucosa and may be activated as opposed to via the lymphatics by DC mediated signals. This illustrates that the pathophysiology of allergic inflammation is more complex than first considered. Moreover, innate associated cytokines have also been described as critical in the pathogenesis of Th2 disease. These include IL-33, IL-25 and thymic stromal lymphopoietin (TSLP) that are primarily produced by epithelial cells and are thought to be early initiators of Th2 inflammation (Hurst, Muchamuel et al. 2002) (Ziegler and Artis 2010) (Lloyd 2010). These more recent findings challenge the conventional model of disease pathogenesis and present a more intricate network of both innate and adaptive pro-inflammatory mediators working in concert to direct inflammatory response of the lung to inhaled environmental stimuli.

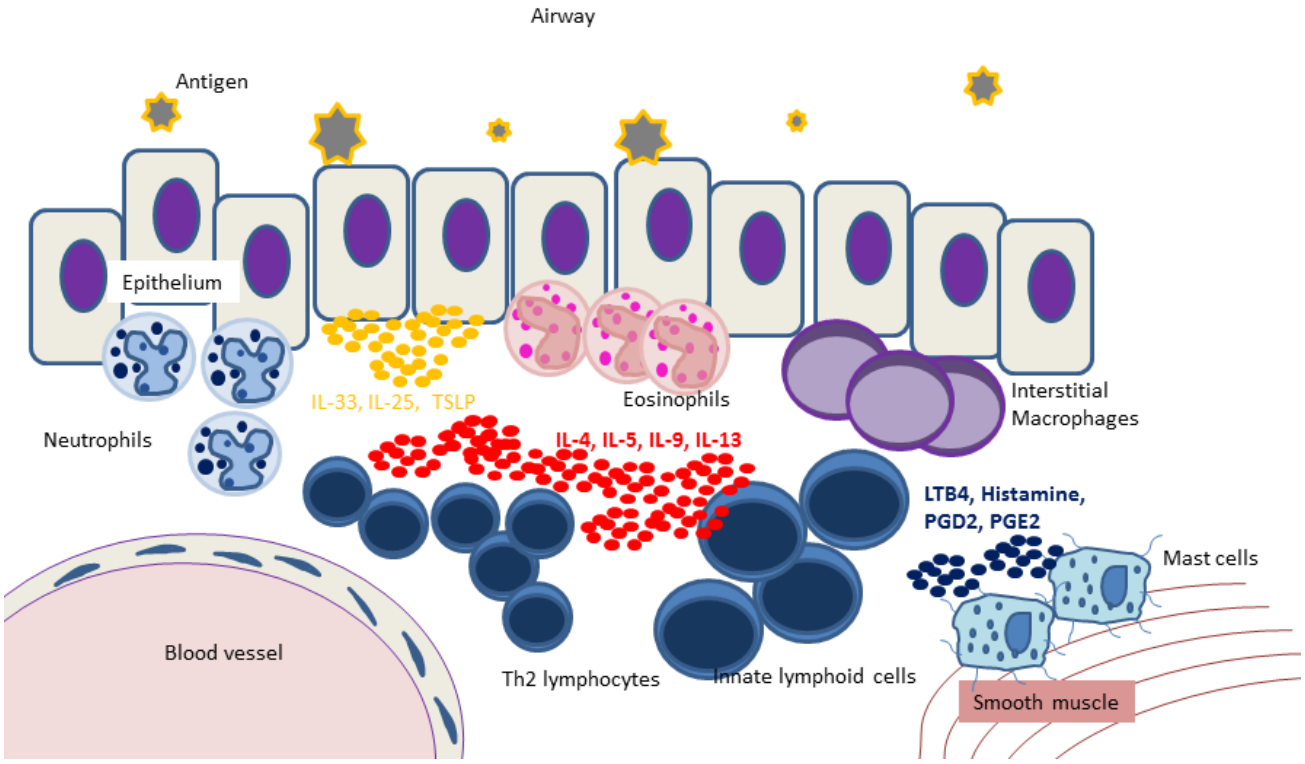


Figure 1.1 Schematic representation of cellular and soluble inflammatory mediators in the allergic airway. Following inhalation of allergen the airway epithelium secretes cytokines IL-25 IL-33 and TSLP to propagate Th2 type inflammation. This includes mast cells, eosinophils, and neutrophils. Lymphocytic populations such as Th2 lymphocytes and ILCs are recruited and amplify the response by secreting Th2 cytokines IL-4, IL-5, IL-9 and IL-13. Antigen binding by IgE and receptor cross linking on mast cells promote degranulation of lipid mediators that drive bronchoconstriction and airway hyperactivity.

1.2.1 Mast cells

Mast cells are widely distributed around the body at connective tissue and mucosal sites and are considered to play an important role in tissue homeostasis, wound healing and host defence against bacterial infections (Bradding and Holgate 1999, Bradding, Walls et al. 2006). Granules situated within the cytoplasm comprise an array of preformed lipids, proteins and nitrous oxide that upon mast cell activation can be rapidly released to exert a potent response to local injury. In the asthmatic lung, mast cells have been found to be located in the bronchial smooth muscle and considered to contribute to the pathophysiology of disease. The dominant signal for mast cell activation is via its high affinity receptor, FcεR1 (Siraganian 2003). They are also an early source of inflammatory mediators following allergen exposure. Atopic asthmatics have enhanced levels of IgE compared to non-asthmatics (Humbert et al 1996). Allergen bound IgE complex binds to its receptor, FCεR1, expressed on mast cells. Cross linking of the receptor promotes a rapid degranulation and release of potent pre-formed autacoids. These include histamines, leukotrienes (LT) and prostaglandins (PG) which promote bronchospasm and feature in the early asthmatic reaction (EAR) (Bradding, Walls et al. 2006).

Mast cells also secrete Th2 cytokines that can prime local DCs to promote an adaptive Th2 immune response (Bradding, Walls et al. 2006) and are a source of the growth factors transforming growth factor β (TGFβ) and fibroblast growth factor 2 (FGF2). These growth factors have been implicated in the remodelling of the airway submucosa and described to have a role in smooth muscle proliferation and collagen deposition in the lung (Tourdot, Mathie et al. 2008, Kariyawasam, Pegorier et al. 2009).

1.2.2 Eosinophils

Eosinophils are traditionally considered to be an important component of the innate immune response to helminth and parasitic infections (Trivedi and Lloyd 2007). These cells, like mast cells, possess pre-formed granules containing cationic proteins that

include major basic protein and eosinophil peroxidase that elicit parasiticidal effects (Kariyawasam and Robinson 2006).

In allergic individuals eosinophils feature in abundance in the allergic inflammatory foci of the lung and airway and are shown to correlate with severity of disease (O'Byrne and Inman 2003). Recruitment of eosinophils to the airway is driven by Th2 facilitated chemokine production of Eotaxin1/CCL11, Eotaxin2/CCL24 and Eotaxin3/CCL26 acting via CCR3 (Gutierrez-Ramos, Lloyd et al. 1999). Eosinophil survival is dependent on the Th2 cytokine IL-5 (Foster, Hogan et al. 1996). Following mobilisation to the tissue, primed eosinophils release their granule contents. This function would be protective to the host in a parasitic infection however in the lung environment this superoxide production damages the surrounding tissue and promotes release of inflammatory mediators thus propagating an inflammatory milieu. Eosinophils are potent sources of Th2 cytokines and autacoids. These include IL-4 and IL-13, prostaglandins and leukotrienes thereby contributing to the chronic Th2 environment. (Trivedi and Lloyd 2007). Eosinophils have been associated with airway remodelling seen in asthma (Humbles, Lloyd et al. 2004) and are able to secrete growth factor TGFβ, which has a role in epithelial cell differentiation and mucous production (Minshall, Leung et al. 1997) (Foster, Hogan et al. 1996).

1.2.3 Neutrophils

Neutrophils are the most abundant leukocytes in the body and are critical for host defence. Neutrophils are continuously and rapidly produced from the bone marrow upon injury or infection. They are short lived but their granule products are potent and are essential for microbial defence (Kolaczkowska and Kubes 2013). In a chronic inflammatory environment, when there is a failure to resolve, neutrophils release their contents which can cause long term damage to local tissue. Unlike mast cells and eosinophils neutrophils are not classically attributed to a Th2 immune response, nonetheless they are frequently found at high levels in severe asthmatics with numbers of

neutrophils in sputum correlating with steroid resistance in adult disease (Woodruff and Fahy 2002) (Jatakanon, Uasuf et al. 1999). Severe asthmatic sufferers have extensive remodelling of the lung and are refractory to corticosteroid treatment, frequently presenting with high levels of neutrophils (Wenzel, Schwartz et al. 1999). Experimentally, a neutrophilic model of asthma, using mice exposed to repeated low dose aerosolised ovalbumin, demonstrated that mice were resistant to amelioration of inflammation following steroid administration, supporting an important role for neutrophils in steroid resistant patients (Green, Brightling et al. 2002, Ito, Herbert et al. 2008)

1.2.4 CD4 type 2 helper lymphocytes

Th2 lymphocytes are a fundamental feature of allergic inflammation (Bousquet, Jeffery et al. 2000). These allergen specific Th2 lymphocytes have been considered as important drivers of allergic disease. Activation and recruitment of these CD4 T cells is thought to occur via DC antigen uptake and presentation, with an accompanying long lived memory response (Hammad, Plantinga et al. 2010). Antigen presentation and co-stimulation from DCs promotes Th2 lymphocyte polarisation that is characterised by expression of the transcription factors GATA3 and STAT6. This disposes Th2 cells to favourably produce the Th2 associated cytokines interleukin 4 (IL-4), IL-5, IL-9 and IL-13. The primary T-helper 2 function of these cytokines is to promote B lymphocyte maturation and IgE production promoting an atopic phenotype (Coyle and Tsuyuki 1995). In a helminth infection this Th2 polarised immunity would provide an appropriate host defence response to an invading parasite. However, in allergic and asthmatic suffers, the effects of this aberrant response in the lung can promote damage and drives airway hyper-reactivity and remodelling. The plethora of Th2 cytokines secreted means that they can drive pathophysiological allergic disease by acting on structural cells, as well as influencing cells of the immune system (Murdoch and Lloyd 2009). The putative surface marker T1/ST2 has been used to define Th2 cells in human and murine models of

disease (Lohning, Stroehmann et al. 1998). In experimental models, blocking T1/ST2 inhibits the development of allergic airways disease (AAD) (Kearley, Buckland et al. 2009). IL-13 has been shown to be critical in driving AHR and mucus production and experimental studies have shown that it is a critical feature of allergic disease (Wills-Karp, Luyimbazi et al. 1998). Research aimed at identifying the cellular source of IL-13 identified CD4 lymphocytes as the primary source of this cytokine (Wills-Karp 2004). However, recently this concept has been challenged (Oliphant, Barlow et al. 2011). Firstly, T1/ST2 was shown to be the receptor for the innate cytokine IL-33 (Schmitz, Owyang et al. 2005). IL-33 is a member of the IL-1 family and has been shown to be important in the initiation of Th2 responses (Kurowska-Stolarska, Kewin et al. 2008). Secondly, IL-33 has been shown to drive IL-13 production, suggesting a critical role in driving AHR. Finally, ILC2 cells have been shown to be induced by IL-33 and produce key Th2 cytokines including IL- 13 (Oliphant, Barlow et al. 2011) (Barlow, Bellosi et al. 2012, Barlow, Peel et al. 2013). These cells have been shown to be important in driving airway hyper-reactivity in the lung (Scanlon and McKenzie 2012). Therefore, in addition to classical adaptive Th2 lymphocytes, new sources of Th2 cytokines, such as ILCs, need to be considered in understanding allergic airways disease

1.2.5 Innate Lymphoid Cells (ILCs)

ILCs were first identified when it was observed that administration of recombinant IL-25 treatment in RAG KO mice, which lack T and B cells, still resulted in potent Th2 production (Fort, Cheung et al. 2001, Hurst, Muchamuel et al. 2002). They represent a lineage distinct from thymic derived CD3 lymphocytes and do not express the regular lineage markers for lymphocytes (Walker, Barlow et al. 2013). Since the discovery of this non-T non-B lymphocyte population the ILC family has expanded to include the already described NK cells and Lymphoid Tissue inducer cells (Spits, Artis et al. 2013). There are 3 subsets of ILCsILC1, ILC2 and ILC3, that are classified by their cytokine and

transcription factor expression, paralleling the classification of Th1, Th2 and Th17 respectively (Walker, Barlow et al. 2013). ILCs are found at mucosal surfaces in the lung and gut. In the gut, these cells are thought act in a protective capacity and have been shown to be important in host defence against helminth infection (Sonnenberg, Fouser et al. 2011). However, in the lung ILC2 lymphocytes have been shown to be important in driving AHR and features of allergic airways disease, (Barlow, Bellosi et al. 2012). These cells express the transcription factors RORα and GATA3 and produce IL-4, IL-5 and IL-13 (Spits, Artis et al. 2013). The evidence for their importance in allergic disease is compelling as it has been shown that innate epithelial associated cytokines TSLP, IL-25 and IL-33, are capable of inducing these type 2 ILCs. There is much more to learn about these cells but it is evident that these cells are important effector cells in the lung.

1.2.6 Th2 associated cytokines

The Th2 cytokines IL-4, IL-5, IL-9 and IL-13 are clustered on chromosome 5 in humans and chromosome 11 in mouse. Expression of the Th2 cytokine genes are co-ordinately regulated during Th2 differentiation via induction of GATA3 transcription factor (Hwang, Kim et al. 2013). These cytokines are associated with chronic Th2 inflammation as seen in asthmatics (Robinson, Hamid et al. 1992, Bousquet, Jeffery et al. 2000, Jones, Gregory et al. 2012). Each of these cytokines has a distinct role in Th2 immunity and has been investigate as potential targets for therapy. IL-4 is important for B-cell survival and the induction of immunoglobulin class switch recombination, in particular the production of allergen specific IgE (Lebman and Coffman 1988). Eosinophil recruitment from bone marrow and survival in tissue is dependent on the cytokine IL-5. (Foster, Hogan et al. 1996). IL-9 has been shown to promote goblet cell hyperplasia and mast cell activation. CD4 lymphocyte secretion of IL-9 has been shown to be early drivers of AHR (Kearley, Erjefalt et al. 2010, Jones, Gregory et al. 2012). IL-13 has been shown to drive goblet cell

mucus production and mast cell activation which are thought to be the key meditator of AHR. (Wills-Karp, Luyimbazi et al. 1998, Wills-Karp 2004).

The majority of allergic asthma research has focussed on the role of these pro-inflammatory mediators initiating the disease phenotype. More recently innate cytokines derived from airway epithelium, TSLP, IL-25 and IL-33, have been implicated in the initiation of the Th2 cascade (Hammad et al, 2009). TSLP has a role in priming DCs for the induction of Th2 immunity (Zhou, Comeau et al. 2005, Tamachi, Maezawa et al. 2006, Angkasekwinai, Park et al. 2007). IL-25 has been shown to be critical in induction of Th2 cytokines and driving AHR and sub mucosal remodelling in murine models of allergic airways disease (Tamachi, Maezawa et al. 2006, Gregory, Mathie et al. 2010, Gregory, Jones et al. 2013). IL-33 has been shown to drive AHR and induces IL-13 secreting ILC2 (Kim, Chang et al. 2012, Barlow, Peel et al. 2013). IL-33 has also been shown to have a role in steroid resistance and is unchanged following budesonide treatment in murine models of asthma (Saglani, Lui et al. 2013).

The number of mediators implicated in the promotion of allergic inflammation is ever expanding and it is now accepted that the description of asthma is far more complex than simply a classic Th2 adaptive immune response. This demonstrates the need to investigate how different innate and adaptive pathways may be regulated.

1.3 Pulmonary homeostasis

The lung is a mucosal surface that is required to maintain a certain threshold of homeostasis to the constant inhaled external environment in order to preserve efficient gas exchange (Wissinger, Goulding et al. 2009). In asthmatic individuals this tolerance to normally innocuous particles is lost, homeostasis is disrupted and chronic inflammation ensues (Lloyd and Murdoch 2010). In normal individuals, homeostasis in the lung is maintained by an intricate network of immune cells and the airway epithelium. The lung environment has a surface area of 70 m^2 and is continuously exposed to a myriad of

environmental particles on a daily basis (Holt et al, 2008). In asthmatic individuals there is an excessive immune response to what would normally be considered an innocuous material. This aberrant immune response may be due to dysregulation of endogenous pulmonary homeostatic mechanisms in the lung. The following section will discuss the role of the airway epithelium and cellular and soluble mediators of pulmonary homeostasis.

1.3.1 Airway epithelium

The airway epithelium is more than a physical barrier to the inhaled environment and divides into two functionally distinct compartments: the conducting airways and the lung parenchyma. Each compartment contains a distinct set of immune cells that reflect differences in levels and type of exposure to inhaled material (Holt, Strickland et al. 2008).

The epithelia of the conducting airways comprises of ciliated cells and secretory goblet cells. IgA secreted from the bronchial associated lymphoid tissue binds to antigens, which in concert with mucus secretion by goblet cells provides a mechanical means of host defence and elimination of inhaled antigens (Rodriguez, Tjarnlund et al. 2005). In addition, alveolar epithelial cells can secrete mucins and surfactant proteins which can offer host defence via opsonisation of infectious agents (Holt, Strickland et al. 2008). Airway epithelial cells are equipped to detect particles from the inhaled environment via pattern recognition receptors (PRR) and toll-like receptors (TLR). Activation of these receptors on epithelial cells promotes the secretion of chemokines and cytokines for the recruitment and activation of innate and adaptive leukocytes (Lloyd and Saglani 2010, Holgate 2011, Lambrecht and Hammad 2012).

Submucosal dendritic cells in the conducting airways extend processes into the airway lumen to sample for potentially harmful pathogens. Their location is strategic and enables them to carry out immune surveillance and initiate an adaptive immune response if

required. Epithelial cells express PRR which recognise damage and pathogen associated molecular patterns (de Heer, Hammad et al. 2005, Hammad and Lambrecht 2011). Activation of TLR signalling by HDM allergen has been shown to be important for inducing DC activation and the priming of a T helper response and triggering of TLR on the epithelium induced secretion of TSLP, GM-CSF, IL-25 and IL-33 which are important in propagating a Th2 response (Hammad, Chieppa et al. 2009).

As branching of the pulmonary airways continues in the lower airway, the lung parenchyma becomes comprised of an alveolar wall that is specialised for gas exchange. The alveolar epithelium consists of alveolar type 1 and type 2 cells. The immune cells in these lower regions are present within both the airway lumen and the interstitial tissue. In normal individuals the predominant immune cell in the lower airway is the alveolar macrophage. The epithelium is central to the asthma pathogenesis and it is thought that impaired epithelial barrier integrity makes the airways susceptible to virus infection which allows a chronic inflammatory environment to ensue and provides the stimulus to prime DCs towards directing a Th2 response and allergen sensitisation (Holgate 2012). The interaction between alveolar macrophages and pulmonary epithelium is important in maintaining pulmonary homeostasis (Wissinger, Goulding et al. 2009).

1.3.2 Alveolar Macrophages

Alveolar macrophages play a central role in maintaining immunological homeostasis and host defence in the lung (Holt, Strickland et al. 2008, Wissinger, Goulding et al. 2009). During homeostasis alveolar macrophages comprise approximately 95% of the lung immune cell population (Wissinger et al 2009). These cells reside in the lower airways and alveolar spaces where gas exchange occurs, and exist in close proximity with alveolar epithelial cells. They are inherently suppressive and their role requires them to eliminate pathogens from the lung whilst ignoring innocuous material. They express receptors for IL-10 and TGFβ that are secreted by the alveolar epithelium to maintain the

lungs in a steady state of homeostasis (Hussell and Cavanagh 2009, Wissinger, Goulding et al. 2009, Hussell 2012). Snelgrove et al (2009) describe an additional mechanism that supports the critical role of the alveolar macrophages in pulmonary regulation. They showed that alveolar macrophages have high basal surface expression of the glycoprotein CD200 receptor (CD200R). Receptor expression unique to alveolar macrophages and a homeostatic loop is activated by CD200R binding its ligand CD200 which is expressed on alveolar epithelial cells (Figure 1.2). Activation of CD200R imparts a uni-directional negative signal which results in regulation of alveolar macrophage activation and function (Holt & Strickland, 2008). Infection with influenza potentiates the induction of an inflammatory response, overriding the CD200R:CD200 interaction. Resolution of inflammation following influenza infection is delayed in CD200 knockout mice compared to wild type (Snelgrove, Goulding et al. 2008). This was accompanied by elevated levels of $IFN\gamma^+$ T lymphocytes, macrophages and dendritic cells which could be reversed by administering CD200 soluble fusion protein. In addition, an up-regulation of CD200R expression on alveolar macrophages following influenza infection during resolution was observed. This increase in CD200R expression suggests an alteration in the innate immune pathway during resolution. This concept of the innate immune rheostat (Wissinger et al, 2009) proposes that both negative and positive immune pathways influence on a variable scale the expression of homeostatic molecules on the alveolar macrophage. This altered pulmonary homeostasis following resolution of inflammation supports the idea that resolution is an adaptable and active process (Figure 1.2).

The role of alveolar macrophages is not very well understood in an allergic inflammatory setting. In a rat model of OVA induced AAD, depletion of alveolar macrophages prior to allergen sensitisation promoted exacerbated AHR and Th2 inflammation with excessive cytokine production (Careau and Bissonnette 2004) (Careau, Turmel et al. 2010). The adoptive transfer of donor alveolar macrophages back into the alveolar macrophage depleted rats improved features of disease. This has also been demonstrated in a murine

model of OVA induced AAD (Bang, Chun et al. 2011). In this study, the group transferred back both naïve and OVA sensitised alveolar macrophages. They demonstrated that naïve but not sensitised alveolar macrophages could rescue the allergic airways disease phenotype suggesting that allergen exposed macrophages have an impaired suppressive ability. A recent study demonstrated that alveolar macrophage depletion resulted in heightened DC activation with increased APC function. This study suggested that alveolar macrophages may function to regulate DC triggered allergic response. (Lauzon-Joset, Marsolais et al. 2013). However, these studies utilised an OVA model of AAD which does not represent how alveolar macrophages would interact with inhaled allergens in humans. This thesis aims to address the role of alveolar macrophages in inflammation and resolution using the perennial allergen house dust mite (HDM) administered to the airways to induce disease.

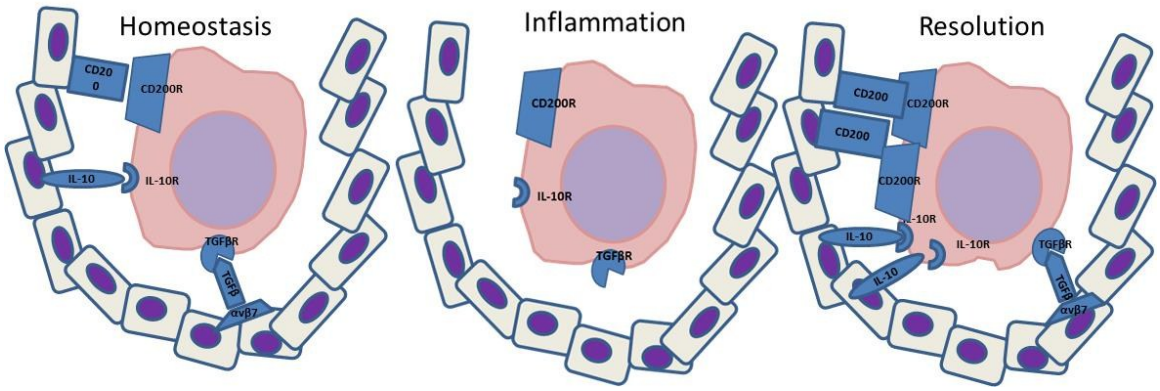


Figure 1.2 Schematic representation of alveolar macrophage homeostatic molecules. Alveolar macrophages express regulatory receptors that tether to ligands expressed on the alveolar epithelium. Following inhalation of infectious or harmful material alveolar macrophages down regulate their receptors and are activated to direct an appropriate immune response. Following resolution of inflammation regulatory receptors are up regulated to promote a return to homeostasis.

1.3.3 Interstitial macrophages

Interstitial macrophages are a less well defined population of pulmonary macrophages. This is most likely due to their anatomical location, which makes it more difficult to purify them compared to alveolar macrophages which can be lavaged directly from the airway. Interstitial macrophages are also thought to have a regulatory function in the lung tissue. Whilst alveolar macrophages exert their regulatory effects via non-specific lines of defence (such as high phagocytic ability, the secretion of antimicrobials, nitric oxide, TNF and IFNγ in addition to CD200R expression), it has been suggested that interstitial macrophages have a greater propensity to release specific cytokines. By virtue of their tissue location they express high levels of MHC with the potential to interact directly with lymphocytes to initiate an appropriate immune response (Franke-Ullmann, Pfortner et al. 1996). There has been comparatively little written regarding the role of interstitial macrophages in disease. However, bone marrow reconstitution studies have demonstrated that interstitial macrophages are intermediaries between blood monocytes and alveolar macrophages (Landsman and Jung 2007). In a murine model of asthma where animals have been peripherally sensitised to OVA, interstitial macrophages were shown to produce the regulatory cytokine IL-10 and down regulate the DC response to allergen (Bedoret, Wallemacq et al. 2009). This was later attributed to HIF1α expression. Interstitial macrophages deficient in HIF1α had reduced IL-10 production and enhanced ability to induce DC proliferation (Toussaint, Fievez et al. 2012). Interstitial macrophages are distinguished from alveolar macrophages by their location and function in the lung; however there role in the pathogenesis of asthma is not very well described.

1.3.4 T regulatory lymphocytes

The dampening of the allergic inflammatory response and tolerance to allergen has been attributed to T regulatory lymphocytes (Tregs) (Shalev, Schmelzle et al. 2011, Holgate 2012). These lymphocytes can either be naturally occurring thymic derived T regulatory lymphocytes or they can be induced extrathymically from conventional CD4 lymphocytes.

Naturally occurring T regs (nTregs) (classed as CD4+, CD25+ and FoxP3+) are allergen specific and mediate regulation and tolerance via secretion of IL-10 and TGFβ. Inducible T regulatory lymphocytes (iTregs) can be subdivided into CD4+ $CD25^{lo}FoxP3^{lo}$ IL-10+ lymphocytes (Tr1), or TGFβ producing Th3 (Shalev, Schmelzle et al. 2011). These Th3 cells were found in the gut mucosa and are able to suppress inflammation in a model of experimental autoimmune encephalomyelitis. T regulatory lymphocytes have been shown to be critical in the maintenance of intestinal barrier function and inflammatory homeostasis to prevent autoimmune inflammatory disease such as colitis (Chen, Kuchroo et al. 1994, Read and Powrie 2001, Izcue, Coombes et al. 2009).

In patients it has been shown that nTregs from non-allergic donors could suppress proliferation and Th2 cytokine secretion in T lymphocytes; however, this was not observed with nTregs taken from allergic donors suggesting there is a defect in suppressive function of Tregs in asthmatic individuals (Ling, Smith et al. 2004). It is thought that T regs function to suppress DC activation, can directly inhibit Th1, Th2 and Th17 responses, suppress IgE, and induce IgG4 (Durham, Walker et al. 1999). In experimental models of allergic airways disease T regulatory lymphocytes have been shown to regulate resolution of AHR which has been demonstrated to be dependent on Treg induced IL-10 (Kearley, Barker et al. 2005). Manipulation of these regulatory lymphocytes to promote tolerance is being used in sublingual and subcutaneous immunotherapy (SLIT) and (SCIT). These therapies work by giving low dose allergen to promote long term tolerance and suppression of clinical symptoms. The beneficial effects are thought to be due to class switching from IgE to a protective IgG4 and increased number of T regulatory lymphocytes are also observed (Till, Francis et al. 2004).

Quite recently a new member of the regulatory lymphocyte family was described. These cells secrete IL-35 which is a heterodimer of EBi3, a downstream product of FoxP3 activation, and the IL-12a subunit (Vignali and Kuchroo 2012). It has been shown that IL-35 is specifically produced by T regulatory lymphocytes, rather than T effector cells and

that IL-35 producing Tregs are a distinct population from IL-10 and TGFβ regulatory cells (Collison, Chaturvedi et al. 2010). IL-35+ T regulatory lymphocytes, but not IL-10 or TGFβ, have been shown to be important in regulating AHR in an OVA murine model of allergic airways disease (Whitehead, Wilson et al. 2012). It is still be elucidated if IL-35 T-regulatory lymphocytes have a role in human allergic disease.

1.3.5 IL-10

IL-10 was first described as a product of Th2 cells but it is now known to be secreted by macrophages, DCs, B cells and CD4+ and CD8+ lymphocytes (Moore, de Waal Malefyt et al. 2001). IL-10 is the archetypal anti-inflammatory cytokine and works to terminate inflammatory events (Moore, de Waal Malefyt et al. 2001). It exerts its inhibitory effects via down regulation of MHCII and co-stimulatory molecules B7-1/B7-2 on monocytes and macrophages, and also inhibits the production of pro-inflammatory cytokines such as IL-6, IFNγ, TNFα, and it can also down regulate chemokine production (Couper, Blount et al. 2008). IL-10 can inhibit pro-inflammatory cytokine production from both mast cells and eosinophils (Takanaski, Nonaka et al. 1994, Arock, Zuany-Amorim et al. 1996). IL-10 has an important regulatory role in the lung and has been reported to modulate effector functions implicated in allergic airways disease, such as Th2 activation and IgE production (Nouri-Aria, Wachholz et al. 2004). In the lung a loss of tolerance to inhaled allergens is attributed to reduction in the levels of IL-10 (Borish, Aarons et al. 1996).

1.3.6 TGFβ

The pleiotropic mediator TGFβ is produced both by immune cells and structural cells and has pro-fibrotic effects (Dennler, Goumans et al. 2002). TGFβ has been implicated in airway smooth muscle remodelling, microvascular changes, subepithelial fibrosis, and epithelial changes in the lung (Lambrecht and Hammad 2012). It also exerts potent anti-inflammatory effect and inhibits T cell proliferation, as well as IgE synthesis and secretion

(Letterio and Roberts 1998). TGFβ is associated with allergic airways disease pathogenesis and is increased in the lung of asthmatics (Aubert, Dalal et al. 1994). Although, whether the concentration of TGFβ correlates with disease severity remains controversial. In an OVA model of chronic allergic airway disease blocking TGFβ with a neutralising antibody reduced allergen induced peribronchial collagen deposition, airway epithelial cell proliferation and mucus production, however, did not affect Th2 cytokines and inflammation. Thus uncoupling a link between allergic inflammation and remodelling (McMillan, Xanthou et al. 2005). Similarly, another study demonstrated that TGFβ neutralisation abrogated airway remodelling but enhance AHR (Alcorn, Rinaldi et al. 2007). In a HDM model of allergic airway disease, epithelial overexpression of pSMAD2, a downstream mediator of TGFβ signalling pathway, promoted exacerbated airway hyper-reactivity accompanied with an increased collagen deposition and peribronchial smooth muscle mass (Gregory, Mathie et al. 2010). It has also been shown that remodelling can develop independently of TGFβ (Fattouh, Midence et al. 2008). The ubiquitous and pleiotropic nature of this cytokine has made it difficult to dissect the specific role of TGFβ in asthma and requires further study.

1.3.7 IL-27 & IL-35

IL-27 is member of IL-6/IL-12 family. It is a heteromeric cytokine consisting of the EBi3 and IL-12 p28 subunits. IL-27 is produced by dendritic cells and macrophages (Hunter and Kastelein 2012). Although it has been described to promote Th1 responses in certain IL-12 induced environments (Villarino, Huang et al. 2004), it has also been shown to exert an anti-inflammatory role via inhibition of Th2 cell development. *In vivo* intranasal administration of IL-27 inhibited AHR in an OVA induced model of Th2 inflammation (Yoshimoto, Yoshimoto et al. 2007). Moreover, IL-27 was increased in the sputum of steroid refractory asthmatics (Li, Wang et al. 2010). An experimental model of LPS and IFNγ induced steroid resistant airway hyper-reactivity highlighted a role for IL-27

producing macrophages. This study demonstrated that IL-27 and IFNγ, via a MYD88 dependent mechanism, inhibited glucocorticoid receptor nuclear translocation, and AHR in mice failed to resolve with treatment with dexamethasone (Li, Wang et al. 2010). IL-27 shares homology and functions with the recently described anti-inflammatory cytokine IL-35 which is a product of a subset of T regulatory lymphocytes (Collison, Chaturvedi et al. 2010). It has also been shown to exert a suppressive role in autoimmune disease settings. In murine model of diabetes, mice expressing IL-35 via the insulin promoter in pancreatic β-cells had decreased numbers of in pathogenic CD4+ and CD8+ lymphocytes (Bettini, Castellaw et al. 2012). In the lung, overexpression of IL-35 attenuated AHR and IgE production in a model of OVA induced allergic inflammation (Huang, Loo et al. 2011). IL-35 secreting lymphocytes were also shown to suppress AHR and inflammation in an OVA model of allergic airways disease (Whitehead, Wilson et al. 2012). Further investigation into IL-27 and IL-35 and their potential role for therapy in asthma is required.

1.3.8 Immunoglobulin A

Immunoglobulin A (IgA) is the predominant immunoglobulin secreted at mucosal surfaces. It is believed to be required for host defence in the gut and lung (Cerutti 2008). IgA is known to sequester and neutralise viruses following infection (Wines and Hogarth 2006). However, the downstream effects of IgA are not clear. It has been suggested IgA can mediate host defence by inducing phagocytosis, respiratory burst activity, and the release of pro-inflammatory cytokines, which may help its role in mucosal defence (Wines and Hogarth 2006). The receptor for IgA, FcaR1, is expressed mainly on cells of the myeloid lineage including macrophage, monocytes, neutrophils and eosinophils (van Egmond, Damen et al. 2001). In an OVA model of AAD, improved lung function in mice deficient in the eicosanoid enzyme 12/15 LOX was associated with higher IgA levels (Hajek et al, 2008).

1.4 Resolution

Research into the mechanisms underlying allergic inflammation has focussed primarily on the cellular and molecular events involved in the initiation and propagation of pro-inflammatory pathways. There has been less investigation into the endogenous “stop” signals that limit the severity of inflammation and promote the process of resolution. The complete resolution of acute inflammation and return to tissue homeostasis is required for continuing health. The concept of inflammatory resolution, whilst driven in part by a decrease in inflammatory mediators, is now recognised as an active process with early signalling pathways that engage biosynthetic circuits for the later formation of counter regulatory mediators (Serhan and Savill 2005, Serhan, Brain et al. 2007). There is increasing evidence that deficiencies in resolution mechanisms can play a part in sustained inflammation observed in chronic inflammatory diseases, including allergic asthma (Haworth and Levy 2007).

Resolution of inflammation results in the restoration of tissue homeostasis or catabasis. The resolution program is distinct from anti-inflammatory mechanisms (Serhan, Brain et al. 2007) (Serhan 2007). In addition to limiting granulocytic infiltration and the inhibition of cytokine expression, pro-resolution molecules enhance the phagocytic activity of macrophages towards apoptotic leukocytes, cellular debris and microbes at inflamed sites and also stimulates anti-microbial activity of mucosal epithelial sites (Canny, Levy et al. 2002). Resolution is also characterised by the non-inflammatory recruitment of monocytes, although these have a role in inflammation they are also critical for wound healing and tissue repair (Serhan, Krishnamoorthy et al. 2011).The use of non-steroidal anti-inflammatory drugs that target lipoxygenase (LOX) and cyclooxygenase (COX) lipid enzymatic pathways to treat inflammatory conditions have revealed an important role for lipid products that have beneficial effects. In particular induction of COX2 in inflammatory conditions can switch from the generation of pro-inflammatory lipids, leukotrienes and prostaglandins to lipoxins, lipids of pro-resolution properties (Stables and Gilroy 2011).

Early research into identifying the mediators responsible for directing resolution utilized self-limiting models of inflammation, such as air pouch and zymosan peritonitis. LS/MS/MS analysis of these exudates identified a new genus of lipid molecules with specialised pro-resolving properties (SPMs) derived from the omega3 acids docosahexaenoic acrd (DHA) and eicosapentaenoic acid (EPA) and arachidonic acid (AA) (Serhan, Krishnamoorthy et al. 2011) (Figure 1.3). Together these lipids influence both immune and structural cells and can drive the program of resolution and a return towards tissue homeostasis (Serhan, Krishnamoorthy et al. 2011).

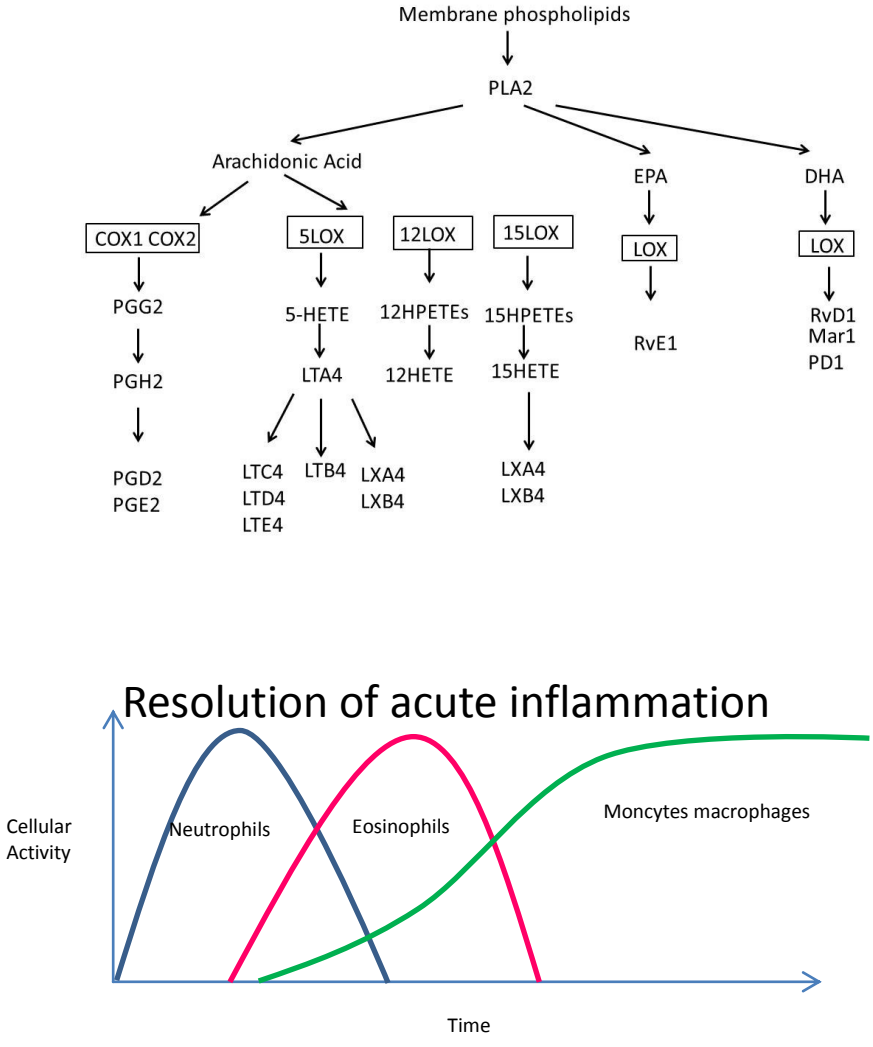


Figure 1.3. Lipid mediators in inflammation and resolution. Resolution and the return to homeostasis via specific group of omega 3 and omega6 derivatives: Specialised pro-resolving mediators (SPMs). Temporally and spatially up regulated during self-limited inflammation. Deficiencies in pro-resolving molecules are implicated in the maintenance of chronic inflammation, leading to fibrosis, scarring and airway remodelling.

1.4.1 DHA and EPA derived lipids

The generation of bioactive lipids from DHA, EPA and AA occurs via enzymatic oxidation by lipoxygenase (LOX) and cyclooxygenase (COX) of membrane phospholipids in circulating immune cells and platelets. Briefly, COX1 is constitutively expressed in most tissues and synthesises prostaglandins at low levels to maintain physiological functions. COX2 is induced under inflammatory conditions enhancing production of prostaglandins and can be expressed in macrophages, fibroblast, endothelium, and human tracheal epithelium (Gilroy and Colville-Nash 2000).5LOX is predominantly expressed by neutrophils and at lower levels in monocytes and can be activated by a variety of agonists (O'Donnell and Murphy 2012). 12/15 LOX is the murine homolog to human 15LOX and is highly expressed in human monocytes and murine macrophages and also in eosinophils and epithelium (Chaitidis, O'Donnell et al. 2005) (Figure 1.3).

DHA and EPA derived members of the SPMs include Maresin1, Resolvins, and Protectins (Serhan and Savill (2005) (Norling & Serhan, 2010). Maresin (MaR1) is a DHA derived lipid biosynthesised by macrophages (Macrophage mediator in resolving inflammation) and was first identified in a peritonitis model of inflammation and shown to promote the resolution of inflammatory mediators (Serhan, Yang et al. 2009). It is yet to be elucidated if maresins are important in the allergic lung and if they are associated with a dysfunction in alveolar or interstitial macrophages.

ResolvinE1 (RvE1), is derived from EPA and its formation occurs during inflammation following endothelial interactions with leukocytes via the 5LOX enzymatic pathway. RvE1 is a potent inhibitor of the transmigration of neutrophils across endothelial and epithelial barriers. RvE1 up regulates CCR5 expression on neutrophils. In the presence of high levels of CCR5 macrophages increase their phagocytic activity and promote clearance of apoptotic neutrophils (Campbell, Louis et al. (2007). ReV1 was shown to attenuate eosinophilia, mucus production and airway hyper-reactivity in an OVA model of allergic airways disease while also suppressing IL-23, and inducing Lipoxin A (4) to promote resolution. (Haworth, Cernadas et al. 2008). The same group demonstrated in a later

study that RvE1 induced resolution occurred concomitant with an increase in NK cells. The receptor for RvE1 ChemR23 is expressed on NK cells and suggested that this pathway may play in role in promoting resolution of OVA induced inflammation (Haworth, Cernadas et al. 2011).

Another member of the resolvin series is Resolvin D1 (RvD1) which is synthesised from DHA via 15LOX and has also been implicated in the resolution of allergic inflammation. (Rogerio, Haworth et al. 2012). It is similar to RvE1 in that it is also induced to promote macrophage phagocytosis of apoptotic neutrophils (Uddin and Levy 2011). The role of RvD1 in resolution was first identified in neural tissues where there are high levels of DHA which has been shown to be important for maintenance of neural cell signalling and proliferation (Salem, Litman et al. 2001). Both RvD1 and RvE1 can be induced by aspirin which acetylates COX2 inhibiting prostaglandin synthesis (Levy 2010). Aspirin triggered RvD1 (AT-RvD) has been shown to be more efficacious than RvD1 in promoting resolution with a quicker reduction in eosinophil numbers and mucus metaplasia in an OVA model of AAD. AT-RvD1 was also shown to enhance macrophage phagocytosis function (Rogerio, Haworth et al. 2012). Like RvD1, Protectin D1 (PD1) is formed from DHA via 15LOX and was first identified after it's generation in glial cells. It has been shown to offer protection against ischaemic brain injury and Alzheimer's disease (Hong, Gronert et al. 2003, Serhan, Gotlinger et al. 2006). It has different stereochemistry from RvD1 where PD1 is distinguished by a triene double bond (Levy 2010). PD1 was found to be present in the exhaled breath condensates from healthy individuals and interestingly was found to be lower in asthmatics. It was also shown that exogenous administration of PD1 in a OVA model of AAD could accelerate the resolution of eosinophils, airway mucus production, IL-13 and AHR (Levy, Kohli et al. 2007).

1.4.2 Arachidonic acid derived lipids

Eicosanoid are a family of lipids that contain 20 carbons and are derived from arachidonic acid (AA). Products of this pathway are generated in a tissue specific manner and synthesised lipids initiate an inflammatory cascade. These bioactive molecules include prostaglandins and leukotrienes which are induced via the COX and LOX families of enzymes. Classically these mediators are known to be released early in response to injury and to activate the inflammatory response. Prostaglandins are widely known to promote vascular permeability and oedema and to induce fever and platelet aggregation (Stables and Gilroy 2011). However, prostaglandins have been shown to have a dual role during inflammation. In addition to promoting inflammatory responses, PGE2 can exert immunosuppressive effects by inhibiting phagocytic function of rat alveolar macrophages via up regulation of intracellular cAMP (Aronoff, Canetti et al. 2004). PGE2 has also been shown to enhance IL-10 production in LPS stimulated murine bone marrow derived macrophages (MacKenzie, Clark et al. 2013). In the lung PGE2 can mediate bronchodilatory effects and inhalation of PGE2 can abolish exercise induced bronchoconstriction. PGE2 can also inhibit early and late phase response with a reduction in eosinophils and AHR (Chung 2005). In a murine model of allergic airways disease deletion of the PGE2 receptor EP3 displayed worse AHR in response to allergen challenge (Kunikata, Yamane et al. 2005).

Prostaglandin D2 (PGD2) has also been shown to exert a dichotomous role in inflammation. PGD2 signals through its receptors DP1 and DP2/CRTH2 (Smyth, Grosser et al. 2009). PGD2 is a major product of mast cells and bronchoconstrictor and contributes to the allergic inflammatory response and has been found to be rapidly induced in the BAL of allergic individuals following allergen exposure (Murray, Tonnel et al. 1986). Signalling via DP1 induces vascular permeability and blood flow. PGD2 can promote Th2 lymphocyte polarisation and activation via Th2 cellular expression of CRTH2 (Pettipher, Hansel et al. 2007). Thus PGD2 receptors are an attractive target for therapy. However, studies in a murine model of asthma showed that activation of DP1 on lung

dendritic cells suppressed airway hyper-reactivity with increased T regulatory lymphocytes and IL-10 (Hammad, Kool et al. 2007) suggesting a complex role for PGD2 in regulating inflammation. Indeed it was shown in a model of carrageenan induced pleurisy, after the initial inflammation and COX2 induced PGE2 subsided, there was a second peak of COX2 expression and PG synthesis that coincided with the resolution phase with PGD2 and its derivative 15deoxy delta(12-14)-prostaglandin J2 (15d-PGJ2) being in predominance (Gilroy, Colville-Nash et al. 1999). Downstream derivatives of PGD2 have been described to mediate suppression of inflammation (Stables and Gilroy 2011).15-d-PGJ2 a non-enzymatically derivative of PGD2 and has high affinity for the anti-inflammatory nuclear factor PPARγ and was shown in macrophages to activate PPARy target genes resulting in an inhibition of inducible nitric oxide synthase, gelatinase B and scavenger receptor A genes which have been shown to be detrimental in atherosclerosis and rheumatoid arthritis (Ricote, Li et al. 1998).

As described above COX2 can promote a switch in eicosanoid production and induce anti-inflammatory PGs and lipoxins therefore promoting resolution of inflammation (Gilroy, Colville-Nash et al. 1999). Moreover, neutrophils producing LTB4 can convert arachidonic acid into Lipoxins that can serve to terminate inflammation and promote resolution (Levy, Clish et al. 2001, Serhan, Chiang et al. 2008).

Lipoxin A4 (LXA4) and Lipoxin B4 (LXB4) were the first identified resolution lipids and represent the archetypal SPMs. LXA4 has been reported to mediate resolution effects in various immune and structural cells and disease settings (Serhan, Chiang et al. 2008). LXA4 can inhibit neutrophil entry into inflamed sites, stimulate monocyte chemotaxis, and non-phlogistic phagocytosis of apoptotic neutrophils. It has also been shown to inhibit TNF and IL-8 on intestinal epithelium and inhibit IL-1β, IL-6 and IL-8 production in fibroblasts (Jia, Morand et al. 2013). In the lung, LXA4 and its receptor are decreased in severe asthmatics (Planaguma & Levy 2008). Exogenous LXA4 can actively promote resolution of allergic airway inflammation and AHR in a murine model of OVA induced

AAD. (Levy et al 2002). LXA4 can be synthesised via multiple pathways. The first involves neutrophil derived 5-LOX conversion of arachidonic acid to LTA4 and then converted to LXA4 in the vasculature by 12-LOX (Kuhn and O'Donnell 2006). LTA4 can also be converted to LXA4 by 15 LOX. Epithelia, eosinophil or monocyte derived AA can also be converted by 15-LOX to 15-HETE which is subsequently converted to LXA4 by the action of 5-LOX. Neutrophils can also convert 15-HETE into LXA4. (Kuhn and O'Donnell 2006).

The COX and LOX enzyme families are a target for NSAIDS which promote pain relief. Aspirin can trigger epimers of lipids, such as AT-RvD1 and E1 mentioned earlier, which can have more potent effects than endogenously synthesised resolvins. Epimers of LXA4 can be synthesised via COX2 acetylation in endothelial or epithelial cells. This can be via aspirin or as an yet unknown endogenous mechanism (Gilroy 2010). Aspirin mediates the enzymatic activity of COX2 such that the products of its action switch from pro-inflammatory prostanoids to anti-inflammatory lipoxins. Aspirin activated COX2 converts AA into 15-R-HETE which is a substrate for 5-LOX which results in the generation of 15-epi-LXA4 or LXB4 in a rapid manner (Claria and Serhan 1995). Thus COX2 has a dual role in inflammation and in the presence of NSAIDs can produce protective lipids (Gilroy, Colville-Nash et al. 1999).

The cantharidin induced skin blister model can be used in human volunteers to assess the anti-inflammatory properties of drugs. Using this technique it has been shown that there are two types of responders to aspirin (Morris, Stables et al. 2010). Early responders have lower levels of LXA4 and AT-LXA during the inflammation phase as inflammation resolved levels of LXA4 and AT-LXA increased. Conversely, delayed responders have high levels of LXA4 and AT-LXA4 early on which results in relatively lower levels of inflammation. However, as inflammation continues LXA and AT-LXA waned and inflammation progressed. This study highlighted that two humans phenotypes exist with respect to responsiveness to aspirin. The anti-inflammatory effects of aspirin

have been attributed to its role in inhibiting pro-inflammatory and promoting pro-resolution lipids. In a subgroup of asthmatics aspirin can induce an exacerbation of disease. The exact mechanisms are not fully understood, however, susceptible individuals have been shown to have lower PGE2 and increased levels of leukotriene C4 synthase and cysLT receptor expression. Therefore, susceptible individuals have elevated production of leukotrienes and increased responsiveness resulting in bronchoconstriction and mucus production. It has also been suggested that aspirin may cause a structural change to COX2 and inhibits the production of pro-resolution lipids (Hamad, Sutcliffe et al. 2004).

15LOX is the most abundantly expressed gene in human monocytes following IL-4/IL-13 exposure. The analogue in mice, 12/15 LOX produces 12- and 15-HETES. 12-HETE has been shown to be induced in an OVA model of allergic airways disease (Morgan et al, 2009). Indeed, mice lacking the 12/15 LOX gene show attenuation of allergic airway disease (Andersson et al, 2008). However, both 12- and 15-HETE have also been implicated in anti-inflammatory actions by modulating the activity of peroxisomal proliferator-activating receptor-γ (PPARγ), (Kunh & O'Donnell, 2006). Activation of PPARγ is associated with anti-inflammatory pathways and is thought to have a role in regulating airway inflammation and remodelling (Park & Lee, 2008). The investigation of these pathways in HDM induced allergic airway inflammation and resolution requires further elucidation.

1.4.3 Resolution lipid receptors

The downstream effects of resolution lipids are mediated by G-protein coupled receptors (GPCR) expressed on myeloid and lymphocytic cells. RvE1 mediates its effects through ChemR23 has been shown to regulate inflammation in a model of peritonitis (Arita, Ohira et al. 2007). RvE1 is also a partial agonist of BLT1, the receptor for LTB4 and can mediate resolution indirectly via dampening of LTB4 signalling (Arita, Ohira et al. 2007). ChemR23 expressed on macrophages, dendritic cells and NK cells and mediates anti-

inflammatory signals from peptides derived from the protein chemerin in addition to RvE1. Chemerin is a chemoattractant protein that has been found in various human inflammatory conditions such as synovial fluid in rheumatoid arthritis sufferers and ascites from the peritoneum (Wittamer, Franssen et al. 2003). Chemerin is produced primarily by macrophages and immature dendritic cells (Samson, Edinger et al. 1998). Chemerin undergos proteolytic cleavage by cysteine and serine proteases to produce peptides that exert anti-inflammatory effects (Cash, Hart et al. 2008). C-15 is one such peptide that has been shown to mediate anti-inflammatory effects in the picogram range. C-15 is composed of the first 15AA of the c-terminal end of the chemerin peptide. ChemR23 is predominantly expressed by myeloid cells, and C-15 has been shown to have potent anti-inflammatory effects on macrophages in vitro.

RvD1 signals through formyl-petide receptor 2, (FPR2) and GPR32 (Krishnamoorthy, Recchiuti et al. 2012). RvD1 can inhibit neutrophilia in a dose dependent manner in a model of peritonitis. Annexin A1 and Lipoxin A4 are also ligands for FPR2. This receptor has been investigated in a variety of disease models (Dufton and Perretti 2010). In the lung synthetic agonists of FPR2, such as Quin-C and 6 amino acid peptide, w-peptide, have beneficial effects on lung disease. In a model of bleomycin induced fibrosis, treatment reduced cytokine expression and leukocyte infiltration leading to protection from bleomycin injury (He, Cheng et al. 2011). Prophylactic treatment with a w-peptide resulted in a reduction in airway hyper-responsiveness, airway inflammation and IL-17 as well as reduction in IL-6 and IL-12 production from dendritic cells levels, in a model of LPS injury (Tae, Park et al. 2012). Targeting these ChemR23 and FPR2 in chronic inflammatory diseases using derivatives that selectively mimic the resolution properties of these endogenous lipids and proteins offers an exciting avenue for therapeutic intervention.

1.4.4 Pro-resolution peptide Annexin A1

Annexin A1 is a 37kDa anti-inflammatory peptide highly expressed in macrophages and neutrophils (Perretti and D'Acquisto 2009). Following cell activation Annexin A1 stored in granules is rapidly mobilised to the cell surface and secreted where it undergoes conformation change exposing the N-terminal region that activates its receptor FPR2 (D'Acquisto, Perretti et al. 2008). The effects of Annexin A1 include inhibition of neutrophil trafficking and the induction of neutrophil apoptosis. Annexin A1 has been shown to reduce inflammation by preventing binding of inflammatory monocytes to endothelial cells (Solito, Romero et al. 2000). Annexin A1 is contained in mast cells granules and can down regulate mast cell histamine and PGD2 secretion (Bandeira-Melo, Bonavita et al. 2005). Annexin A1 and its function in eosinophils is not well investigated however it has been shown to be localised in rat eosinophils (Oliani, Damazo et al. 2002). Structural cells have also been reported to express Annexin A1. A recent study described Annexin A1 expression in intestinal epithelial cells that was increased in colitis patients compared to normal patients and suggested a role for Annexin A1 in the regulation of epithelial wound repair (Leoni, Alam et al. 2013). Annexin A1 and LXA4 has been shown to be up regulated in colitis patients suggesting this pro-resolving circuit and the receptor FPR2 are critical for resolution at mucosal surfaces (Vong, Ferraz et al. 2012). Annexin A1 may have a role in fibroblast function, since siRNA silencing increased the TNF induced proliferative capacity of normal human fibroblasts as well as their capacity to secrete IL-6 (Jia, Morand et al. 2013). In the lung, a model of bleomycin induced fibrosis mice lacking Annexin A1 displayed increased pulmonary inflammation and exacerbated indices of fibrosis in a model of compared to WT mice (Damazo, Sampaio et al. 2011). The use of a Annexin A1 mimetic, Ac-26, which is the n-terminal sequence from 2-26, prevented bleomycin induced fibrosis and inflammation. In another study LPS induced inflammation could also be regulated by Ac-26 administration (da Cunha, Oliani et al. 2012). It has also been shown in a model of AAD, that Annexin A1 KO exhibit increased AHR in an OVA induced model of AAD (Ng, Wong et al. 2011). Annexin A1 is closely linked with lipid

resolution pathways. In vitro studies with mast cells showed that cleaved Annexin A1 failed to bind with PLA2 and resulted in increased activation and subsequent eicosanoid production (Kwon, Lee et al. 2012). Thus intact cytosolic Annexin A1 is required to regulate PLA2 and downstream eicosanoid production.

1.4.5 Resolution lymphocytes in allergic inflammation

Removal of pro-inflammatory mediators and induction of resolution is also characterised by the repopulation of inflamed tissue with regulatory leukocytes (Rajakariar, Lawrence et al. 2008). As mentioned above, monocytes with a pro-resolving fate are recruited via pro-resolving signals. However, lymphocytes of the innate and adaptive immune response may also play a role in resolution and are thought to be important in maintaining pulmonary homeostasis. In a model of zymosan induced peritonitis, repopulating lymphocytes were shown to confer protection with a secondary bacterial infection (Rajakariar, Lawrence et al. 2008). These lymphocytes responded to PGD2, linking resolution lipids and lymphocytic interaction. This observation highlights that failure for effective resolution delays proper restoration of homeostasis and increases susceptibility to secondary infection (Rajakariar, Lawrence et al. 2008). At mucosal sites, innate lymphocytes such as γδ T cells and ILCs have been shown to regulate the local inflammatory response. In the context of allergic airways disease, our group has identified lymphocytic pathways that are critical in the resolution of allergic inflammation and AHR. T-regulatory lymphocytes have been shown to direct resolution of allergic airways disease (Kearley, Barker et al. 2005). Transfer of CD4+ CD25+ FoxP3+ T regs reversed OVA induced AHR and this was mediated by anti-inflammatory IL-10. In a chronic model of disease therapeutic adoptive transfer of Tregs promoted a reduction in eosinophils, Th2 cytokines and TGFβ. Mucus production and collagen deposition was also reduced (Kearley, Robinson et al. 2008). Adoptive transfer of γδ IL-17+ lymphocytes have also been shown to promote resolution of AHR and inflammation in an OVA model

of AAD (Murdoch and Lloyd 2010). Disruption of the Th2 cell signalling pathway between IL-33 and T1/ST2 has also been shown to promote resolution of allergic airways disease (Kearley, Buckland et al. 2009).

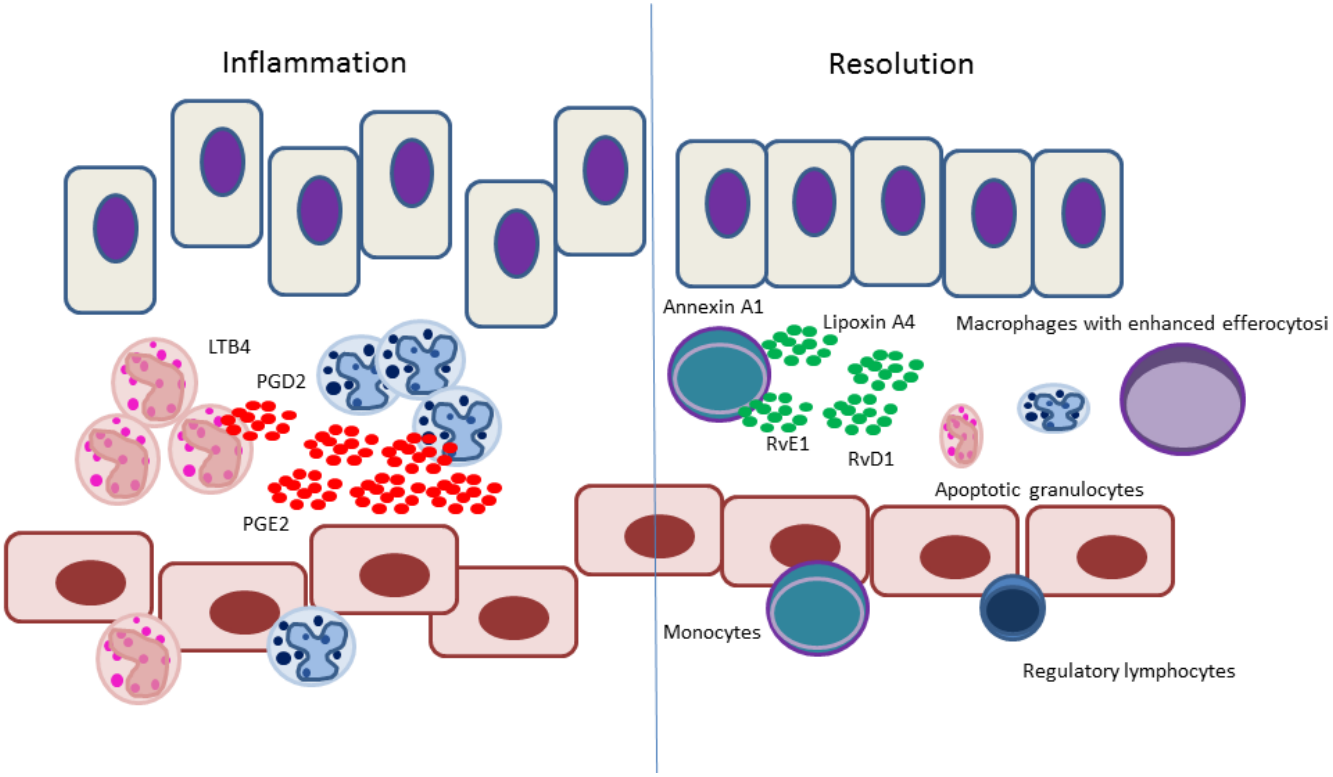


Figure 1.4. Schematic of ideal outcome following acute inflammation. COX and LOX derived lipids produced by structural and immune cells undergo class switching during resolution to promote a return to homeostasis. This involves the induction of granulocyte apoptosis, the recruitment of monocytes and regulatory lymphocytes, and the up regulation of resolvins protectins and lipoxins. Dysregulation of resolution processes can lead to chronic inflammation and excessive wound healing and scarring

1.5 Animal models of disease

Animal models of disease have been widely used to investigate the molecular and cellular mechanisms underlying allergic asthma. There are a variety of methods used to establish murine models of allergic airways disease (Lloyd 2007) and one of the most common models uses the surrogate allergen ovalbumin (OVA). Sensitisation to this antigen occurs via intra-peritoneal injection of OVA with the adjuvant alum followed by a period of airway challenge with aerosolised OVA. Exposure of sensitised mice to aerosolised or soluble OVA promotes the development of features of disease that mimic the human clinical parameters such as IgE, Th2 allergic inflammation, mucus production, induction of airway hyper-reactivity and airway remodelling (Lloyd 2007). Whilst this model provides a polarised Th2 inflammatory environment to study, it does not fully reflect how sensitisation via the airways and the subsequent manifestation of disease occurs in humans.

More recently experimental mouse studies have shown that antigens of more clinical relevance, such as house dust mite (HDM) have inherent biological properties that promote allergenicity (Johnson, Wiley et al. 2004, Gregory and Lloyd 2011). HDM is a common allergen in asthmatics with up to 85% of asthma patients in the UK being sensitive (Nelson, DiNicolo et al. 1996) and extracts have been found to contain antigens with proteolytic properties. These can disrupt tight junction integrity of the respiratory epithelium and promote a loss in barrier integrity (Wan, Winton et al. 1999, Wan, Winton et al. 2001). Proteases can also react with protease activated receptors (PARs) which are expressed on airway epithelial cells, and shown to be up regulated on the epithelium of asthmatics, to induced leuckoyte infiltration and amplify the response to allergen. The activation of PAR on eosinophil and mast cells result in their degranulation (Reed and Kita 2004). Extracts are also composed of ligands derived from microbial compounds that can be recognised by pattern recognition receptors (PRR) of the innate immune system such as lipospolysaccharide (LPS), which can activate Toll-like receptors (TLRs) expressed at mucosal surface and on innate immune cells (Hammad, Chieppa et al.

2009). TLRs and PARs activated by components of inhaled allergen promote dendritic cell activation that initiates and adaptive allergic inflammatory response (Lambrecht and Hammad 2012) (Hammad and Lambrecht 2011).

The antigenic complexity of HDM and its ability to induce a multifactorial response means that adjuvant is not required and repeated intranasal challenge induces HDM sensitisation and promotes an allergic asthma phenotype (Johnson, Wiley et al. 2004, Gregory, Causton et al. 2009) (Hammad and Lambrecht 2011). This method of mucosal exposure and sensitisation may mimic the route by which humans encounter allergen and provides a more realistic model for studying the mechanisms involved in the inception, propagation and resolution of allergic asthma. This project utilised the HDM model for the study of resolution pathways. The route of administration is particularly critical in studying endogenous homeostatic mechanisms such as alveolar macrophages, which are a first line defence against the inhaled environment.

1.6 Hypothesis

In certain individuals deficiencies in pro-resolving mediators have been associated with disease. However, there are few models of resolution particularly in the lung thus the aim was to establish a model of resolution and identity mediators of resolution with the working hypothesis that resolution of HDM induced allergic airways disease is an active process with specific pro-resolving pathways

1.6.1 Aims

1. To establish a model of resolution and characterise the pulmonary immune interactions within the airway during the resolution of HDM induced inflammation

2. To determine the role of alveolar macrophages following HDM exposure and during the resolution of allergic inflammation.

3. To determine the molecules important in resolution of inflammation in response to HDM.

Chapter 2.

Materials and Methods

2.1 Mice

Female C57BL/6 and BALB/c mice were purchased from Harlan Ltd (Bicester, UK). Mice deficient in CD200R on a C57BL/6 background mice were a kind gift from Tracy Hussell (Imperial College London, UK). Annexin A1 knock-out mice were purchased from Charles River (Margate, UK). Animals were housed at Imperial college animal facility and used at 6-8 weeks of age. Food and water were supplied ad libitum. UK Home Office guidelines for animal welfare based on the Animals (scientific procedures) act 1986 were observed.

2.2 Induction of HDM induced allergic inflammation

2.2.1 Preparation of house dust mite extract

Whole house dust mite (HDM) extract (Greer Laboratories, Lenoir USA) was prepared for *in vivo* use, as originally reported (Gregory, Causton et al. 2009). Throughout this PhD 3 batches of HDM were used, details of protein, antigen and endotoxin tested by Greer Laboratories below:

(1) 113178, Derp1 content 146ug/vial, dry weight 14.3mg/vial, total protein 3.58mg/vial, endotoxin 125 EU/vial

(2) 151776 Derp1 content 149.02ug/vial, dry weight 11.9mg/vial, total protein 4.29mg/vial, endotoxin 125 EU/vial

(3) 189257 Derp1 97.43ug/vial, dry weight 12.93mg/vial, total protein 4.21mg/vial, endotoxin 217 EU/vial.

Lyophilised HDM extract was reconstituted with sterile PBS to a concentration of total protein at 1mg/ml. Mice received repeated intranasal instillation while under isofluorane anaesthesia. Specific dosing regimens are detailed below.

2.2.2 Acute HDM exposure and resolution

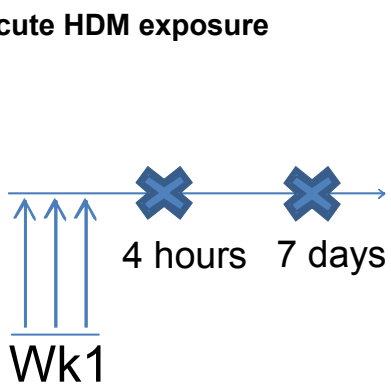
Female mice were given 3 intranasal doses of 25ug in 25ul volumes of prepared HDM in one week (Figure 2.1 A). As a negative control mice received 25ul of sterile PBS (Sigma, Poole, UK). Mice were sacrificed by exsanguinations under terminal anaesthesia (Pentobarbital, Ketamine 100mg/kg) at 4 hours and 7 days post final HDM challenge. Measurements of disease were carried out at 4 hours during peak inflammation and during resolution at 7 days post HDM exposure.

2.2.3 Induction of allergic airways disease and resolution

Female mice were given 3 intranasal doses of 25ug in 25ul volume of prepared HDM for 3 weeks (Figure 2.1 B). As a negative control mice received 25ul of sterile PBS (Sigma, Poole, UK).

Mice were sacrificed by exsanguinations under terminal anaesthesia (Pentobarbital, Ketamine 100mg/kg) at 4 hours, 7 days and 13 days post HDM challenge. Measurements of disease were carried out at 4 hours during peak inflammation and during resolution at 7 days and 13 days post HDM exposure.

A. Model of non-allergic resolution of allergic inflammation



B. Model of allergic airways disease

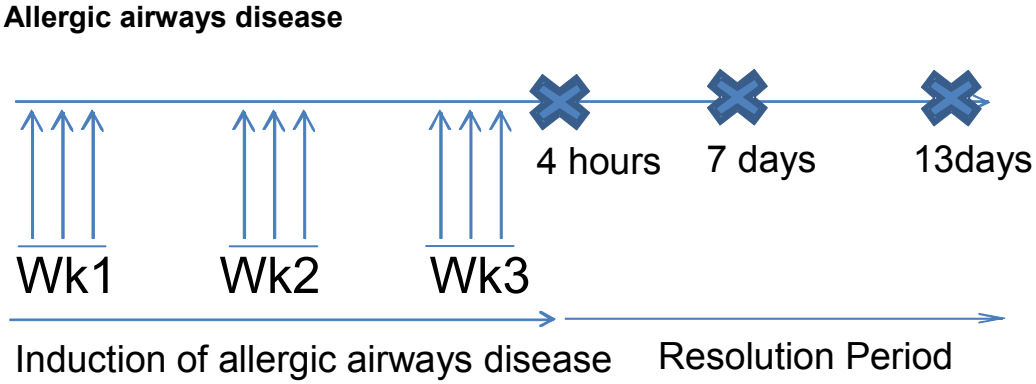


Figure 2.1 Schematics of acute HDM exposure protocol and allergen induced allergic airways disease. (A) Acute HDM exposure (B) Allergen induced protocol of HDM induced allergic airways disease.

2.3 Manipulation of mouse models

2.3.1 Alveolar Macrophage depletion during resolution

Female mice were given 3 doses of 25ug HDM a week for 3 weeks. As a negative control mice received the same volume of PBS (Sigma, Poole, UK). For alveolar macrophage depletion mice received 50ul of clodronate encapsulated liposomes (ClodronateLiposomes.org, Amsterdam, The Netherlands) i.t at 1 day and 5 days post last HDM challenge. Control mice received the same volume of empty liposomes. Mice were sacrificed by exsanguinations under terminal anaesthesia (Pentobarbital, Ketamine 100mg/kg) at 7 and 13 days post HDM challenge. Measurements of disease were measured during resolution at 7 days and 13 days post HDM exposure. Dosing regime and protocol in Chapter 5 (Figure 5.1).

2.3.2 Adoptive transfer of alveolar macrophages during resolution

Alveolar macrophages were derived from naive female BALB/c mice. Mice were given terminal anaesthesia (Pentobarbital 20% i.p) and intubated with a cannula in the trachea. Airways were flushed with 3 x 0.5 ml 1% EDTA/HBSS. Cells were pooled and centrifuge for 4mins, 1400 rpm @ 4^{o}C. Cells were incubated with vibrant dye (Invitrogen, UK) for 10 mins on ice. Approximately 2x10^{5} cells were instilled i.t into recipient mice at 1 day and 5 days post last HDM challenge. Female mice were given 3 doses of 25ug HDM a week for 3 weeks. As a negative control mice received the same volume of PBS (Sigma, Poole, UK). Mice were sacrificed by exsanguinations under terminal anaesthesia (Pentobarbital, Ketamine 100mg/kg) at 7 and 14 days post HDM challenge. Measurements of disease were carried out during resolution at 7 days and 14 days post HDM exposure. Dosing regime and protocol in Chapter 5 (Figure 5.1).

2.3.3 CD200R antibody agonist

Rat anti-mouse CD200R:low endotoxin was purchased from AbD Serotec,Oxford,UK, cat no:MCA2281EL. Mice received either 100µg of CD200R agonist or 100µg of Ig control Rat IgG2a i.p. Dosing regime and protocol in Chapter 4 (Figure 4.1) (Bio express cell culture, West Lebanon, USA) cat no: BE0089

2.3.4 C-15 peptide and C-15 scrambled peptide

The synthetic chemerin-derived peptide C-15 (^{141}AGEDPHGYFLPGQFA^{155}) and C-15 (GLFHPQAGPPAGYEF) scrambled peptide was purchased from Genecust Europe, Luxembourg. Mice challenge with HDM for 3 weeks or PBS control received either 6.4pg C-15 peptide or C-15 scrambled i.p at day 1, 3 and 5 after the last allergen challenge and culled 7 days after last HDM exposure. Dosing regime and protocol in Chapter 4 (Figure 4.1)

2.3.5 WRW4 Formyl-peptide-recptor-2 (FPR2) antagonist and AnxA2-50 Annexin A1 mimetic.

WRW4 and Anx-A2-50 were provided by Mauro Perretti, William Harvey Research Institute, London UK. WRW4 was prepared with PBS to 100µg/ml. AnxA2-50 was prepared to X concentration. Mice received 5µg in 50µl intranasally of either WRW4 or AnxA2-50 daily for 7 days during the final week of 3 week HDM challenge protocol. Control mice received 50µl PBS vehicle. Dosing regime and protocol in Chapter 6 (Figure 6.2)

2.4 Measurements of lung function

Airway hyper-reactivity was measured using Flexivent™ as previously reported (Gregory, Causton Lung resistance and compliance were measured in response to increasing doses of nebulised methacholine (3-100mg/ml, Sigma, Poole, UK) in

tracheotomised anaesthetised mice using an EMMS system (Electro-Medical Measurement Systems, UK). Mice were ventilated according to weight with perturbations measuring resistance and compliance.

2.5 Blood removal and serum isolation

Mice were bled under terminal anaesthesia by cardiac puncture. Approximately 0.5 – 1ml blood was collected from each mouse. Blood was allowed to clot overnight at 4^{o}C. Blood samples were centrifuged at 14000RPM for 15 minutes. Serum was removed and stored at -80.

2.6 Cell recovery

2.6.1 Airway lumen

Bronchoalveolar lavage (BAL) was performed by flushing out the airway three times with 0.4ml of cold PBS via the tracheal cannula as originally reported (Gregory, Causton 2009). BAL fluid was centrifuged (1400 RPM, 4mins, 4^{o}C) and the cell pellet re-suspended in 0.5ml complete RPMI (RPMI + 10% FCS, 2mM L-glutamine, 100U/ml penicillin/streptomycin) and counted using a haemocytometer. Cells were pelleted onto glass slides by cytocentrifugation (5 x10^{4} cells/slide). Differential cell counts were performed on Wright-Geimsa (Sigma, Poole, UK) stained cytospins. Percentages of macrophages, eosinophils, neutrophils and lympho-mononuclear cells are determined by counting ~ 400 cells for each sample and dividing this by the total number of cells counted. To obtain absolute numbers the percentage was multiplied by the total cell counts.

2.6.2 Lung parenchyma

One lobe of lung tissue was mechanically chopped and incubated at 37^{o}C for 1hr in RPMI complete media containing 0.15mg/ml collagenase (Roche Diagnostics, Lewes, UK) and 25ug/ml DNase (Roche Diagnostics, UK). The cells were recovered by filtration through 70µm nylon sieves (VWR Laboratories, UK) washed twice, re-

suspended in 1ml complete media and counted in haemocytometer (SLS, UK). Cells were pelleted onto glass slides by cytocentrifugation (5 x10^4 cells/slide). Differential cell counts were performed on Wright-Geimsa stained cytospins as for BAL.

2.7 Lung tissue histopathology

After removal from the animal, one lobe of the lung was inflated with PBS. Lungs were fixed in 10% normal buffered formalin. Paraffin embedded lung tissue sections (4um) were stained with Haematoxylin & Eosin (H&E) or periodic acid-Schiff (PAS) stain. Paraffin embedding, sectioning and staining were performed by Lorraine Lawrence.

2.7.1 Assessment of mucus production

Goblet cells were counted on Periodic acid Schiff stained lung sections using a semi-quantitative scoring system. Slides were assessed for levels of PAS staining,0 = no staiing, 1= 5-25%, 2 = 25-50%, 3=50-75%, 4= >75%. Scores were divided by the number of airways examined and recorded in arbitrary units.

2.7.2 Assessment of inflammatory foci

A semi-quantitative scoring system was used to grade the size of lung infiltrates, where +5 signified a large (>3 cells deep) widespread infiltrate around the majority of vessels and bronchioles, and +1 signifies a small number of inflammatory foci: 1 = small pocket of infiltrate2 = small pocket (<3 cells deep) in > 1 airway or vessel3= 1>, less than 50% of airways and vessels have large infiltrates (>3 cells deep)4= most airways and vessels have large infiltrates (>50% < 75%)5= majority of airways and vessels have large infiltrates and cells present in alveolar bed (> 75% airways).

All histological analysis were analysed blind and in a randomised order by Sara Mathie.

2.8 Gating and staining protocols for leukocytes by flow cytometric analysis.

Table 2.1. Antibodies for flow cytometry

Molecule	Manufacturer	Isotype	Conjugated dye	Clone
CD11b	BD Biosciences, Oxford, UK	Rat IgG2b	e450	M1/70
CD11c	e-Bioscience Ltd, Hatfield, UK	Hamster IgG1	PerCP-CY5.5	N418
CD200	e-Bioscience Ltd, Hatfield, UK	Rat IgG2a	APC	OX-90
CD200R	AbSerotec, Oxford, UK	Rat IgG2a	APC	OX-110
CD3	BD Biosciences, Oxford, UK	Rat IgG2b	e450	145-2C11
CD4	BD Biosciences, Oxford, UK	Rat IgG2a	PerCP-CY5.5	RM4-5
CD68	Biolegend, San Diego, USA	Rat IgG2a	FITC	FA-11
F4/80	Biolegend, San Diego, USA	Rat IgG2a	PeCY7	BM8
Gr-1	BD Biosciences, Oxford, UK	Rat IgG2b	PeCY7	RB6-8C5.
IL-10	BD Biosciences, Oxford, UK	Rat IgG2b	PE	JES5-16E3
IL-13	e-Bioscience Ltd, Hatfield, UK	Rat IgG1	PE	eBio13A
IL-17	BD Biosciences, Oxford, UK	Rat IgG1	APC	TC11-18H10
Siglec F	BD Biosciences, Oxford, UK	Rat IgG2a	PE	E50-2440
T1/ST2	Morwell Diagnostics GMBH, Switzerland	Rat IgG1	FITC	DJ8
γδ T cell receptor	BD Biosciences, Oxford, UK	Hamster IgG2	FITC	GL3
Mouse Hematopoietic Lineage cocktail	e-Bioscience Ltd, Hatfield, UK	Rat IgG1	e450	17A2, RA3-6B2, M1/70, TER-119, RB6-8C5
ICOS	Biolegend, San Diego, USA	Hamster IgG1	PeCY7	C398.4A
CD45	e-Bioscience Ltd, Hatfield, UK	Rat IgG2b	PerCP-CY5.5	30-F11

PE:R-Phycoerythrin; FITC:Fluorescein; APC: Allophycocyanin; PerCP: Peridinin chlorphyll protein; PE-Cy7: Tandem dye- R-Phycoerythrin with Cy7.e450: efluor450.

2.8.1 Gating strategy for CD3 CD4 lymphocytes by flow cytometric analysis

Whole lung digest and BAL cell pellets were gated for lymphocyte populations as below, (Figure 2.2). γδ TCR+ IL-17+ lymphocytes were determine from lymphocyte gate. CD3 and CD4 populations were identified and analysed for expression of T1/ST2, IL-17, IL-13, IL-10 and FoxP3.

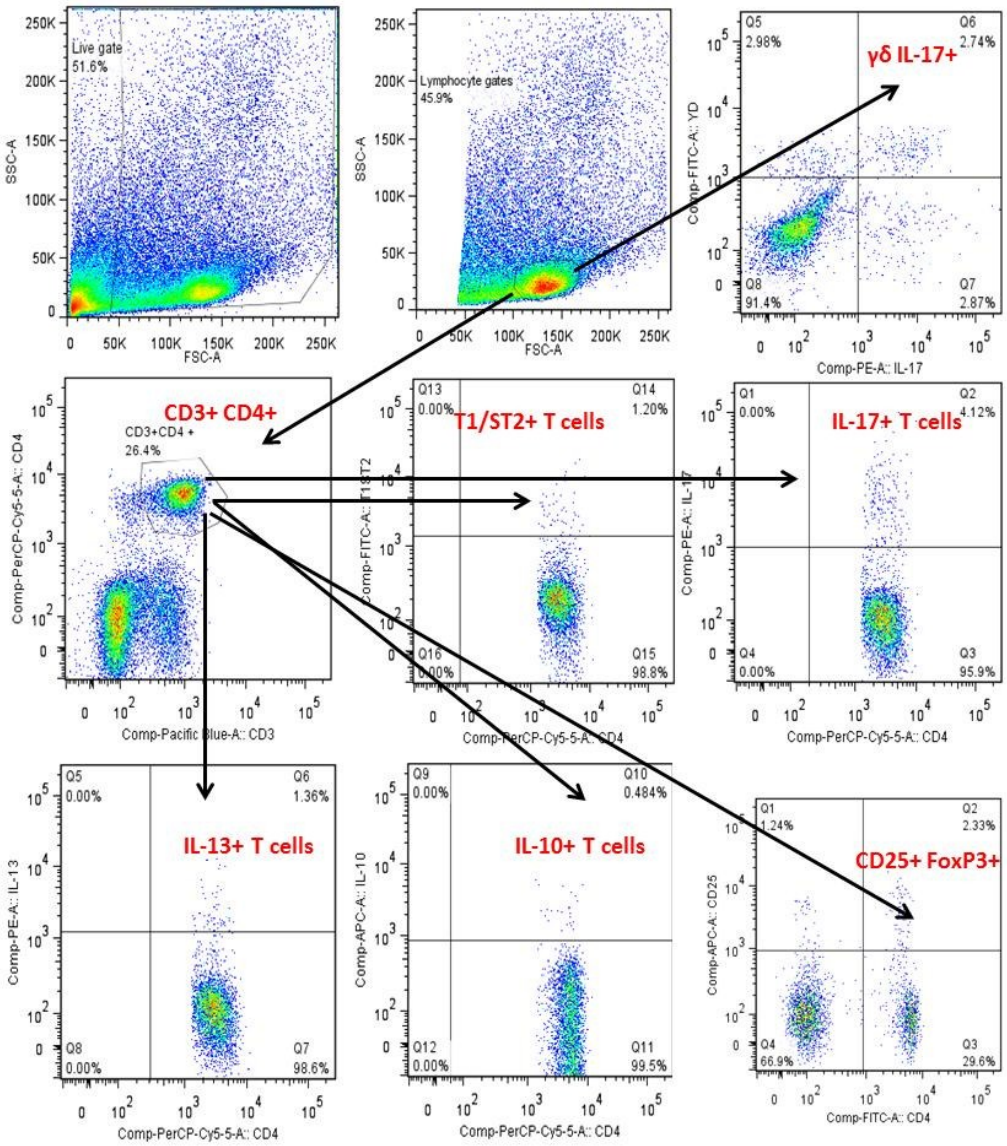


Figure 2.2 Lymphocyte gating strategy. Prepared lung digest and BAL cells were selected in low FSC vs. SSC to isolate lymphocyte population. γδ TCR+ IL-17+ lymphocytes were determined from this gate. CD3 and CD4 populations were identified and analysed for expression of T1/ST2, IL-17, IL-13, IL-10 and FoxP3. Representative FACS plots are shown.

2.8.2 Gating strategy for innate helper cells by flow cytometric analysis

Whole lung digest were gated for innate helper cells (IHC) populations as below, (Figure 2.3). Lineage negative antibody cocktail was used to isolate IHC gate. CD45 and ICOS were used to determine the total IHC population. IL-13 and IL-17 detection was used to determine ILC2 and ILC3, respectively, IHC sub-populations.

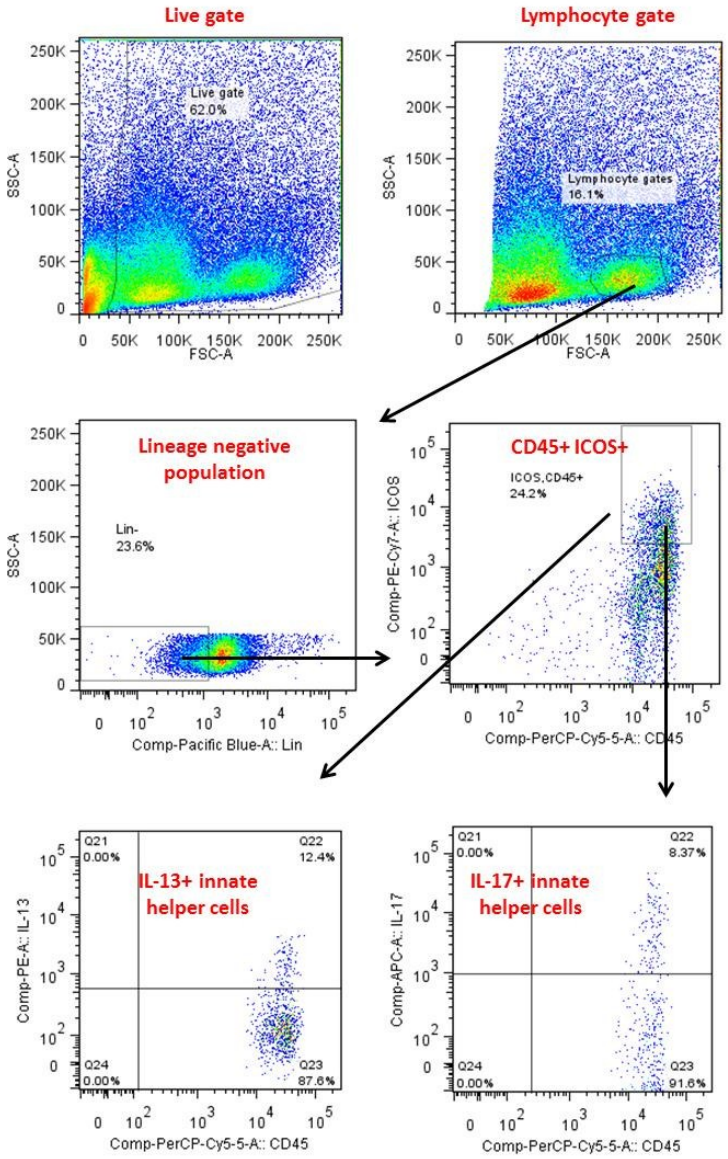


Figure 2.3 Innate helper cell gating strategy. Prepared lung tissue digests were incubated with antibody cocktail for lineage negative markers and antibodies for ICOS, CD45, IL-13 and IL-17 and innate helper cell populations determined as above. Representative FACS plots are shown.

2.8.3 Gating strategy for granulocytes by flow cytometric analysis

Whole lung digest and BAL were gated for eosinophils and neutrophils as below, (Figure 2.4). SiglecF+ CD11c- were classified as eosinophils. Neutrophils were CD11b ^{hi} Gr-1 ^{hi}.

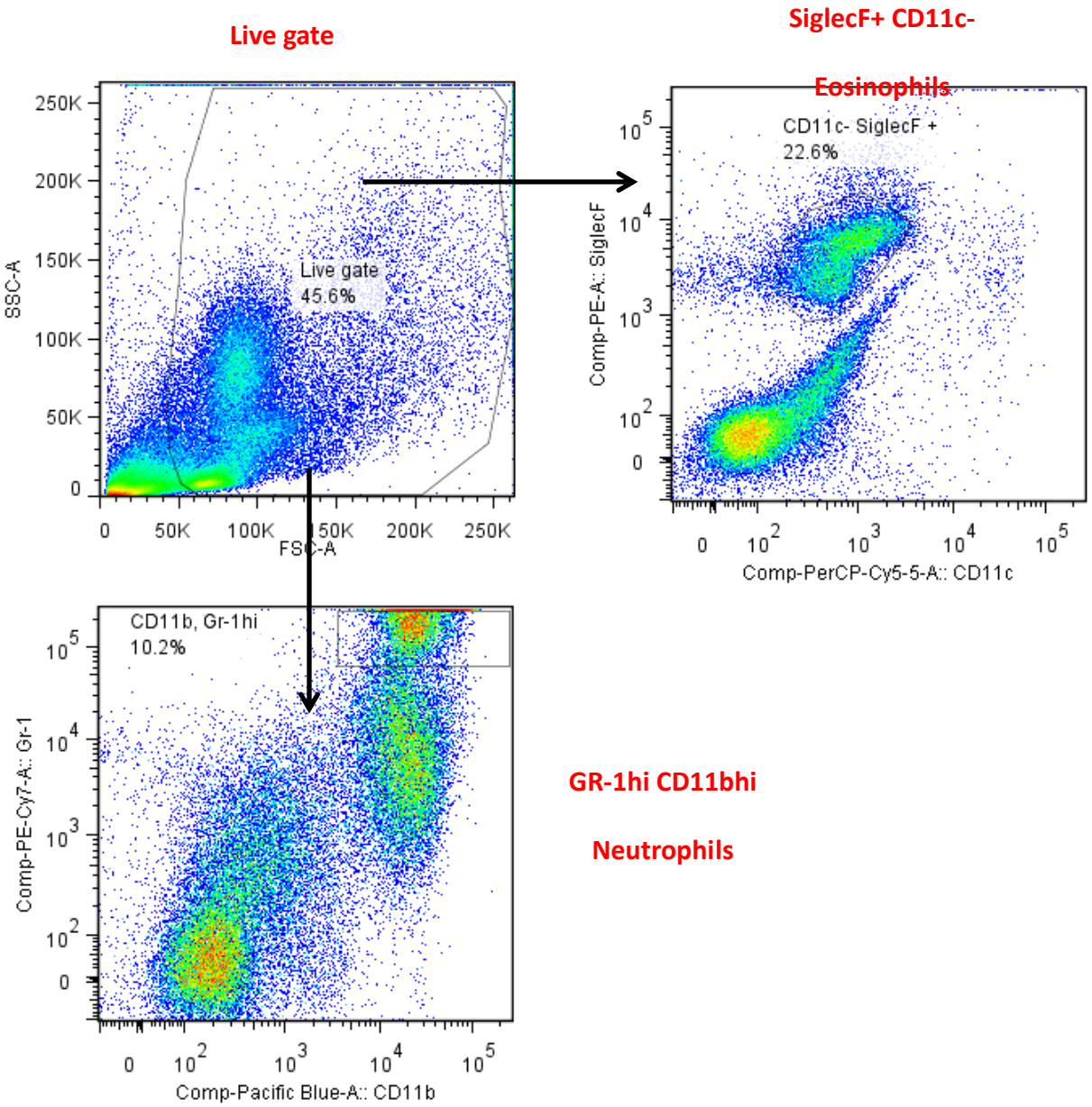


Figure 2.4 Granulocytic gating strategy. Prepared lung tissue digests and BAL cells were incubated with CD11b, CD11c, SiglecF and Gr-1. Representative FACS plots are shown.

2.8.4 Gating strategy for lung macrophage populations by flow cytometric analysis

Whole lung digest and BAL cell pellets were gated for macrophage populations as below, (Figure 2.4). CD68+ F4/80+ cells from whole lung where gated for CD11c expression to determine CD11c+ alveolar macrophages. Interstitial macrophages were determined as CD11c- and CD11b+

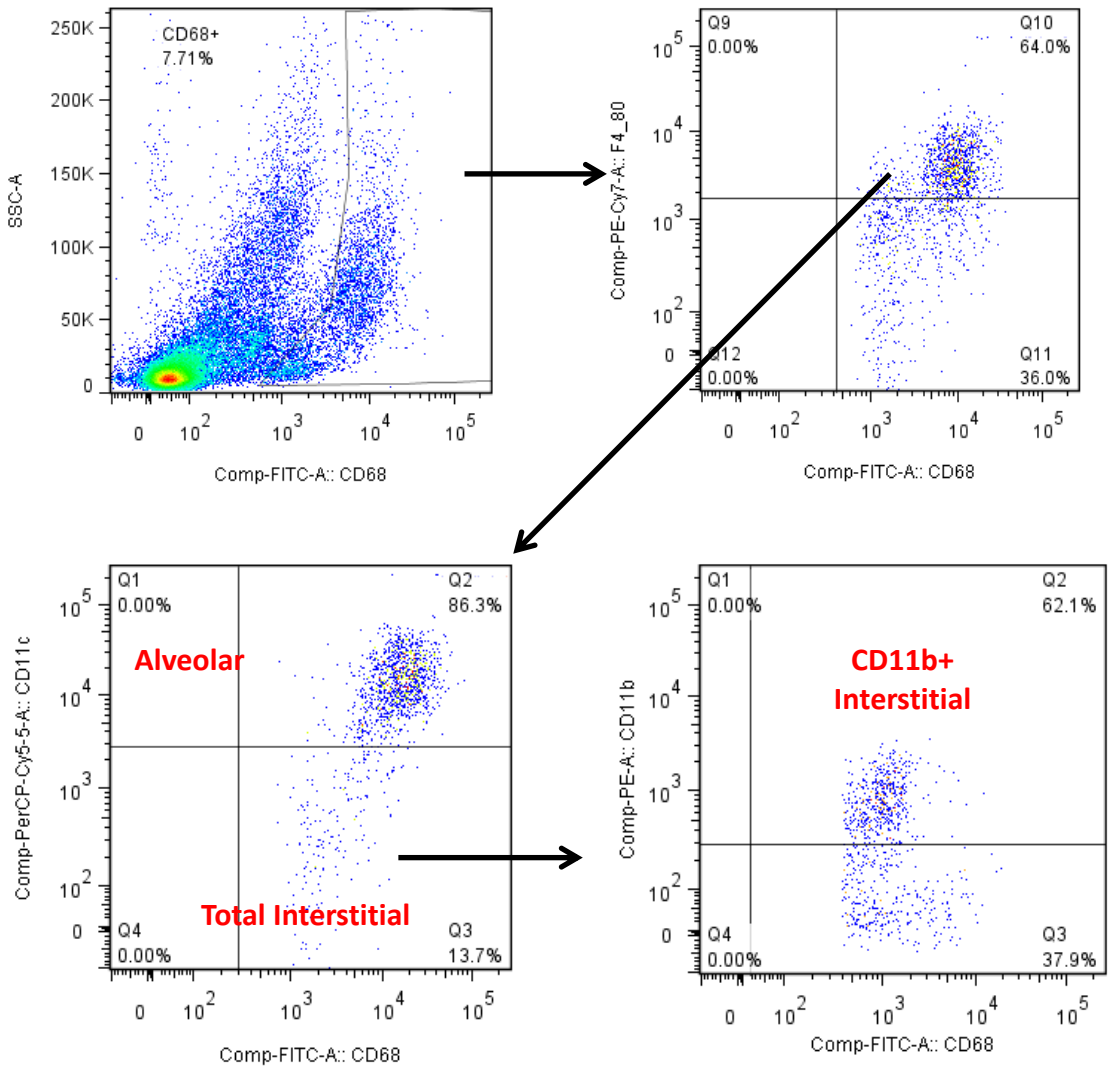


Figure 2.5 Macrophage gating strategy. Prepared lung tissue digests and BAL cells were incubated with antibodies for F4/80, CD68, CD11c, and CD11b. Alveolar macrophages and Interstitial macrophages were determined as shown above. Representative FACS plots are shown.

2.8.5 Extracellular staining protocol

BAL and Lung digest cells were plated in a 96 well plate at a concentration between 2-4x10^5cells/ml. Cells were incubated with rabbit serum (Sigma, UK) to prevent non-specific binding for 15mins prior to staining. Cells were then incubated with fluorescently conjugated extracellular antibodies in filtered FACS buffer for 20minutes @ 4^{O}C, washed twice and fixed in cell fix (BD Biosciences, Oxford, UK). Flow cytometry was performed by FACS Aria (BD Biosciences, Oxford, UK) and analysed using Flowjo software (Treestar, US).

2.8.5 Intracellular staining protocol

Cells were stimulated with PMA/ionomycin in the presence of brefeldin A (Sigma, Poole, UK) for 3 hours prior to extracellular staining. After extracellular staining and fixing as described about cells were permeabilised using Perm buffer (BD Biosciences, Oxford, UK) and stained with intracellular antibodies or isotype control. CD4 CD25 FoxP3 staining for T regulatory lymphocytes was performed using a kit according to manufacturer's instructions (ebioscience, San Diego, US). Flow cytometry was performed by FACS Aria (BD Biosciences, Oxford, UK) and analysed using Flowjo software (Treestar, US).

2.9 Immunoglobulin (Ig) expression, cytokines

2.9.1 Samples

Cytokines were analysed in BAL supernatants and lung tissue homogenate supernatants. Lung tissue was homogenised @ 50mg/ml with HBSS (Invitrogen, Paisley, UK) containing protease inhibitor tablets (Roche Diagnostics, Lewes, UK) centrifuged and the supernatant collected. Total IgE and IgA and HDM-specific IgE and IgA levels were measured in serum homogenate tissue.

2.9.2 Immunoglobulins

Levels of IgE in the serum and IgA in the serum, lung and BAL were measured using paired antibodies according to manufacturer's instructions (BD Biosciences, Oxford, UK). Levels of HDM-specific IgE in the serum and IgA in the serum, lung and BAL were measured. ELISA plates were coated with HDM (1mg/ml) in PBS and blocked. Samples were added and HDM-specific IgE and IgA was detected using biotinylated anti-mouse IgE and IgA ((BD Biosciences, Oxford, UK)

2.9.3 Cytokines and chemokines

Cytokines and chemokines were analysed in BAL samples and lung tissue homogenate supernatants. Paired antibodies for murine IL-4, IL-5 and IFNγ were used in a sandwich ELISA (BD Biosciences, Oxford, UK). IL-10, IL-33, MDC/CCL2, KC/CXCL1, TARC/CCL17, MCP1/CCL2, MDC/CCL22, Eotaxin1/CCL11 & Eotaxin2/CCL24 were measured using R&D duoset. (R&D systems, Abingdon, UK). IL-13 was quantified using Ready-Set-Go! ® paired antibody kit (ebioscience, Hatfield UK), with the exception of IL-13 measurements reported in Figure 3.7C, where Quantikine™ pre-coated ELISA plates (R&D Systems, Abingdon, UK) was used. IL-27 were measured using Ready-SET-Go® ELISA kit, according to manufacturer's instructions, (ebioscience, Hatfield UK)

2.9.4 General ELISA protocol

Enhanced protein binding ELISA plates were coated with the appropriate antibody in coating buffer overnight. Plates were blocked with blocking buffer and samples diluted (1% Bovine Serum albumin + PBS 0.05% Twee) added. A 7 point curve was also generated in duplicated using recombinant antibody, IgE or IgA in assay buffer. After overnight incubation, biotinylated antibody in assay buffer was added. Bound antibody was detected using streptavidin-HRP (Vector Laboratories, Peterborough, UK) in assay buffer. Captured cytokines, IgE or IgA were quantified using K-blue substrate (Skybio, Bedford, UK) and the reaction stopped with 0.19M H_2SO_4.

2.10 Bone marrow derived macrophages

Femurs and tibias from both WT and Annexin KO BALB/c mice were excised, and flushed with sterile complete media to extract the bone marrow. BM was centrifuged, and the supernatant removed. Cell pellets were resuspended in medium containing 10% FCS, 1% pen/strep, 500ul beta-mercaptoethanol in RPMI with L-Glutamine. Cells were diluted to 5*10^6 per ml, and cultured with either GM-CSF to produced M1-like macrophages or M-CSF for M2-like macrophages, at 37°C for 8 days. Fresh GM-CSF & BM medium was added to cells cultured with GM-CSF at day 3 and day 6. Cells cultured with M-CSF were left for 8 days without change At day 8, cells were harvested with EDTA to detach adherent cells. Cell counts were carried out, and diluted to 1*10^6 before being plated. Cells were left at 37°C for one day, before being stimulated with either 50ul of PBS (control), HDM, or LPS for either 4 hours, 24 hours, or 40 hours. After each time point, cells were centrifuged, and the supernatant removed and frozen before cytokine and chemokine analysis, using kits described above.

2.11 RT –PCR

Real-time PCR was used to quantify expression of Annexin A1 and FPR2 mRNA in murine lung tissue. The medial lobe was preserve in RNAlater and homogenised to total RNA isolated using RNeasy plus mini kits (Qiagen,Crawley UK) according to manufacturer's instructions. 1µg of RNA was reversed transcribed using high capacity reverse transcription kit (Applied Biosystems, Warrington, UK).Specific cDNA was detected using pre-designed probes (Applied Biosystems, Warrington, UK). 7µl real-time PCR reactions were carried out with Taqman advanced master mix (Applied Biosystems, Warrington, UK) and performed on ViiA7 system (Applied Biosystems, Warrington, UK).

2.12 Annexin A1 and FPR2 immunohistochemistry

Paraffin-embedded mouse lung sections were used for immunolocalisation of Annexin A1 and FPR2. Sections were acquired from mice sensitised and challenged to HDM then sacrificed at 4 hours and 7 days after last HDM challenge. Sections were treated with sodium citrate, and blocked with 10% donkey serum, 1 % BSA, 0.025% triton. Sections were incubated with the primary antibody Annexin A1 (R&D Systems) or FPR2 antibody (Santa Cruz,Inc, Heidelberg, Germany). Annexin A1 primary was, followed by a secondary detection antibody (donkey anti-goat 488). FPR2 antibody was incubated with a secondary biotin anti-rabbit then tertiary detection antibody, streptavidin conjugated with Alexa Fluor 488.

Data analysis

All analysis of HDM-treated groups compared with PBS control groups were tested using Mann Whitney U test. Primary statistical endpoint determining resolution event was day 13 vs. PBS control group. Secondary statistical endpoint determining inflammatory events was 4 hours vs. PBS control group. Data expressed as mean ± SEM, n=6-12 mice/group, with significance of less than p<0.05. The numbers of mouse used per treatment are based on original experiments from our group as required by the Home Office for project licence application. These were designed to calculate the minimum number of animals for maximum significance. Power calculations were performed to calculate a sample size necessary per treatment group to be 90% confident to correctly detect differences of at least 30%. Graph generation and statistical analysis were performed using GraphPad Prism software (version 4; GraphPad, La Jolla, CA, USA).

Chapter 3.

Generation of a model of the resolution of allergic airways disease using house dust mite allergen

3.1 Introduction

This chapter describes the resolution of the features of house dust mite induced allergic airways disease. The purpose of this study was to develop a protocol to study the resolution of allergic inflammation induced by inhaled allergen challenge. This was carried out by characterising the progression of the allergic airways disease phenotype and regulatory mediators following cessation of HDM exposure. We have previously shown that continual exposure to inhaled HDM for 3 weeks promotes a decline in lung function and induces Th2 allergic airway inflammation (Gregory, Causton et al. 2009). Using this model we have used the read out at 4 hours after last HDM challenge as a reference for peak disease. In BALB/c mice, at this time point mice exhibit significant airway hyper-reactivity, pulmonary inflammation and Th2 cytokine production. Here, to determine the resolution profile of HDM induced allergic airways disease, mice were culled at 7 and 13 days post HDM challenge and parameters of allergic airways disease measured and compared with 4 hours post final challenge.

3.1.1 Hypothesis

The resolution of HDM induced allergic airways disease is an active process characterised by specific cellular and molecular mediators.

3.1.2 Aims

To characterise the temporal resolution of allergic airways disease following cessation of allergen challenge.

To determine the regulatory cells and molecules involved in the resolution process.

3.2 Method

3.2.1 Experimental protocol of time course post HDM challenge

Female BALB/c mice aged between 6-8 weeks received repeated intranasal instillation of 25µg house dust mite 3 times a week for 3 weeks. Mice were culled at 4 hours, 7 days and 13 days post final challenge. Lung function measurements and collection of blood, BAL and lung tissue samples were carried out, Figure 3.1.

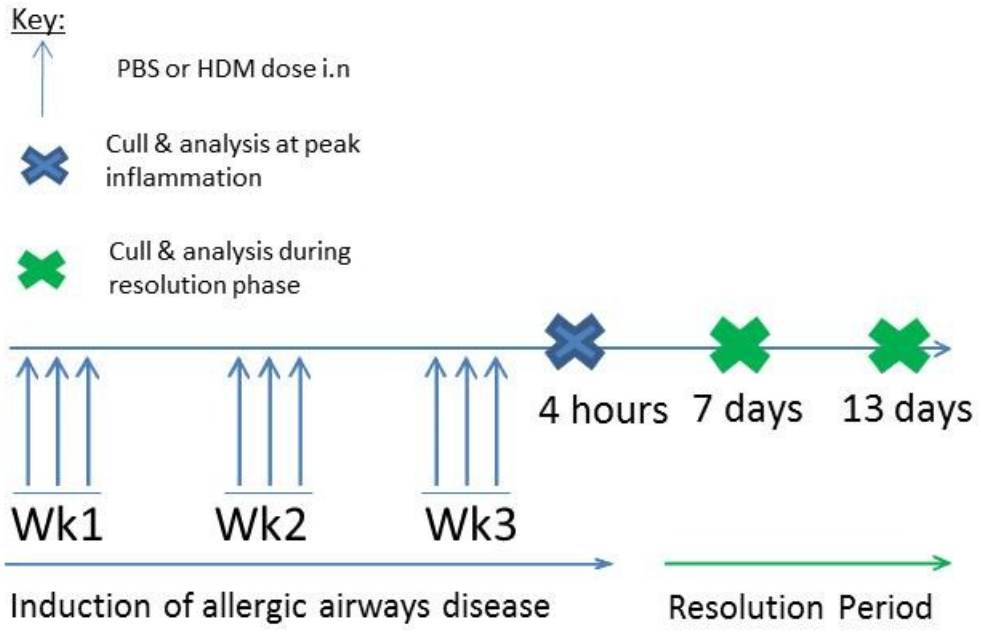


Figure 3.1 Experimental protocol after exposure to HDM.
Mice were culled at 4 hours, 7 days and 13 days after final HDM challenge for lung function measurements and collection of blood, BAL and lung samples.

3.3 Results

3.3.1 Airway hyper-reactivity (AHR) is maintained up to 7 days after last HDM challenge and resolves by 13 days.

Airway resistance induced by HDM, remained significantly elevated at 7 days. This reduced to baseline levels 13 days post allergen challenge, (Figure 3.2 A & B). Lung compliance is significantly decreased following allergen challenge and remains significantly lower at 7 days, resolving to levels comparable to PBS by 13 days, (Figure 3.2 D & E). These results demonstrate that AHR is restored to baseline by 13 days.

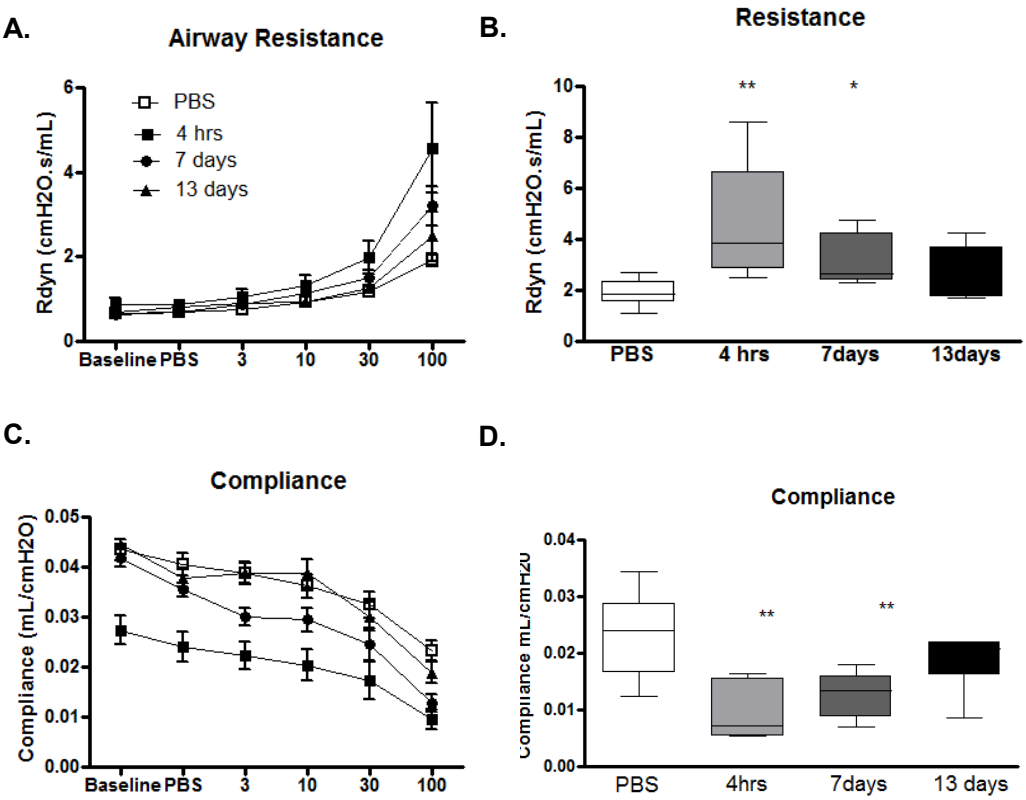


Figure 3.2 Airway hyper-reactivity during resolution of HDM induced allergic airways disease. AHR was measured following increasing doses of Methacholine (MCh) challenge. Changes in Resistance (A) and Compliance (C) were measured 4 hours, 7 days and 13 days after HDM challenge. Mean levels of resistance (B) and (D) compliance at 100mg/ml MCh. n = 4-8 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ and ** $p<0.01$ relative to PBS control group by Mann Whitney test. $^{+}$ $p<0.05$ $^{++}p<0.01$ and $^{+++}$ $p<0.001$ relative to peak inflammation, 4 hour time point group by Mann Whitney test.

3.3.2 Lung tissue leukocytes resolve by 13 days whilst residual levels remain in the airway at 13 days

Pulmonary inflammation was quantified in both the lung tissue compartment and in the airway. Leukocytes in the lung tissue egress into the airway where it is thought that they undergo apoptosis and efferocytosis by alveolar macrophages. Thus to determine the movement of leukocytes during the resolution of allergic inflammation both compartments were analysed. Lung tissue leukocytes were isolated by digest and airway leukocytes were retrieved by broncho- alveolar lavage (BAL) as described in materials and methods.

Following HDM exposure, the numbers of leukocytes in the lung tissue and airway were significantly increased, (Figure 3.3 A & B). Total lung tissue leukocytes remained increased at 7 days and resolved to levels comparable to PBS by day 13 post challenge, (Figure 3.3 A). Cell numbers in the BAL decreased compared to peak inflammation by 7 and 13 days but remained significantly higher compared to PBS controls up to 13 days, (Figure 3.3 B). The resolution of pulmonary inflammation was also demonstrated by H&E staining of paraffin embedded lung sections, (Figure 3.3 C). Dense cellular infiltrate can be seen surrounding the airways and pulmonary vasculature at 4 hours. Sections were scored for inflammation using semi-quantitative analysis, (Figure 3.3 D). Inflammation recedes from the airway at 7 days and by day 13 inflammation has significantly resolved. Scoring of sections correlates with total BAL cell counts where residual low levels of cells can be observed. Lung tissue cells counts demonstrate resolution by 13 days which correlates with the resolution of AHR.

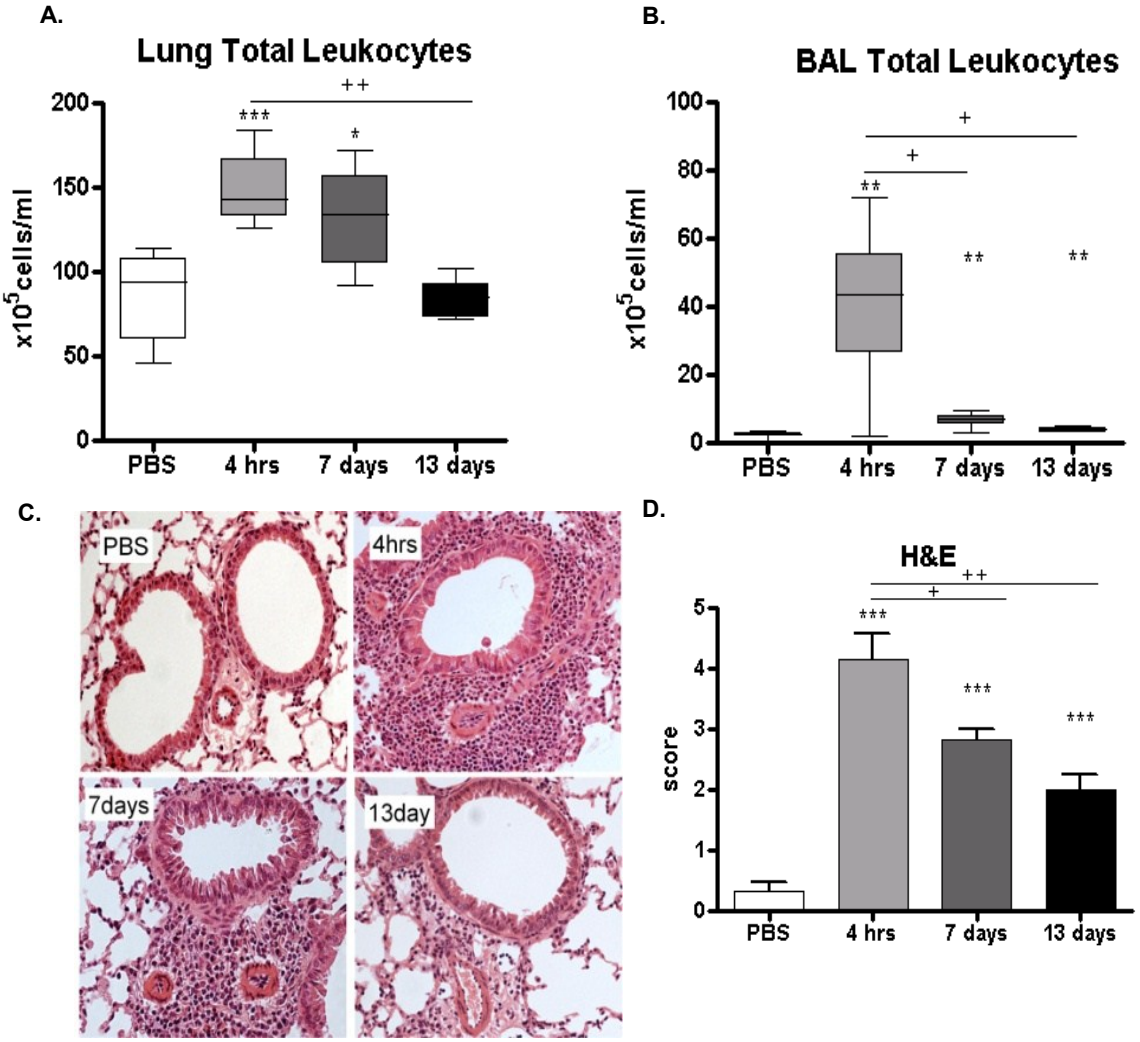


Figure 3.3. Pulmonary inflammation following HDM exposure. BAL and lungs were quantified at 4 hours, 7 days and 13 days following 3 weeks of HDM challenge. Inflammation was measured by total cell counts (A & B) and scoring of H&E stained lung sections x20 magnification (C&D) n = 4-8 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ relative to PBS control group by Mann Whitney test.$^{+}$ $p<0.05$ $^{++}p<0.01$ and $^{+++}$ $p<0.001$ relative to peak inflammation, 4 hour time point group by Mann Whitney test.

3.3.3 Lung tissue and airway differential cell counts

Differential cell counts analysis was carried out on wright giemsa stained cytospins, (Figure 3.4A). Analysis demonstrated a significant increase in granulocytes following HDM exposure in the lung tissue and BAL, (Figure 3.4 B-E). Eosinophils remain elevated at 7 days and return to baseline by day 13 whereas neutrophils return to baseline more quickly and are not different from PBS controls 7 days after cessation of allergen challenge. In the lung tissue, lympho-mononuclear and macrophage populations were not altered following HDM exposure (Figures 3.4 F & H). However, by 7 days a significant increase in the lympho-mononuclear population was observed, which then returned to baseline levels by 13 days, (Figure 3.4 F). In the airway, there was a significant increase in macrophages and lympho-mononuclear cell numbers following HDM exposure. (Figure 3.4 G & I). Macrophage numbers return to PBS levels by 13 days. However, the lympho-mononuclear population remained significant elevated in the airway up to 13 days post HDM challenge. These results illustrate that HDM induced inflammation resolves in the lung tissue by 13 days with residual levels of lymphocytes remaining in the airway.

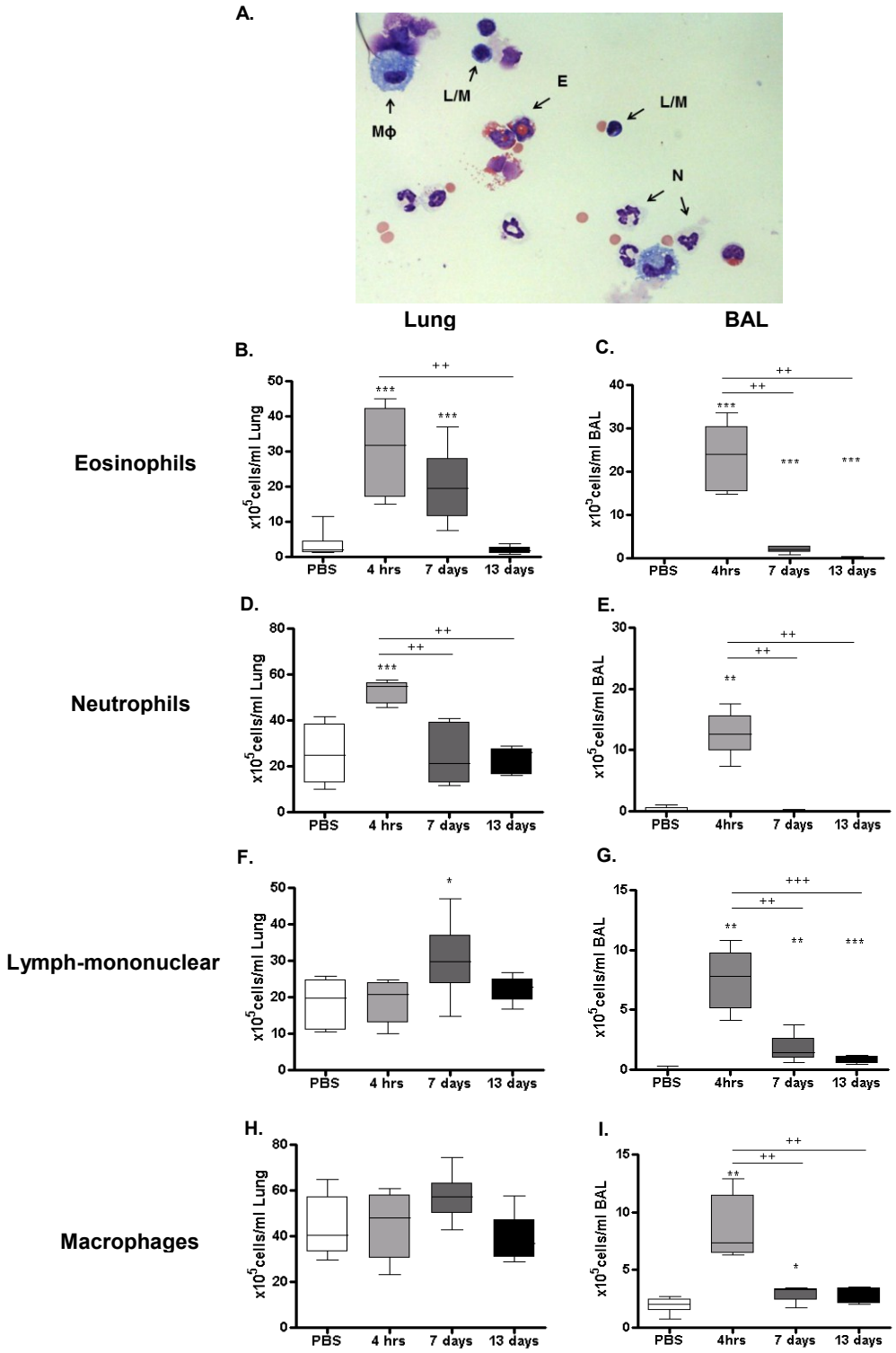


Figure 3.4 Differential BAL and Lung tissue cell counts during resolution. BAL and lungs were taken at 4 hours, 7 days and 13 days following 3 weeks of HDM challenge. Representative photo of cytospins depicting differential cell populations; E: Eosinophils, N: Neutrophils, L/M: Lympho-mononuclear and Mϕ: Macrophage for quantification (A). Eosinophils (B&C), Neutrophils (D&E) lympho-mononuclear cells (F&G) and macrophages (H&I) were identified and quantified on Wright Giemsa stained cytospins prepared form lung tissue digest and BAL. n = 4-6 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ relative to PBS control group by Mann Whitney test. $^{++}p<0.01$ and $^{+++}p<0.001$ relative to peak inflammation, 4 hour time point group by Mann Whitney test.

3.3.4 Epithelial mucus production persists up to 13 days post HDM exposure

In our experimental model, HDM promotes a change in epithelial cells of the conducting airways to a mucous secreting phenotype, demonstrated by Periodic-acid Schiff (PAS) staining of paraffin embedded lung tissue sections, (Figure 3.5 A). Semi-quantitative analysis of PAS stained lung sections show an increase in the numbers of mucous producing cells. This feature of HDM induced airway remodelling was not resolved by 13 days post final allergen challenge.

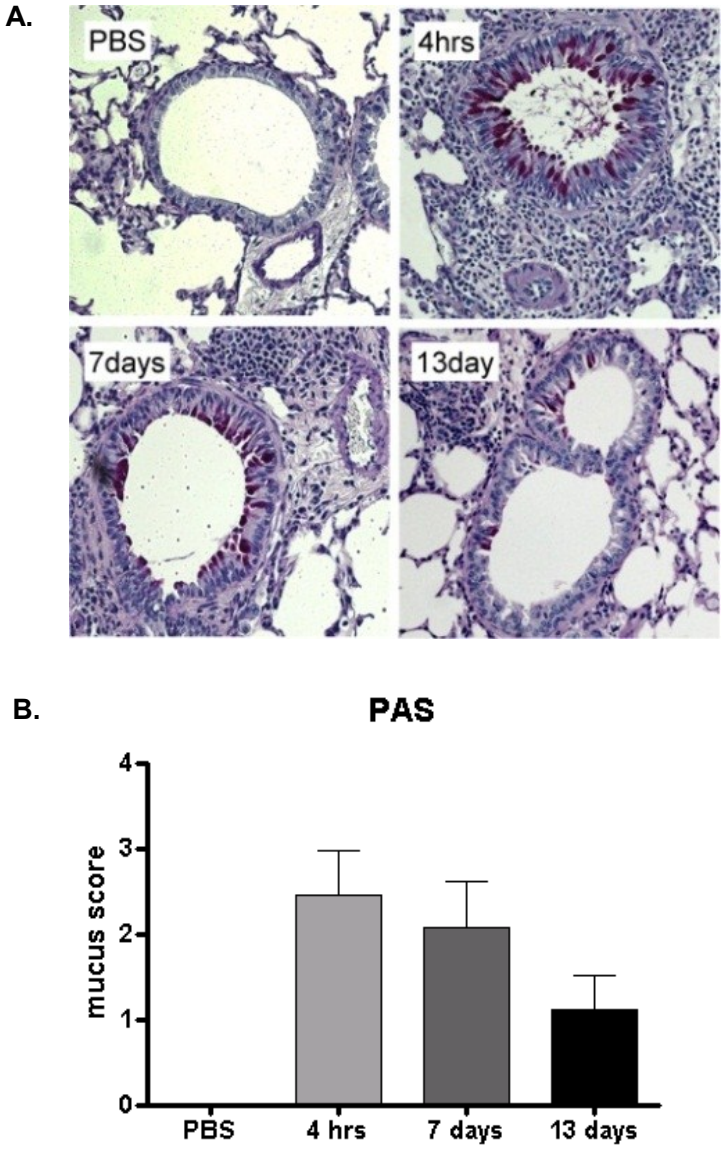


Figure 3.5 Epithelial mucus production. Lungs were taken at 4 hours, 7 days and 13 days following 3 weeks of HDM challenge. (A) Representative photomicrograph of airway sections stained with periodic acid Schiff (PAS) x20 magnification. (B) Mucus production was determined by semi-quantitative scoring of PAS stained lung tissue sections n = 4-8 mice/group, bar represents mean +/- SEM. * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ relative to PBS control group by Mann Whitney test. $^{+}$ $p<0.05$ $^{++}p<0.01$ and $^{+++}$ $p<0.001$ relative to peak inflammation, 4 hour time point group by Mann Whitney test.

3.3.5 Flow cytometric analysis of lung tissue lymphocytes during resolution

FACS analysis of lung tissue cells was employed to further characterise the inflammatory lymphocyte subsets during resolution. Analysis of the CD4+ lymphocyte sub-population demonstrated increased levels of lymphocytes expressing the putative Th2 marker, T1/ST2+. Th2 cell numbers were significantly reduced by 7 days compared to peak inflammation and did not return to baseline levels until 13 days post final allergen challenge, (Figure 3.6 A). Th17 and γδ+ IL-17 T cells were induced following HDM exposure. Levels of Th17 lymphocytes but not γδ IL-17+ T cells remained significantly elevated up to 13 days following HDM exposure, (Figure 3.6 B& C). CD200 expression on CD4 lymphocytes has been linked with the resolution process and therefore we determined levels of CD4 CD200+ lymphocytes following allergen challenge. CD4 lymphocytes expressing CD200 were not significantly elevated at peak inflammation, however, levels were significantly up regulated at 7 days after last HDM exposure and remained elevated at day 13, (Figure 3.6 D). In contrast, the numbers of natural occurring CD4+ CD25+ FoxP3+ and CD4+ IL-10+ T regulatory lymphocytes in the lung peaked at 4 hours and followed a similar resolution profile to Th2 lymphocytes, remaining significantly elevated at 7 days post challenge and returning to baseline by 13 days, (Figures 3.6 E & F). These results demonstrate that subsets of CD4 lymphocytes have a distinct temporal role during resolution.

3.3.6 Lung tissue Th2 cytokines resolve by 13 days

To corroborate the increase in Th2 type inflammation following HDM, levels of Th2 associated cytokines were quantified in the lung by ELISA. IL-4, IL-5, IL-13 and IL-33 were all elevated at peak inflammation. IL-4 and IL-5 remained increased at 7 days and resolved by 13 days, (Figure 3.7 A & B), IL-13 was intimately linked to HDM exposure and returned to baseline levels by 7 days post allergen challenge (Figure 3.7 C). IL-33 induced by HDM although reduced significantly in the lung by 7 days remained at significantly higher levels compared to PBS controls up to 13 days, (Figure 3.7 C & D).

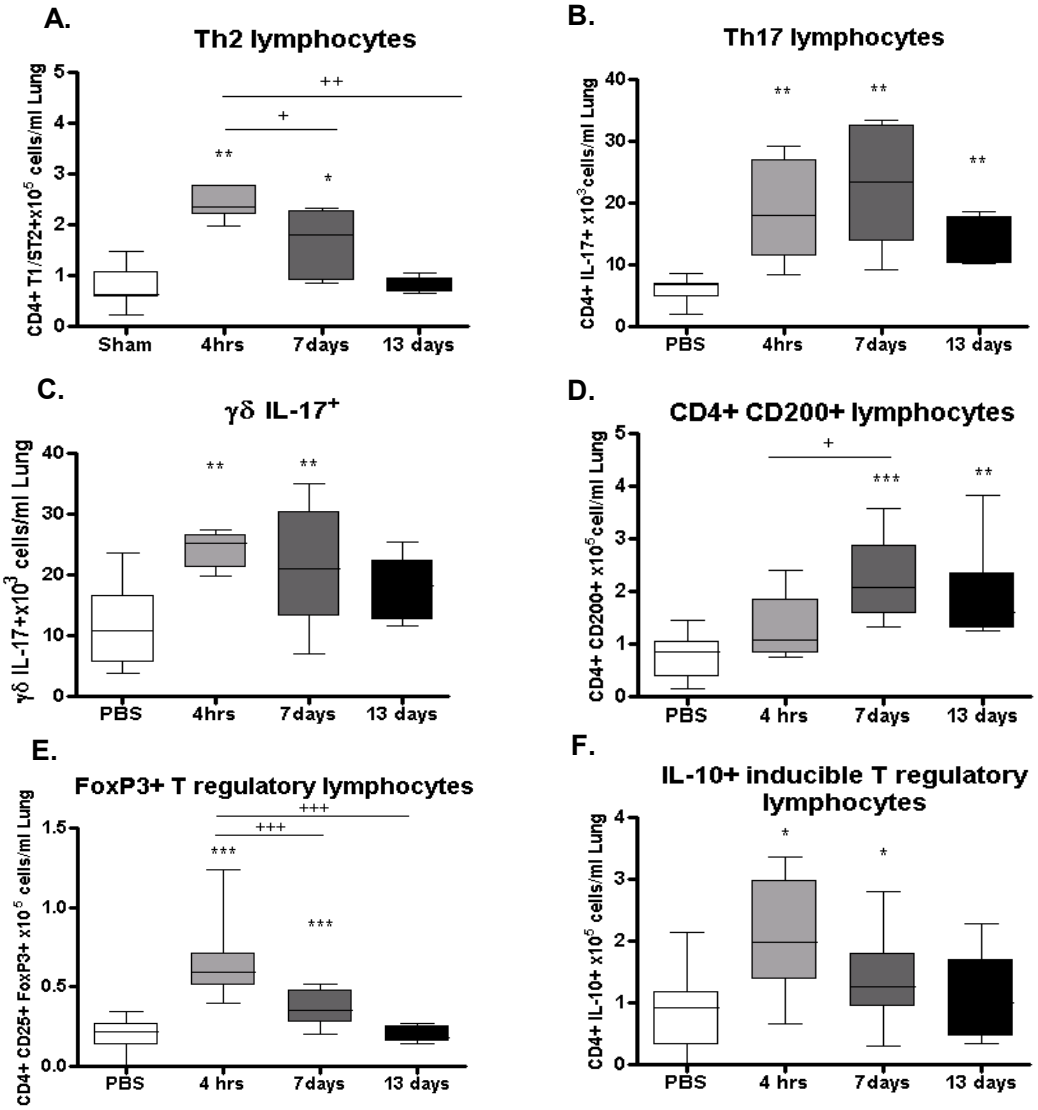


Figure 3.6 Induction of T lymphocytes following HDM exposure. Lungs were taken at 4 hours, 7days and 13 days following 3 weeks of HDM challenge. Levels of (A) CD4+ T1/ST2+ (B) CD4+ IL-17+, (C) YD+ IL-17+ (D) CD4+ CD200+, (E) CD4+ CD25+ FoxP3+ T regulatory and (F) CD4+ IL-10+ lymphocytes were identified and quantified by flow cytometry. n = 4-8 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ relative to PBS control group by Mann Whitney test. $^{+}$ $p<0.05$ $^{++}p<0.01$ and $^{+++}$ $p<0.001$ relative to peak inflammation, 4 hour time point group by Mann Whitney test.

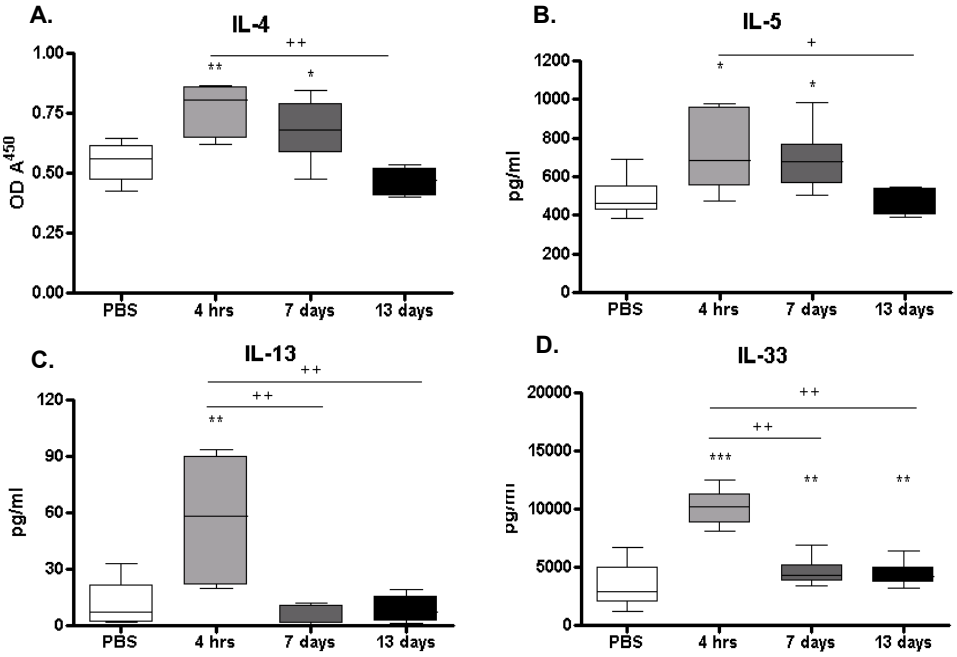


Figure 3.7 Mediators of Th2 inflammation during resolution. Lungs were taken at 4 hours, 7 days and 13 days following 3 weeks of HDM challenge. Cytokines were quantified in lung homogenate supernatant using ELISA. Apart cytokines are expressed as pg/ml calculated from the standard curve, IL-4 levels are expressed as optical density by absorbance at 450nm. (A) IL-4 (B) IL-5, (C) IL-13 (D) IL-33. n = 4-6 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ relative to PBS control group by Mann Whitney test. $^{+}$ $p<0.05$ $^{++}p<0.01$ and $^{+++}$ $p<0.001$ relative to peak inflammation, 4 hour time point group by Mann Whitney test.

3.3.7 Humoral immunity during resolution of HDM induced inflammation

To determine the systemic effects following HDM exposure and during the resolution phase, serum total and HDM specific IgE were measured by ELISA. At peak inflammation, total and HDM specific IgE were significantly elevated. IgE remained significantly increased up to 13 days post allergen challenge (Figure 3.8 A & B). HDM specific IgG1 and IgG2a were also induced by HDM and serum levels remained significantly elevated up to 13 days, (Figure 3.8C & D).

To examine the humoral mucosal defence in the lung during resolution, total and HDM specific IgA were measured in the lung and BAL. Total IgA and HDM specific IgA measured in the BAL and lung increased following HDM exposure and remained elevated during resolution, (Figure 3.8 E - H). Interestingly, at 7 days after cessation of allergen exposure, levels of total IgA and the production of HDM specific IgA, in both the lung and BAL, was further increased and remained significantly elevated up to 13 days following final HDM challenge. This up regulation during the resolution phase suggests IgA is playing an active role in restoring homeostasis.

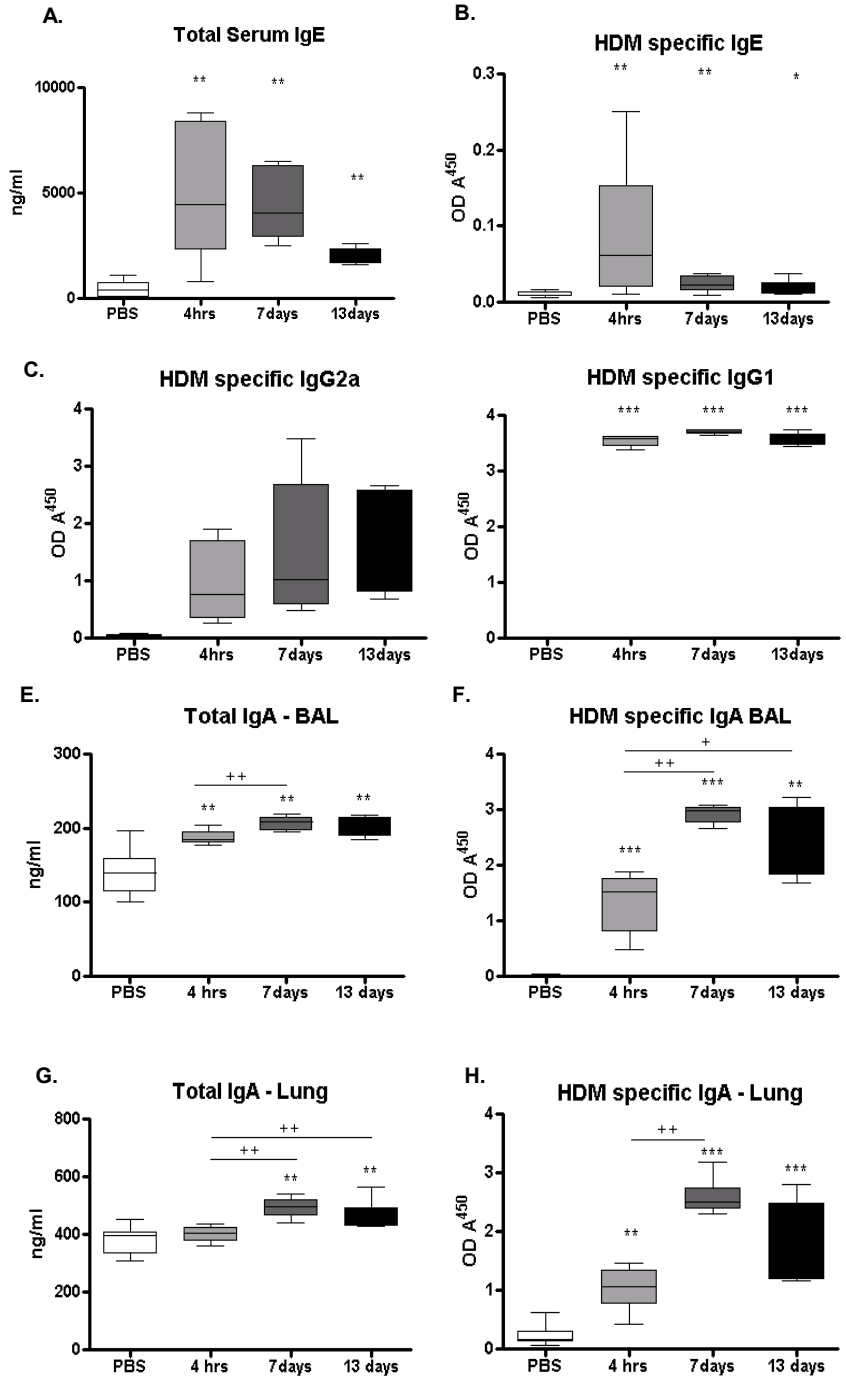


Figure 3.8 Humoral immunity during resolution of HDM inflammation. Levels of IgE IgG1 and IgG2a were quantified in serum by ELISA. (A) Total IgE, (B) HDM specific IgE, (C) HDM specific IgG2a, (D) HDM specific IgG1. Total and HDM specific IgA were measured in BAL and Lung. (E) Total IgA BAL, (F) HDM specific IgA BAL, (G) Total IgA lung and (H) HDM specific IgA Lung. Serum, BAL and lung tissue were taken at 4 hours, 7 days and 13 days following 3 weeks of HDM challenge n = 4-8 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ relative to PBS control group by Mann Whitney test. + $p<0.05$ ++$p<0.01$ and +++ $p<0.001$ relative to peak inflammation, 4 hour time point group by Mann Whitney test.

3.3.8 Alveolar macrophages are increased concomitant with the resolution of HDM induced inflammation

Alveolar macrophages are the most predominant leukocyte in the healthy airway and are required to maintain pulmonary homeostasis (Holt, Strickland et al. 2008, Wissinger, Goulding et al. 2009). Therefore, it was of interest to investigate their role in the resolution of HDM induced inflammation. The numbers of alveolar macrophages in the lung during resolution were quantified using flow cytometry. Levels of CD11c+ F4/80+ macrophages increased at 4 hours with numbers significantly elevated at 7 days post challenge, remaining significantly elevated at day 13, (Figure 3.9 A). Expression of the homeostatic molecule CD200R is significantly up regulated on alveolar macrophages at 7 days and remains elevated at 13 days, (Figure 3.9 B). This temporal up regulation indicates that alveolar macrophages are important during the resolution phase of HDM inflammation. This also implies that AMs are important in promoting the return to homeostasis after allergic airway insult.

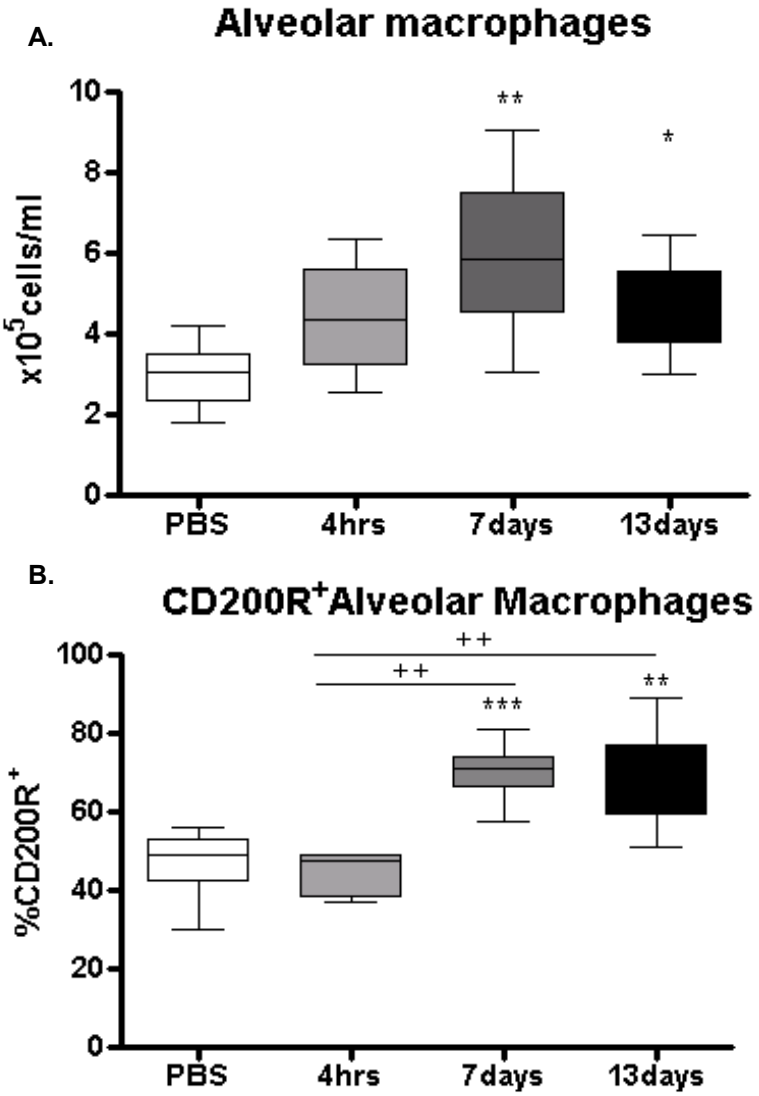


Figure 3.9 Alveolar macrophages are increased concomitant to resolution of AAD Lungs were taken at 4 hours, 7 days and 13 days following 3 weeks of HDM challenge**.** Levels of alveolar macrophages and expression of CD200R were identified and quantified by flow cytometry. n = 4-8 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ relative to PBS control group by Mann Whitney test. $^{+}$ $p<0.05$ $^{++}p<0.01$ and $^{+++}$ $p<0.001$ relative to peak inflammation, 4 hour time point group by Mann Whitney test.

Table 3.1 Summary of features of disease and regulatory mediators during the resolution of house dust mite induced allergic airways disease

Disease parameters compared to PBS controls	Time post last allergen challenge		
	4 hours	7 days	13 days
Airway Resistance	↑	↑	—
Lung Compliance	↑	↑	—
Th2 lymphocytes	↑	↑	—
Eosinophils	↑	↑	—
Neutrophils	↑	—	—
IL-4	↑	↑	—
IL-5	↑	↑	—
IL-13	↑	—	—
IL-33	↑	↑	↑
IgE	↑	↑	—
Regulatory Parametres			
Macrophage	—	↑	↑
CD200R	—	↑	↑
Lympho-mononuclear cells	—	↑	↑
T regulatory lymphocytes	↑	↑	—
IgG	↑	↑	↑
mucosal IgA	↑	↑	↑

↑ = increase relative to PBS

—— = equivalent to no change from baseline

3.3.9 Schematic of resolution of allergic airways disease

Table 3.1 summarises the temporal expression of the features of allergic airways disease and regulatory mediators during resolution. Overall, as the main features of AAD resolve there is a parallel elevation of regulatory parameters which remain elevated after cessation of allergen challenge. This is depicted in a schematic below.

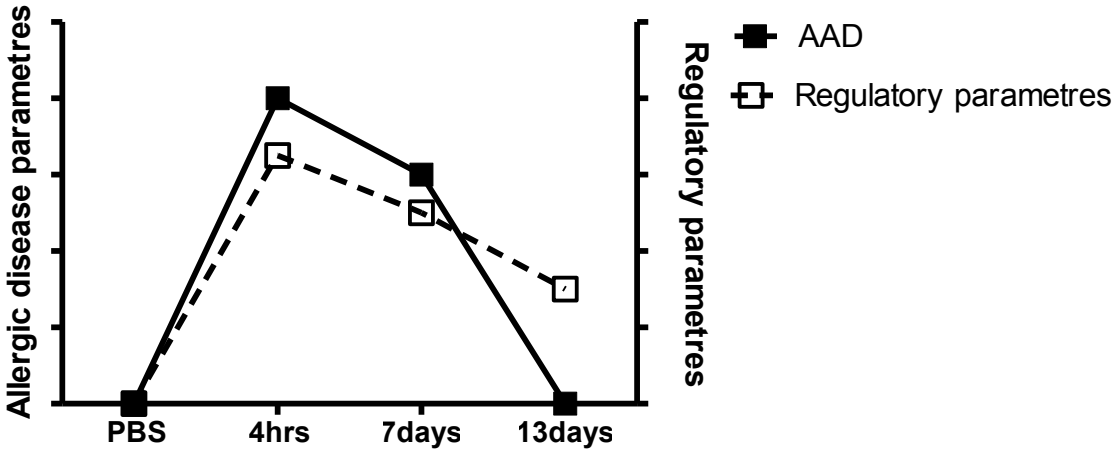


Figure 3.10 Schematic to show resolution of allergic airways disease

Filled squares and complete line represent features of AAD. Regulatory parameters are represented by open squared and dash line.

3.3.10 Rechallenge with HDM in the resolved lung induces a rapid allergic airways disease phenotype

The data above illustrates that regulatory mediators are upregulated in parallel with the waning of the features of allergic airways disease. This upregulation suggests that the homeostatic state of the lung following resolution of HDM induced inflammation is altered. This is particularly demonstrated by an increase in the number of alveolar macrophages and their increased expression of the myeloid negative regulatory molecule CD200R. To determine if the resolved lung after HDM induced disease demonstrated altered homeostasis, mice were rested for a period of 28 days after last HDM challenge then rechallenged with a single dose of HDM and culled 4 hours after. A control group received rechallenge with PBS. A second control group consisted of PBS challenged mice receiving a single dose of HDM at day 28 and culled 4 hours after.

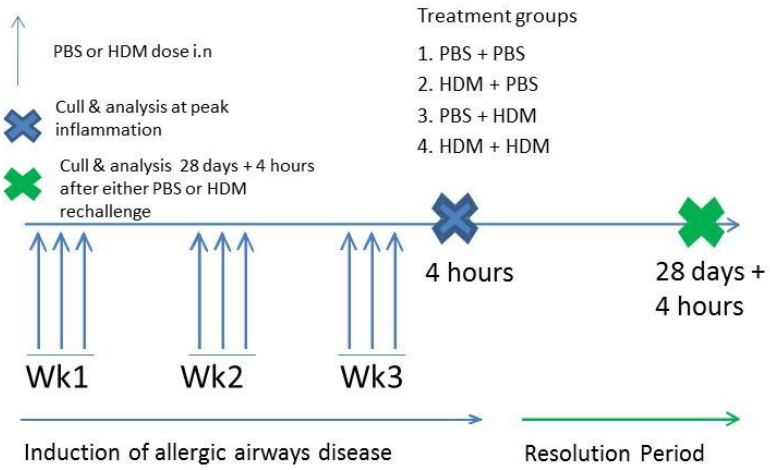


Figure 3.11 Experimental rechallenge protocol

Mice were culled at 4 hours and 28 days after final HDM challenge for lung function measurements and collection of blood, BAL and lung samples. Groups at HDM challenged mice at 28 days either received rechallenge with a single dose of 25ug of HDM or 25ul PBS and culled 4 hours after. A second control group of PBS challenge mice received a single dose of 25ug of HDM at 28 days.

3.3.11 Rechallenge with HDM in the resolved lung induces significant airway hyper-reactivity

A single rechallenge with HDM exhibited a significant induction of AHR as illustrated by an increase in airway resistance and a reduction in lung compliance, (Figures 3.12). This was due to prior exposure to HDM as mice receiving a single dose of HDM did not exhibit significant AHR. Baseline compliance in mice at 28 days + 4 hours is higher than PBS groups at 4 hours. This is due to the mice being older and bigger. We have previously shown that compliance values increase with age and size of the mice (Saglani, Mathie et al. 2009).

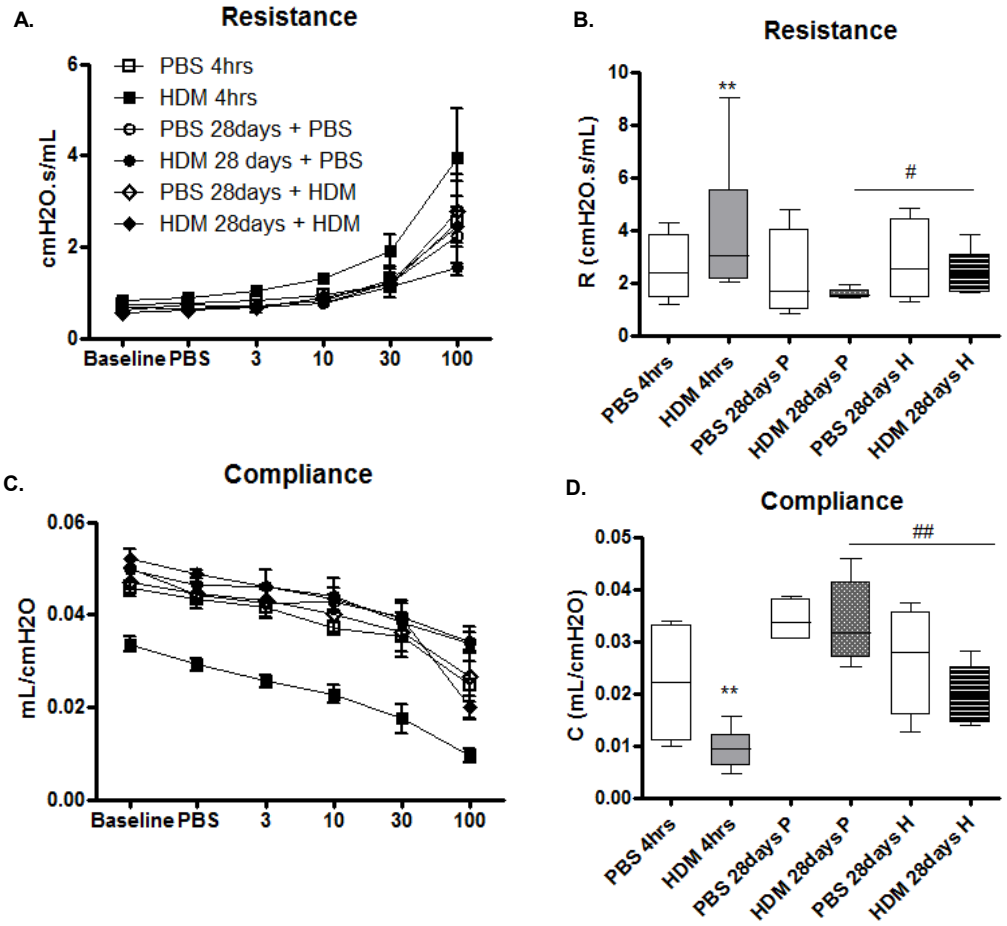


Figure 3.12 Rechallenge with HDM after a period of resolution induces AHR. AHR was measured following increasing doses of Methacholine (MCh) challenge. Changes in Resistance (A) and Compliance (C) were measured 4 hours and 28 days + 4 hours after last challenge. Mean levels of Resistance (B) and (D) Compliance at 100mg/ml MCh. n = 4-8 mice/group, plots depict the median and interquartile range and minimum and maximum values. ** $p<0.01$ relative to HDM at 4 hours vs PBS at 4 hours by Mann Whitney test. $^{\#}p<0.05$ and $^{\#\#}p<0.01$ relative to HDM challenged + HDM rechallenge vs HDM challenged by Mann Whitney test.

3.3.12 Rechallenge with HDM in the resolved lung induces pulmonary inflammation

Total and differential cell counts demonstrated that leukocyte numbers resolved to baseline levels by 28 days. This was expected as earlier in this chapter the resolution of pulmonary cellular infiltrate occurred by 13 days. At 28 days, a rechallenge with HDM did not induce any changes in total lung and BAL, eosinophils & macrophage numbers, (Figures 3.13 A-D, I &J). However, there was a significant increase in the number of lung and airway neutrophils that were comparable with levels at peak disease. This demonstrates a rapid recruitment of neutrophils to the lungs even after a single dose of HDM (Figure 3.13E & F). This was due to prior exposure to HDM as a single dose of HDM did not induced neutrophilia. A small but significant induction of airway lympho-mononuclear cells was observed following rechallenge, (Figure 3.13 H).

FACS analysis revealed that within the lymphocytic population, there was an increase in lung tissue CD4 +T1/ST2+ lymphocytes in the HDM rechallenge treated group compared to mice that had rested for 28 day, (Figure 3.13 A). This was due to prior exposure to HDM as a single dose of HDM did not induced Th2 lymphocytes. CD4+ IL-13+ and CD4+ IL-17+ lymphocytes numbers did not differ following rechallenge (Figure 3.13 B & C). Interestingly, γδ IL-17 producing lymphocytes were significantly induced following rechallenge as were inducible regulatory CD4+ IL-10+ lymphocytes, (Figures 3.13 D &E).

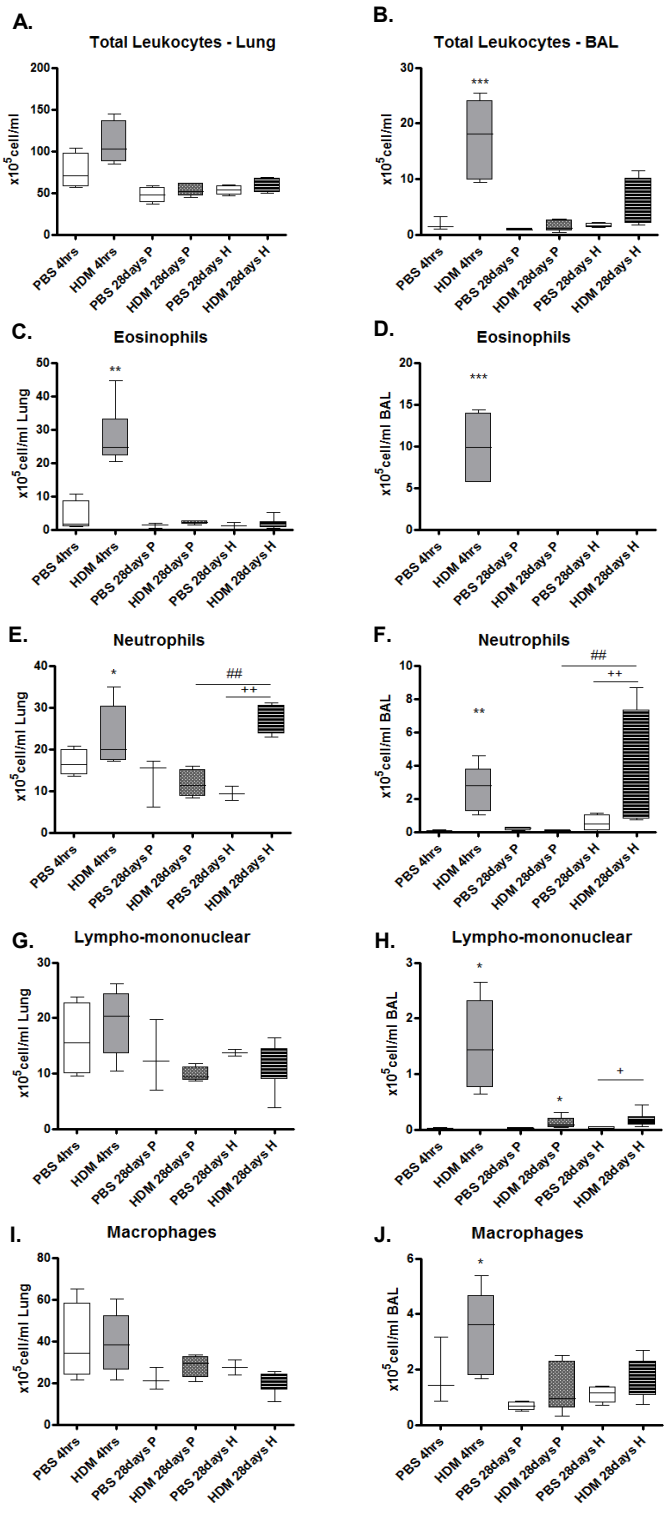


Figure 3.13 Rechallenge with HDM after a period of resolution induces inflammation. Measurements were carried out at 4 hours and 28 days + 4 hours after last challenge. Total cell counts (A) lung tissue (B) BAL. Lung and BAL differential counts identified and quantified by WG staining: (C&D) Eosinophils, (E&F) Neutrophils (G&H) Lympho-mononuclear and (I&J) macrophages. n = 3-6 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ relative to HDM at 4 hours vs PBS at 4 hours by Mann Whitney test. $^{\#}p<0.05$ and $^{\#\#}p<0.05$ relative to HDM challenged + HDM rechallenge vs HDM challenged + PBS rechallenge group by Mann Whitney test. $^{+}p<0.05$ and $^{++}p<0.01$ relative to HDM challenged + HDM rechallenge with PBS + single HDM challenge by Mann Whitney test.

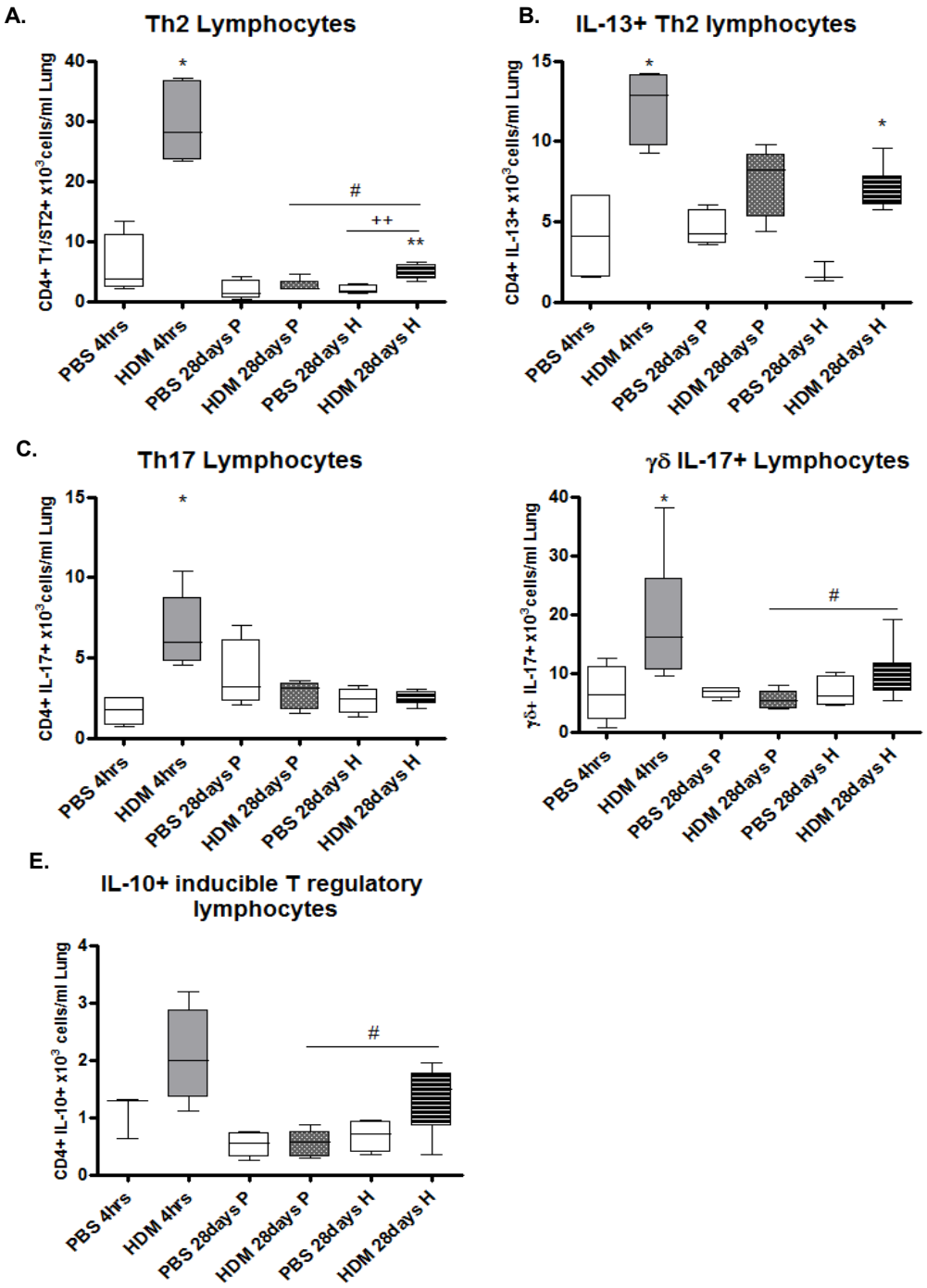


Figure 3.14 Induction of T lymphocytes following HDM rechallenge. Lung tissue was acquired at 4 hours, 7 days and 28 days after HDM challenge. A group of mice was rechallenged with HDM at 28 days were sacrificed at 4 hours. Control mice received PBS. CD3+ lymphocyte subsets: (A) CD4+ T1/ST2+, (B) CD4+ IL-13+ (C) CD4+ IL-17 + (D) γδ IL-17+ (E) CD4+ IL-10+ were quantified by flow cytometry. n = 3-8 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ relative to HDM at 4 hours vs PBS at 4 hours by Mann Whitney test. $^{\#}p<0.05$ relative to HDM challenged + HDM rechallenge vs HDM challenged + PBS rechallenge group by Mann Whitney test. $^{+}$ $p<0.05$ and $^{++}p<0.01$ relative to HDM challenged + HDM rechallenge with PBS + single HDM challenge by Mann Whitney test.

3.3.13 Rechallenge with HDM in the resolved lung Th2 cytokines to levels comparable with peak disease

Strikingly, Th2 cytokines, IL-4, IL-5 and IL-33 were significantly induced after rechallenge with HDM with IL-4 and IL-33 reaching levels comparable with those observed at peak disease (Figure 3.14 A &D). IL-5 in the lung tissue was elevated to levels higher than that seen at peak disease (Figure 3.14 C). After rechallenge, IL-13 in the lung was significantly higher compared to mice treated with single dose of HDM. However, this was not statistically higher compared to HDM treated group that had rested for 28 days, (Figure 3.14B). This increase in cytokine production occurs as a result of the prior exposure to HDM as a single dose of HDM did not promote an induction of Th2 cytokines.

These results show that a single dose of HDM can restore the features of allergic airways disease in mice that have had prior exposure to HDM allergen.

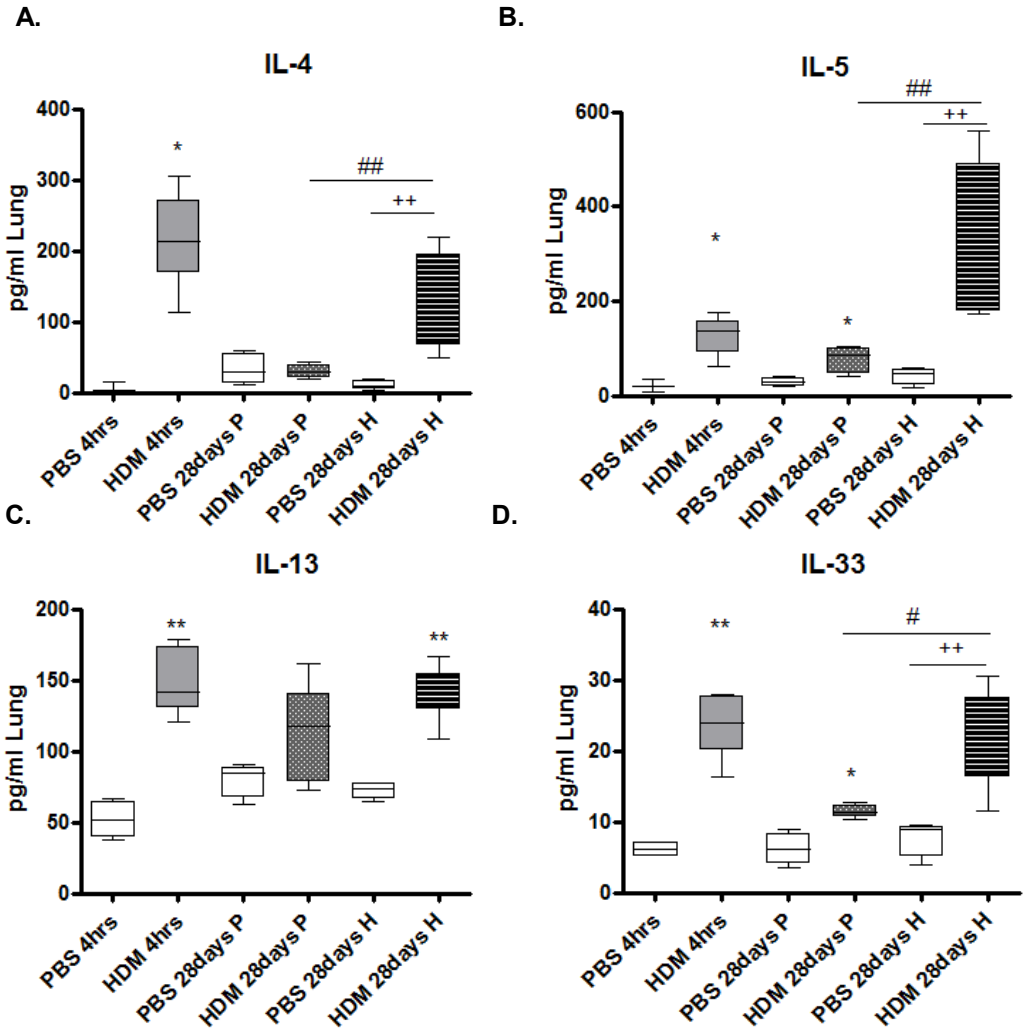


Figure 3.15 Th2 cytokines are rapidly induced following rechallenge with HDM. Lung tissue was acquired at 4 hours, 7 days and 28 days after HDM challenge. A group of mice rechallenged with HDM at 28 days were sacrificed at 4 hours. Control mice received PBS. IL-4 (A & B), IL-5 (C & D) IL-13 (E & F) and IL-33 (G & H). Levels of cytokines were quantified by ELISA. n = 4-8 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ and ** $p<0.01$ relative to HDM at 4 hours vs PBS at 4 hours by Mann Whitney test. $^{\#}p<0.05$ and $^{\#}p<0.05$ relative to HDM challenged + HDM rechallenge vs HDM challenged + PBS rechallenge group by Mann Whitney test. $^{++}p<0.01$ relative to HDM challenged + HDM rechallenge with PBS + single HDM challenge by Mann Whitney test.

3.4 Discussion

The primary aim of this chapter was to characterise the features and mediators of allergic airways disease at peak inflammation and at different time points after house dust mite allergen challenge to establish a model of resolution of pulmonary inflammation.

3.4.1 HDM induced AAD resolves by 13 days following cessation of allergen challenge

Disease parameters were measured at 4 hours post final HDM challenge to characterise peak inflammation in the lung and also at days 7 and 13 following cessation of allergen challenge to track the resolution of airway inflammation. Airway hyper-reactivity, allergic inflammation and Th2 cytokines induced by HDM remained elevated at 7 days and resolved by 13 days post allergen challenge. The resolution of disease associated parameters was associated with concomitant increase in the levels of regulatory mediators. At peak inflammation, mice exhibit features of allergic airway disease, as previously reported, (Gregory et al, 2009). At 7 days post HDM challenge, airway resistance remained significantly elevated returning to baseline by 13 days. The resolution of AHR was accompanied by decreasing numbers of lung and BAL eosinophils and Th2 lymphocytes. HDM induced an increase in pulmonary levels of the Th2 cytokines IL-4 and IL-5 which also resolved in parallel with AHR. IL-4 correlated with Th2 lymphocytes numbers and levels of total IgE at these time points. IL-5, the survival factor for eosinophils, in the lung correlated with the eosinophils profile during resolution. Levels of IL-13 in the lung were dependent on continued HDM challenge. IL-13 concentrations peaked at 4 hours but by 7 days after cessation of allergen challenge levels were not different from baseline. IL-13 is a key inducer of mucus production (Wills-Karp et al, 1997) and is essential for the maintenance of HDM induced chronic inflammation airway hyper-reactivity and remodelling (Tomlinson et al, 2010). Although IL-13 levels do not correlate with AHR exhibited at 7 days, its downstream effects may be longer lasting. In addition to the classic Th2 cytokines described above, the epithelial derived cytokine IL-33 has been identified as a key driver of Th2 inflammation and

mediates its effects via its receptor, the putative Th2 lymphocyte marker, T1/ST2 (Schmitz et al 2005). This IL-33-T1/ST2 signalling pathway has been shown to be necessary for maintaining AHR in an OVA model of AAD. (Kearley et al, 2009). IL-33 has been shown to be rapidly up regulated in a neonatal model of allergen rechallenged correlating with AHR (Saglani et al 2013). In our model, IL-33 was induced at peak disease. In the lung levels of IL-33 decreased by day 7 days but remained significantly elevated compared to PBS controls up to 13 days post allergen challenge. IL-33 is member of the IL-1 family. Following epithelial injury IL-33 is released and is thought to be an early danger signal to initiate a local pulmonary inflammatory response. In addition to its importance in asthma, a similar alarmin role has been shown to be relevant in in skin epithelium in atopic dermatitis and in the gut mucosa. (Chung 2005, Schmitz, Owyang et al. 2005, Moussion, Ortega et al. 2008). This cytokine has been shown very recently to be a critical mediator of allergen induced pulmonary remodelling and mediator of steroid resistance in asthmatics (Saglani et al 2013). Here, IL-33 was shown to persist throughout resolution. This apparent failure to resolve supports a pathogenic role for IL-33 in driving chronic allergic inflammation in asthma patients. IL-33 has also been described to be chemotactic for human Th2 lymphocytes (Mousa, Damo et al. 2007). Although in our model Th2 cell numbers returned to baseline by 13 days post allergen challenge despite continued, albeit at a low level, IL-33 expression. IL-33 has been associated with newly discovered innate lymphoid cells (ILCs), (Barlow et al 2012). IL-33 induces a population of lineage- IL-13+ cells that can maintain AHR independent of adaptive immunity (Barlow et al 2012). ILCs, which were not characterised in the literature at the start of this study, have not been determined in the model of resolution. Total and HDM specific IgE was increased following HDM exposure and levels were maintained up to 13 days. Thus sensitisation to HDM persists even after AHR and allergic inflammation has resolved.

3.4.2 Specific mediators are up regulated during resolution

3.4.2.1 Mucosal defence Immunoglobulin IgA

During the resolution of allergic airways disease, specific mediators were found to be up regulated or remained elevated up to 13 days after last HDM challenge. Total and HDM specific levels of the mucosal immunoglobulin, IgA, was significantly elevated during the resolution phase. HDM specific IgA levels in the lung and BAL were elevated in response to HDM challenge and were further increased 7 days after cessation of allergen challenge and remained elevated during the resolution time course. This induction of HDM specific IgA in the lung and BAL during resolution highlights that following mucosal insult there is induction of humoral homeostatic pathways. In an OVA model of AAD, improved lung function was associated with higher IgA levels, (Hajek et al, 2008). TGFβ and IL-5 are implicated in the class switching of IgA and survival of IgA secretory cells. (Sonoda, Matsumoto et al. 2009), Lee et al 2008). IL-5 levels are maintained at day 7 post challenge, which may support induced IgA secretion observed during the resolution phase. The receptor for IgA, FcαR1 is expressed mainly on cells of the myeloid lineage: macrophage, monocytes, neutrophils and eosinophils. (van Egmond, Damen et al. 2001). IgA is known to sequester and neutralise virus following infection, however, the downstream effects of IgA are not clear. It has been suggested to mediate host defence by inducing effective induction of phagocytosis, respiratory burst activity and the release of pro-inflammatory cytokines, which may help its role in mucosal defence. Activation of FcαR1 has also been shown to induce antibody mediated tumor cell lysis by neutrophils, monocytes and macrophages (Wines and Hogarth 2006). The induction of IgA in the lung during resolution of allergen induced AAD indicates activation of mucosal host defence. However, its function requires further investigation.

3.4.2.2 Regulatory lymphocytes

Regulatory lymphocytes, both FoxP3+ naturally occurring (FoxP3+ Tregs) and IL-10+ inducible T regulatory lymphocytes (IL-10+Tregs), are well accepted to maintain tolerance in the lung. In humans the induction of regulatory lymphocytes to promote tolerance and suppression of clinical symptoms in allergic rhinitis sufferers has been shown successfully following either sublingual (SLIT) or subcutaneous (SCIT) immunotherapy to repeated low dose allergen. These beneficial effects have been associated with immunoglobulin class switching from IgE to a protective IgG4 (Till, Francis et al 2004). We observed an induction of HDM specific IgG at peak inflammation where levels were maintained during the resolution phase supporting a protective role for IgG in our model.

Experimentally, T regulatory lymphocytes have also been shown to be critical in mediating resolution in a peripheral sensitisation model of allergic inflammation utilising OVA as a surrogate allergen (Kearley et al, 2005, 2008). IL-10 is a key anti-inflammatory cytokine that has been shown to control airway inflammation (Ogawa, Duru et al. 2008). HDM exposure induced significant numbers of FoxP3+ Tregs and IL-10+ Tregs in the lung. IL-10+ Tregs remained significantly elevated at 7 days with FoxP3+ Tregs resolving by 7 days. The different profiles in the persistence of these two T regulatory cell populations suggest a disparate function during resolution. It has been reported that in a model of transcutaneous immunisation, IL-10+ Tregs and FoxP3+ acted independently to suppress cytotoxic effects from CD8+ lymphocytes (Stein, Weber et al. 2011). It could be that in the context of HDM inflammation, similar independent regulation is occurring with, FoxP3+ T regs increase and resolve earlier to limit allergic response whereas IL-10+ Tregs persist to mediate resolution. γδ IL-17+ lymphocytes have also been shown to promote resolution in an OVA model of AAD). In this model, transfer of IL-17 deficient γδ lymphocytes failed to resolve allergic disease parameters (Murdoch et al 2009) In the model presented here, γδ IL-17+ cells remained significantly increased at 7 days post allergen challenge. Interestingly, Th17 lymphocytes also showed similar kinetics,

remaining elevated up to day 13. This suggests a role for Th17 in mediating resolution. The role of IL-17 in regulating allergic inflammation remains controversial. IL-17 has been shown to correlate with severity of disease and associated with the neutrophilia observed in severe asthmatics (Tan and Rosenthal 2013). However, the role for IL-17 in the development and resolution of lung inflammation is not entirely clear. Different lymphocyte subsets associated with both adaptive and innate immunity have been described to secrete IL-17 and the function of this cytokine may depend on the leukocyte it is secreted from, as illustrated by γδ IL-17+ study (Murdoch and Lloyd 2010). It may be in this model that IL-17 is regulating the resolution of HDM induced inflammation. The role of IL-17 in the respiratory mucosa requires further investigation and is beyond the scope of this project.

3.4.2.3 Alveolar macrophages are elevated during resolution

In this HDM study, numbers of alveolar macrophages in the lung peaked at 7 days and remain significantly elevated to 13 days. This increase during resolution of disease supports an important role for macrophages in pulmonary homeostasis. The myeloid receptor CD200R has been shown to restrain the pulmonary immune response to influenza infection. Mice deficient in CD200 have exacerbated response to infection and delayed resolution (Snelgrove et al, 2008). The levels of alveolar macrophages in the lung expressing CD200R following 3 weeks of HDM exposure peaked at 7 days and remained significantly elevated during resolution. CD3 + CD4+ lymphocytes expressing CD200, the ligand for the homeostatic molecule CD200R were also significantly increased at 7 days and remained elevated at 13 days. This data demonstrates that during the resolution of AAD regulatory lymphocytes remain elevated up to 7 days after last challenge and may be influencing the resolution of inflammation. CD200R is activated by CD200, but the downstream signalling events are not fully elucidated. This suggests a potential role for these CD200+ cells, via interaction with CD200R to promote resolution. It also demonstrates altered level of this homeostatic pathway during resolution. This is

consistent with the innate immune rheostat hypothesis where homeostatic molecules expressed on alveolar macrophages exhibit an altered phenotype following resolution of inflammation after epithelial injury, resulting from influenza infection. Alveolar macrophages increase their expression of homeostatic markers which shifts their threshold for activation. (Wissinger, Goulding et al. 2009). The implications are that a subsequent infection or epithelial injury may not illicit a suitable protective inflammatory response. This has been exemplified in the mortalities of the Spanish influenza endemic in 1918. It was shown that the majority of deaths were not down to the pathologies of primary influenza infection but by secondary bacterial s. pneumonia infection (Morens, Taubenberger et al. 2008). Experimentally, the HDM allergic lung was shown to have impaired response to s. pneumonia infection due to desensitised TLR response via HDM exposure (Habibzay, Saldana et al. 2012). In asthmatic sufferers, particularly children, hospitalisations following recurrent wheezy episodes are largely due to viral or bacterial exacerbations. Thus, the understanding the underlying homeostatic changes following resolution of allergen exposure may provide an insight into the mechanisms of susceptibility to viral and bacterial asthma exacerbations.

3.4.3 Rechallenge with single exposure to HDM promotes rapid induction of AAD

As described above, concomitant to the waning of disease there was an up regulation of the myeloid negative regulatory CD200R expressed by AMs. Thus we aimed to see if this change in homeostasis after resolution would affect a subsequent challenge with HDM. We exposed mice to HDM for 3 weeks before a rest period of 28 days and the features of disease had resolved. Mice were rechallenged with a single dose of HDM and culled 4 hours after. We found mice that had prior exposure to HDM exhibited an AAD phenotype with an increase in airway resistance and reduction in lung compliance. There was a rapid increase in neutrophil numbers in the lung. Th2 lymphocytes were also elevated following HDM rechallenge. Most strikingly, the Th2 cytokines, IL-4, IL-5 and IL-33 were induced to levels comparable to that seen at peak disease. This was attributed to prior exposure to HDM as a

single dose of HDM did not illicit an inflammatory response. These observations were also reported in neonatal mice exposed to HDM that were allowed to rest for 28 days, until 7 weeks of age, and then as adults rechallenged with HDM (Saglani, Lui et al. 2013). This data demonstrates that after resolution of HDM induced inflammation there is a return to an altered state of pulmonary homeostasis. This renders the mice highly susceptible to one challenge of HDM which restores the features of AAD to that observed at peak disease, after continuous allergen exposure. Thus, the resolved lung may have an altered lung homeostasis that predisposes the lung to chronic inflammatory disease.

3.5 Conclusion

This chapter demonstrates that as the features of allergic airway disease resolve there is a concurrent up regulation of humoral and lymphocytic regulatory pathways as exemplified by the increase of IgA and T regulatory lymphocytes subsets during the resolution phase. Of particular interest, there is a temporal increase in the number of alveolar macrophages which suggests they play a distinct role. In addition, resolution shows an alteration of pulmonary homeostasis as demonstrated by up regulation of CD200R and CD200. This was exemplified in mice that received a single rechallenge with HDM and exhibited the features of disease similar to the degree seen at peak inflammation. The next chapter will investigate pro-resolution lipids in the HDM model and the effects of targeting lipid and homeostatic pathways.

Chapter 4.

Targeting pro-resolution pathways in house dust mite induced allergic airways disease

4.1 Introduction

In the previous chapter, it was demonstrated that the features of allergic airways disease induced by HDM remained significantly elevated at 7 days and resolved to baseline levels by the 13 day time point. In parallel, regulatory markers were elevated during the resolution phase. In this chapter the aim was to identify and target pro-resolution mediators that may be important in controlling allergic inflammation. Firstly, fatty acids synthesised from Docosahexaenoic (DHA), Eicosapentaenoic acid (EPA) and Arachidonic acid (AA) were measured in the lung tissue in the resolution model. Secondly, the aim was to determine if specialised pro-resolution mediators (SPMs) derived from fatty acids were important in resolving inflammation. The receptor ChemR23 mediates anti-inflammatory signals from peptides derived from the endogenous protein chemerin. Chemerin is a chemoattractant protein that has been found to be present in various human inflammatory conditions such as synovial fluid in RA sufferers and ascites from the peritoneum (Wittamer, Franssen et al. 2003). It is an endogenous protein produced primarily by macrophages and immature dendritic cells (Samson, Edinger et al. 1998). Chemerin is required to undergo proteolytic cleavage by cysteine and serine proteases to produce peptides that exert anti-inflammatory effects (Cash, Hart et al. 2008). C-15 is one such peptide that has been shown to mediate anti-inflammatory effects in the picogram range. C-15 is composed of the first 15AA of the c-terminal of the protein chemerin. The specialised pro-resolving mediator RvE1, synthesised from EPA, also signals through ChemR23 and has been described to interact with ChemR23 to regulate inflammation in a model of peritonitis (Arita, Ohira et al. 2007). ChemR23 is predominantly expressed by myeloid cells, and C-15 has been shown to have potent anti-inflammatory effects on macrophages in vitro. In a zymosan model of peritonitis, C-15 peptide administration limited neutrophil and monocytic recruitment and reduced pro-inflammatory cytokines, (Cash, Hart et al. 2008). Thus in our model, we used C-15 to investigate the role of ChemR23 in mediationg resolution of HDM induced inflammation.

Thirdly, the role of the homeostatic molecule CD200R was also investigated. In chapter 3 it was shown to be up regulated on alveolar macrophages during the resolution phase. OX-110 has previously been reported to be important in regulating pulmonary homeostasis (Snelgrove, Goulding et al. 2008). A CD200R antibody agonist was administered to mice during the resolution phase. The importance of CD200R expression in the resolution of HDM induced AAD was also assessed using a CD200R knock-out mouse.

4.1.1 Hypothesis

Activation of pro-resolution pathways will restore homeostasis in a model of allergic airways disease.

4.1.2 Aims

To detect and quantify pro-resolution lipids in the HDM model.

To determine whether C-15 peptide ligation of ChemR23 can promote resolution.

To assess the effect of activating the myeloid negative regulator CD200R on the resolution of HDM induced AAD.

4.2 Method

In collaboration with Val O'Donnell at Cardiff University, UK, essential fatty acids were measured by LS/MS/MS in the lung tissue in the resolution model at 4 hours, 7 days and 13 days post final HDM challenge.

In protocols A & B, female BALB/c mice aged between 6-8 weeks received repeated intranasal instillation of 25 ug HDM 3 times a week for 3 weeks. Mice were culled at 4 hours and 7 days post final allergen challenge. Control mice received PBS (Figure 4.1).

In protocol A, PBS and HDM groups of mice received either 6.4pg of C-15 peptide or the control scrambled peptide i.v at 1 day, 3 days and 6 days post last HDM challenge and were culled on day 7. A control group was culled at 4 hours following the final HDM exposure to determine the level of peak inflammation.

In protocol B, PBS and HDM treated mice received 100ug of OX-110, the agonistic antibody for CD200R, or Rat IgG control i.p at 1 day prior to and 3 days and 6 days post final HDM challenge. A control group was culled at 4 hours following the final HDM exposure to determine the level of peak inflammation.

In protocol C, CD200R KO mice were treated with HDM for 3 weeks and culled at peak inflammation, 4 hours post final HDM challenge. The CD200R KO mice were on a C57BL/6 background and C57BL/6 WT mice were used as a control, (Figure 4.1).

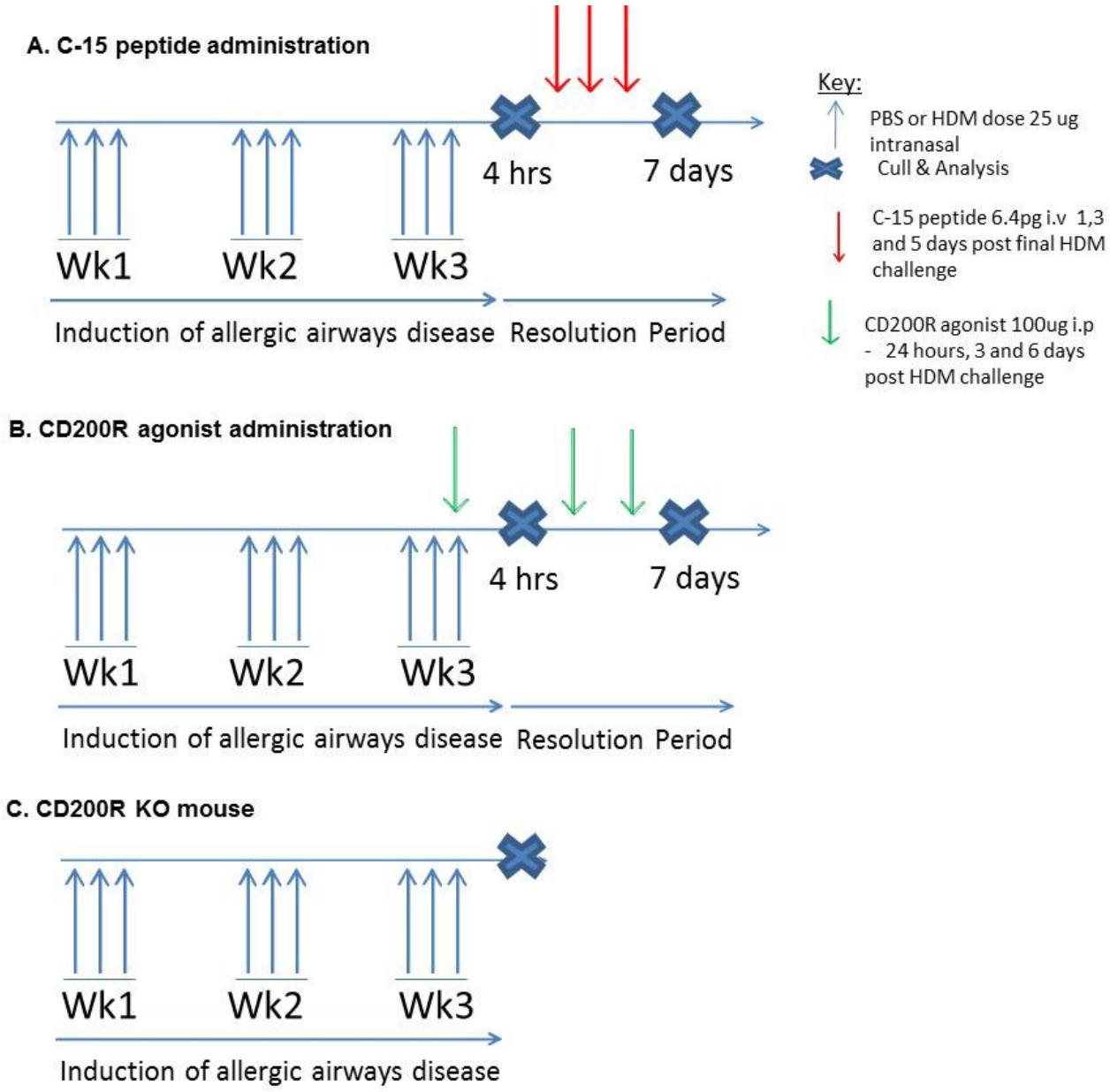


Figure 4.1 Experimental protocols
Female BALB/c mice were culled at 4 hours and 7days after final HDM challenge for lung function measurements and collection of blood, BAL and Lung samples. (A) C-15 peptide treatment (B) CD200R agonist treatment (C) CD200R KO mouse.

4.3 Results

4.3.1 Eicosanoid profile during the resolution phase of HDM induced inflammation

In collaboration with Val O'Donnell at Cardiff University, UK, essential fatty acids were measured by LS/MS/MS in the lung tissue at peak inflammation and during the resolution phase, 7 days and 13 days post HDM challenge. The primary aim was to quantify specialised pro-resolving lipids (SPMs) derived from the omega3 fatty acids, EPA and DHA. Unfortunately in our tissues, levels of Resolvin D1, Protectin D1 and Maresin 1, were found to be below the limit of detection. However it was possible to measure a panel of eiconasoids derived from arachidonic acid. Prostaglandin E2 was significantly induced at peak disease following HDM exposure and remained significantly elevated during the resolution phase, (Figure 4.2 A). Fatty acids produced by lipoxygenases (LOX) pathways such as Lipoxins and HETES were also measureable in the lung. Lipoxin A (4) was not detectable in the lung in PBS control mice but was induced after HDM exposure and remained detectable up to 13 days post challenge (Figure 4.2 B). LOX derived lipids also include the hydroxy eicosatrienoic acids (HETES). HETEs are named by the position of the hydroxyl group. In the lung, 5, 8, 11, 12, and 15-HETE were measured. HDM exposure induced a significant increase in HETEs, which all remained significantly elevated to 7 days post final exposure (Figure 4.2 C-G). 12-HETE was one of the most abundant HETEs present in the lung tissue. It is highly induced by IL-4 and following HDM challenge, levels of 12-HETE were increased to 5 fold compared to PBS control. Levels remained 3 fold higher than PBS during the resolution phase. 15-HETE is induced via the same enzyme as 12-HETE; 12/15 LOX. 15-HETE is produced in lower amounts than 12-HETE, however, levels were increased 25 fold following HDM challenge and remained significantly elevated 13 days post final challenge. Levels of 11-HETE were increased to similar magnitude as 15-HETE and similarly remained elevated following cessation of allergen challenge. These data show that lipids from both COX and LOX enzymatic pathways are rapidly induced following HDM

challenge and persist in the airway after cessation of allergen exposure, suggesting they play an important role in the development and resolution of AAD.

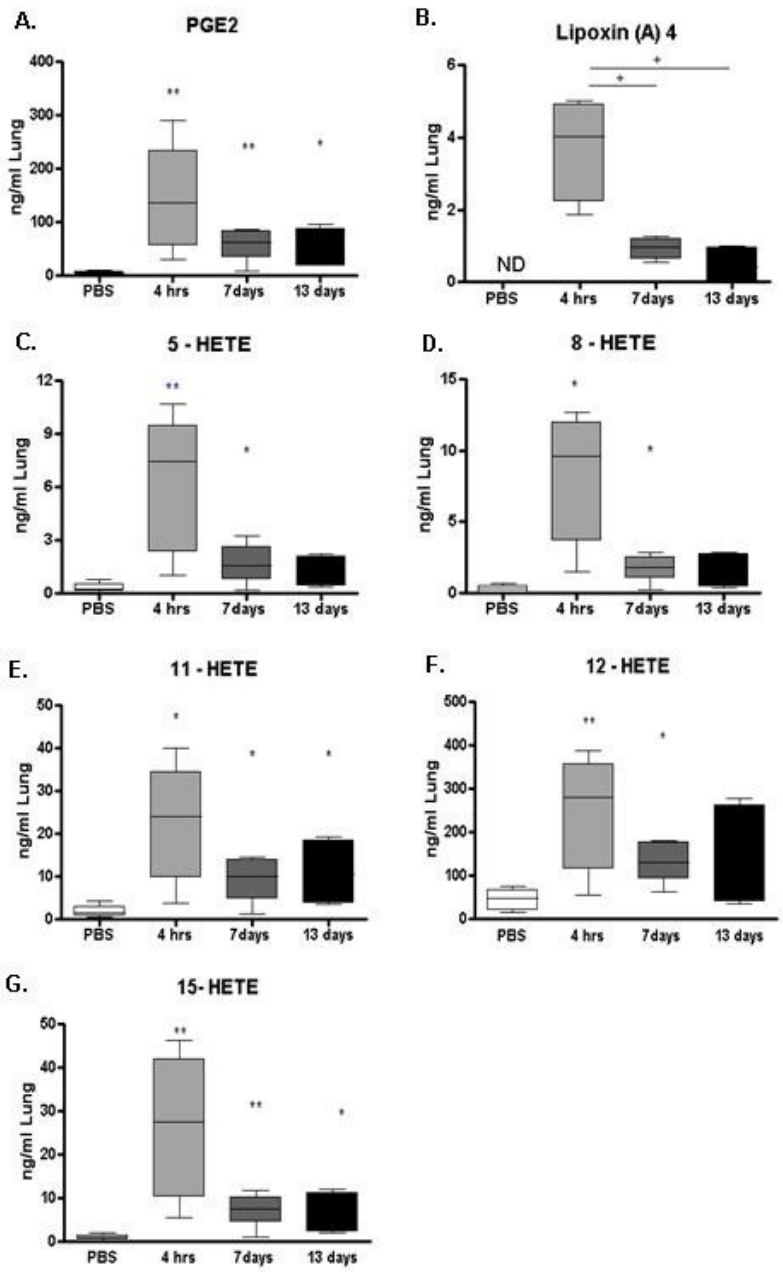


Figure 4.2 Eiconasoids during resolution of HDM induced inflammation. Lungs were taken at 4 hours, 7days and 13 days following 3 weeks of HDM challenge. Lipid levels were quantified in lung homogenate supernatant by LC/MS/MS. (A) PGE2 (B) Lipoxin A(4) (C) 5-HETE (D) 8-HETE (E) 11-HETE (F) 12-HETE (G) 15-HETE. n = 4-6 mice/group, bar represents median, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ and ** $p<0.01$ relative to PBS control group by Mann Whitney test. $^{+}$ $p<0.05$ relative to peak inflammation, 4 hour time point group, by Mann Whitney test. ND = value not detected.

4.3.2 Therapeutic treatment with C-15 did not promote resolution of inflammation

In the lung tissue we could not detect SPMs derived from EPA and DHA and were found to be below the limit of detection. To determine whether Resolvin E1 (RvE1) influences the resolution of HDM induced AAD, mice were treated with the chemerin peptide derivative C15 that binds to and activates ChemR23, the RvE1 receptor. As previously reported in Chapter 3, HDM challenged mice exhibited significantly increased airway resistance and decreased compliance compared to PBS control treated mice (Figure 4.3). At 7 days post final challenge mice treated with HDM still exhibited significant AHR comparable to that observed at peak inflammation. There was no resolution of lung function parameters in HDM treated mice which received the C-15 peptide.

Total and differential cell counts demonstrated that HDM induced a significant increase in lung tissue and airway leukocytes at peak disease (Figure 4.4). At day 7 of the resolution phase, levels of eosinophils and lympho-mononuclear cells remained significantly increased, although reduced compared to peak inflammation as shown previously (Figure 4.4D & H). Treatment with C-15 peptide did not induce any further reduction in total or differential cells counts, (Figure 4.4).

T1/ST2+, IL-13+, IL-17+ and IL-10+ CD4+ lymphocytes were recruited to the lungs of HDM treated mice (Figure 4.5 A-D). Administration of C-15 did not enhance clearance of T cells from the lung and there was no difference in the numbers of pulmonary T cells quantified by FACS in HDM treated mice which received either scrambled or C-15 peptide.

Similarly, there were no changes observed in the levels of pulmonary Th2 cytokine IL-4, IL-5, IL-13 and IL-33 between C-15 treatment and scramble peptide control (Figure 4.6 A-D).

Thus, the chemerin derivative C-15 did not affect resolution of HDM induced inflammation.

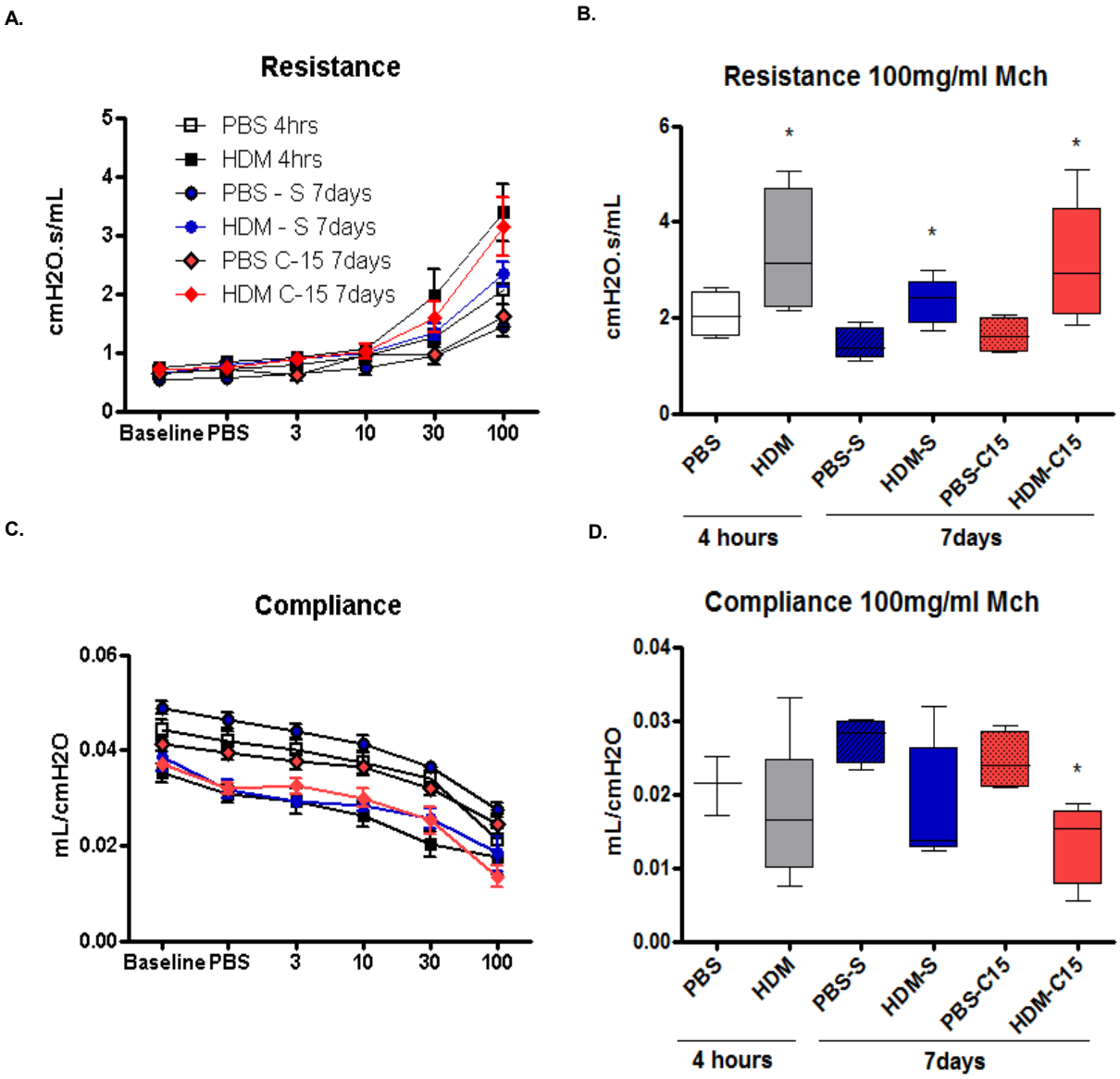


Figure 4.3 Therapeutic treatment with C-15 did not promote the resolution of airway hyper-reactivity. AHR was measured following increasing doses of methacholine (MCh) challenge. Control mice received PBS. PBS and HDM groups culled at 7 days received either C-15 (red bars) or scrambled C-15 peptide (blue bars). Dose response curve demonstrating Resistance (A) and Compliance (C) were measured 4 hours and 7 days after HDM challenge. Mean levels of resistance (B) and compliance (D) at 100mg/ml MCh. n = 3-8 mice/group, bar represents median, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ relative to PBS control group by Mann Whitney test.

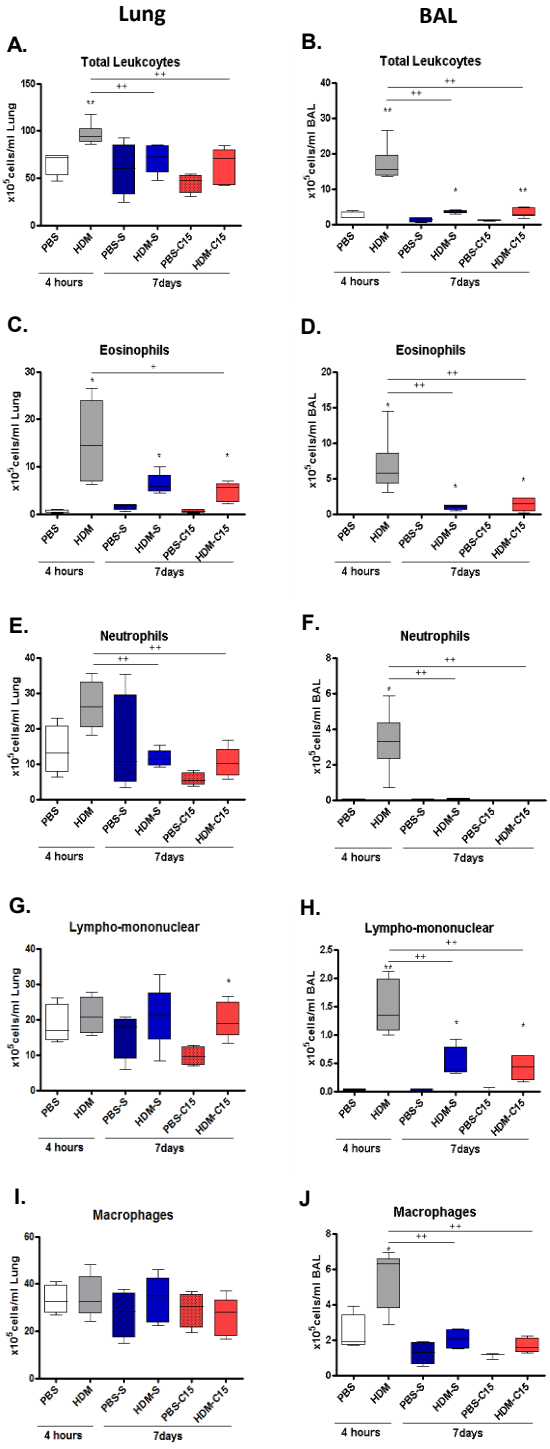


Figure 4.4 Total and differential lung tissue and airway cell counts after C15 treatment. Lungs and BAL were taken at 4 hours and 7 days following 3 weeks of HDM challenge. Control mice received PBS. PBS and HDM groups culled at 7 days received either C-15 (red bars) or scrambled C-15 peptide (blue bars). (A) Total lung counts, (B) total airway counts. Differential cell counts: (C&D) Eosinophils, (E&F) Neutrophils, (G&H) lympho-mononuclear (I&J) macrophages. Cells were identified and quantified using WG staining. n = 4-6 mice/group, bar represents median, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ ** and $p<0.01$ relative to PBS control group by Mann Whitney test. $^{+}$ $p<0.05$ and $^{++}p<0.01$ relative to peak inflammation, 4 hour time point group by Mann Whitney test.

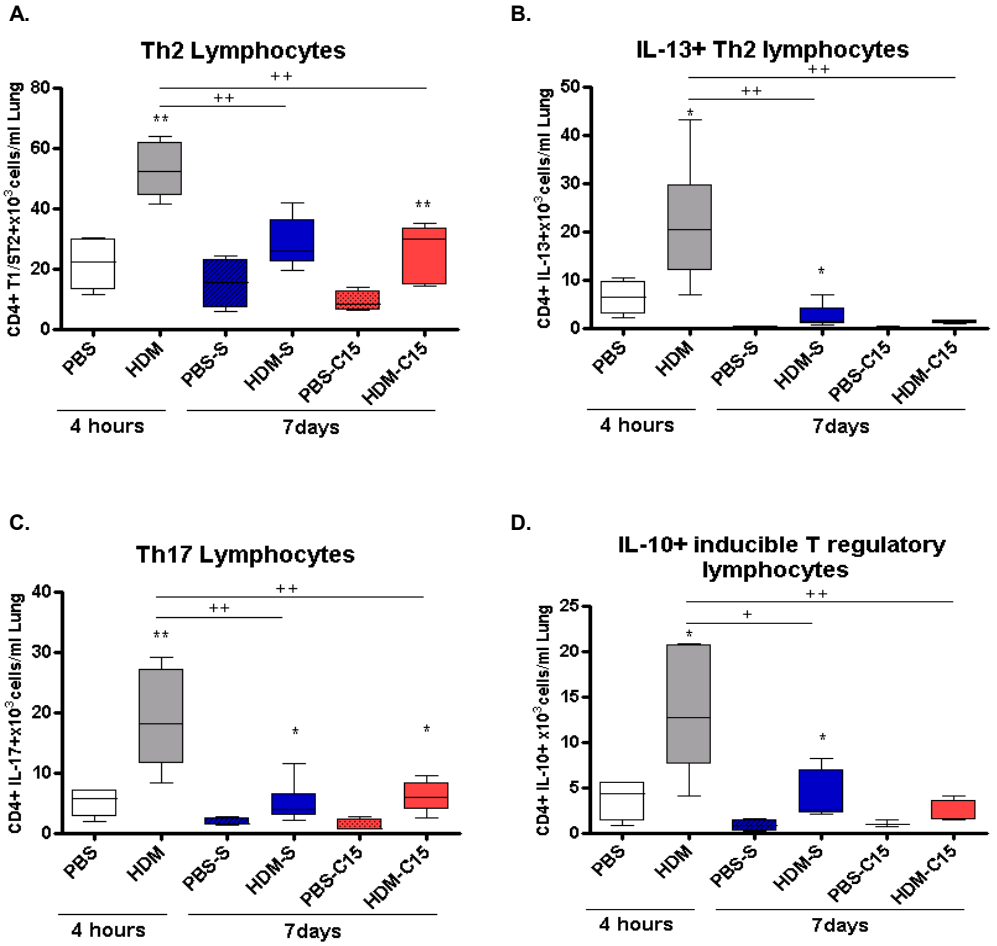


Figure 4.5 Lung tissue lymphocytes after C-15 treatment. Lungs were taken at 4 hours and 7 days following 3 weeks of HDM challenge. Control mice received PBS. PBS and HDM groups culled at 7 days received either C-15 (red bars) or scrambled C-15 peptide (blue bars). (A) CD4 CD3 T1/ST2+, (B) CD4 CD3 IL-13+ (C) CD4 CD3 IL-17+ (D) CD4 CD3 IL-10 +. n = 3-6 mice/group, bar represents median, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ and ** $p<0.01$ relative to PBS control group by Mann Whitney test. $^{+}$ $p<0.05$ and $^{++}p<0.01$ relative to peak inflammation, 4 hour time point group by Mann Whitney test.

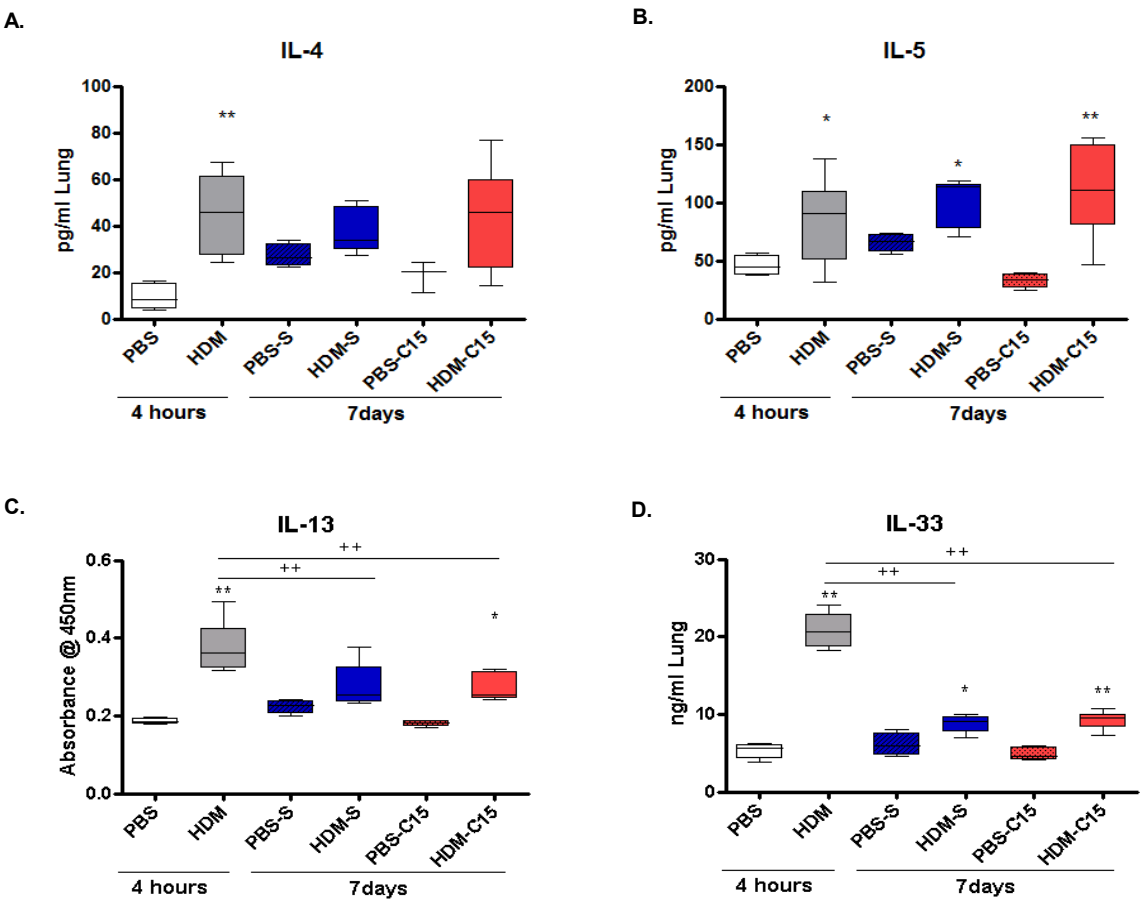


Figure 4.6 Th2 lung tissue cytokines after C15 treatment. Lungs were taken at 4 hours and 7 days following 3 weeks of HDM challenge. Control mice received PBS. PBS and HDM groups culled at 7 days received either C-15 (red bars) or scrambled C-15 peptide (blue bars). (A) IL-4, (B) IL-5 (C) IL-13 (D) IL-33. n = 3-6 mice/group, bar represents median, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ and ** $p<0.01$ $p<0.001$ relative to PBS control group by Mann Whitney test. $^{+}$ $p<0.05$ relative to peak inflammation, 4 hour time point group by Mann Whitney test.

4.3.3 Treatment with CD200R agonist did not affect resolution of inflammation

In Chapter 3, it was observed that CD200R expression was up regulated on alveolar macrophages during the resolution phase. To explore the function of CD200R during resolution, mice received OX-110, an antibody agonist for CD200R, during the week following last allergen exposure. Control mice received rat IgG control.

At 7 days post allergen exposure, HDM challenged mice treated with Rat Ig control exhibited significant airway resistance and compliance comparable to that displayed at peak inflammation, as shown previously in chapter 3 (Figure 4.7). Lung function parameters remained elevated in CD200R agonist treated HDM challenged mice at day 7 and there was no significant differences between HDM challenged mice that had received either CD200R agonist or Rat Ig control. Total cell counts were significantly increased in response to HDM in both the lung tissue and airway lumen at peak disease. 7 days after the final allergen challenge leukocytes numbers remained significantly elevated. Treatment with CD200R agonist did not alter BAL inflammation. However, total lung tissue leukocyte numbers were significantly increased after CD200R agonist administration (Figure 4.8 A & B). The increase in pulmonary inflammation was predominantly due to recruitment of lympho-mononuclear leukocyte population (Figure 4.8 G). In contrast, eosinophil, neutrophil and macrophage numbers were unaltered following CD200R agonist treatment (Figures 4.8 C-F, H-J). FACS analysis revealed that treatment with CD200R agonist induced an increase in Th2 lymphocytes (Figure 4.9A). Interestingly CD200R agonist treatment also induced an increase in CD200+ T lymphocytes and FoxP3 + T regulatory lymphocytes (Figure 4.9 B & C). There were no changes to inducible IL-10+ lymphocytes (Figure 4.9D). Measurements of Th2 cytokines levels in the lung at day 7 revealed an increase in IL-4 following CD200R agonist treatment (Figure 4.10A). This effect was not observed of the other Th2 cytokines IL-5, IL-13 and IL-33 (Figure 4.10 B-D)

CD200R agonist treatment did not improve the features of allergic airways disease. In fact the treatment with the CD200R agonist appeared to elevate some features of disease. There was an increase in total lung tissue cells following CD200R agonist treatment and an increase in Th2 lymphocytes and IL-4. Contrary to expectations, there was no evidence of the promotion of resolution of AAD.

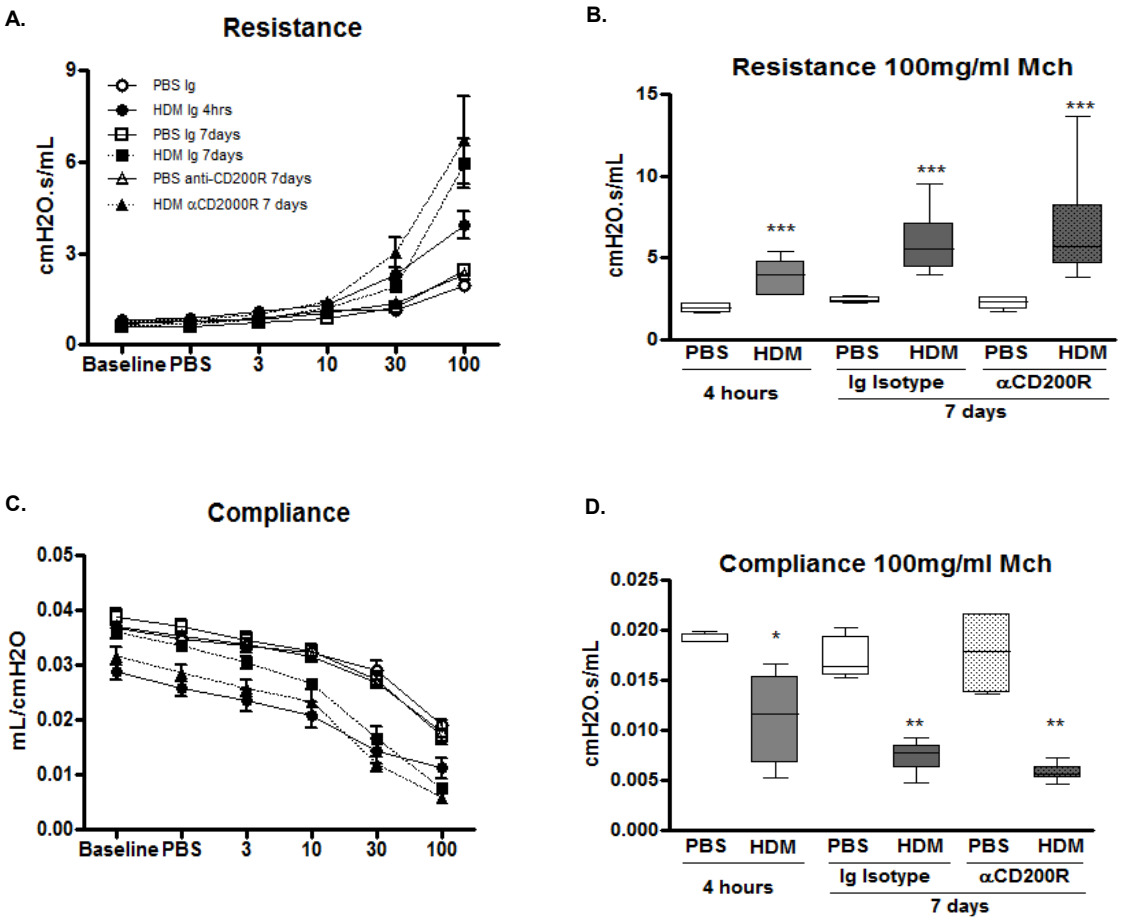


Figure 4.7 Therapeutic treatment with CD200R agonist did not promote the resolution of airway hyper-reactivity. AHR was measured following increasing doses of Methacholine (MCh) challenge. Dose response curve demonstrating Resistance (A) and Compliance (C) were measured 4 hours and 7 days after HDM challenge. Mean levels of resistance (B) and compliance (D) at 100mg/ml MCh. n = 3-8 mice/group, bar represents median, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ relative to PBS control group by Mann Whitney test.

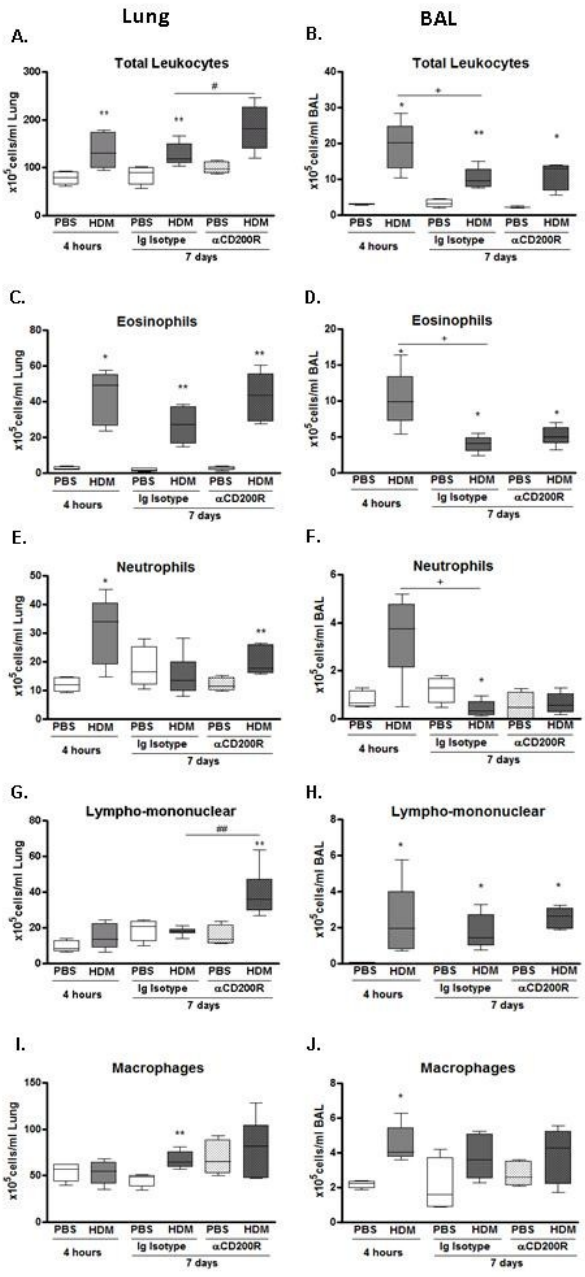


Figure 4.8 Total and differential lung tissue and airway cell counts after CD200R agonist. Lungs and BAL were taken at 4 hours and 7 days following 3 weeks of HDM challenge. Control mice received PBS. PBS and HDM groups culled at 7 days received either OX-110 or Rat IgG as control. (A) Total lung counts, (B) total airway counts. Differential cell counts: (C&D) Eosinophils, (E&F) Neutrophils, (G&H) lympho-mononuclear (EF (G&H), cells were identified and quantified using WG staining. n = 4-6 mice/group, bar represents median, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ and ** $p<0.01$ relative to PBS control group by Mann Whitney test. $^{+}$ $p<0.05$ relative to peak inflammation, 4 hour time point group by Mann Whitney test. # $p<0.05$ and ## $p<0.01$ HDM challenge CD200R agonist treated group relative to HDM Rat IgG control by Mann Whitney test.

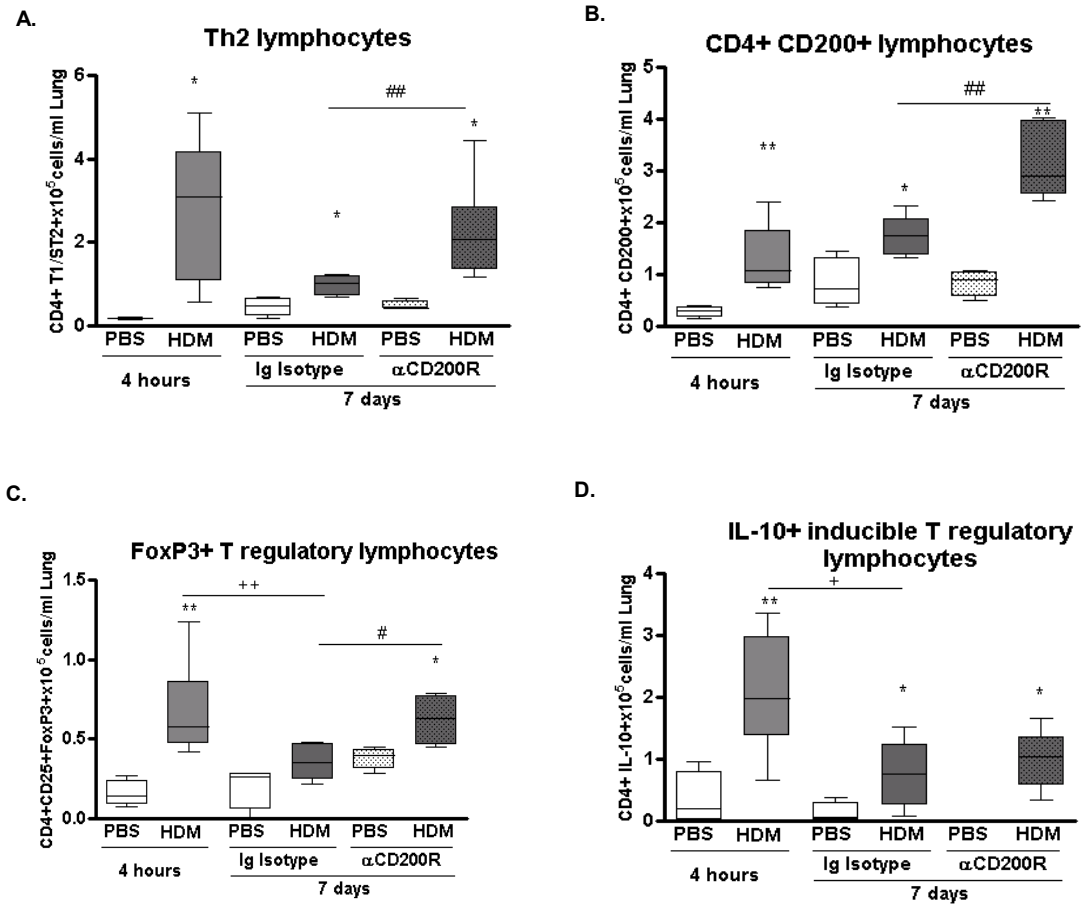


Figure 4.9 Th2 lymphocytes and T regulatory lymphocytes after CD200R agonist treatment. Lungs were taken at 4 hours and 7 days following 3 weeks of HDM challenge. Control mice received PBS. PBS and HDM groups culled at 7 days received either OX-110 or Rat IgG as control (A) CD4+ T1/ST2+, (B) CD4+ CD200+ (C) CD4+ CD25+ FoxP3+ and (D) CD4+ IL-10+ lymphocytes were determined by flow cytometry. n = 3-6 mice/group, bar represents median, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ and ** $p<0.01$ relative to PBS control group by Mann Whitney test. $^{+}$ $p<0.05$ and $^{++}$ $p<0.01$ relative to peak inflammation, 4 hour time point group by Mann Whitney test. # $p<0.05$ and ## $p<0.01$ HDM challenge CD200R agonist treated group relative to HDM Rat IgG control by Mann Whitney test.

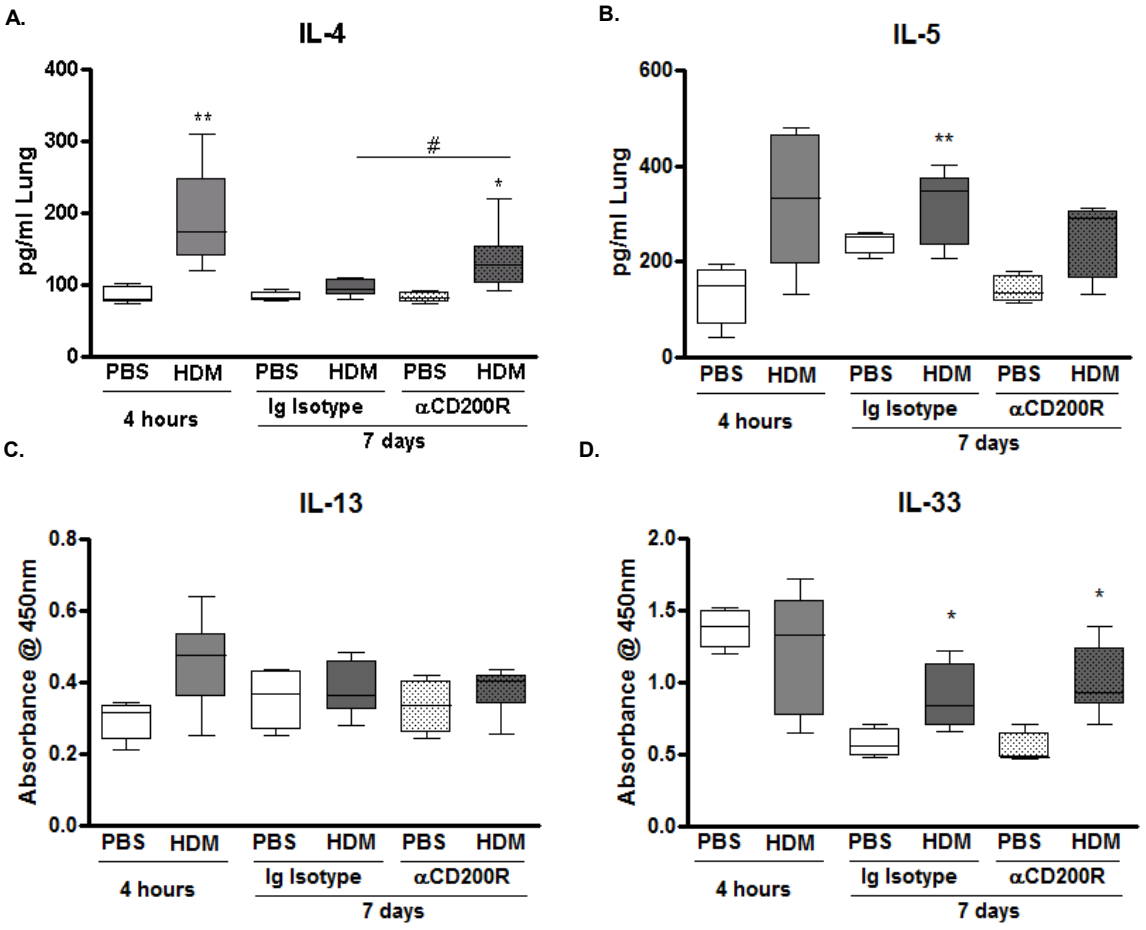


Figure 4.10 Th2 lung tissue cytokines after CD200R agonist treatment. Lungs were taken at 4 hours and 7 days following 3 weeks of HDM challenge. Control mice received PBS. PBS and HDM groups culled at 7 days received either OX-110 or Rat IgG as control. Cytokines were quantified in lung homogenate by ELISA. (A) IL-4, (B) IL-5 (C) IL-13 (D) IL-33. n = 3-6 mice/group, bar represents median, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ and ** $p<0.01$ relative to PBS control group by Mann Whitney test. # $p<0.05$ HDM challenge CD200R agonist treated group relative to HDM Rat IgG control by Mann Whitney test

4.3.4 CD200R KO mice do not exhibit exacerbated HDM induced allergic airways disease

The treatment with CD200R agonist did not promote resolution of disease. To ascertain if CD200R is required to restrain the pulmonary immune response to HDM, mice deficient in CD200R were sensitised and challenged to HDM for 3 weeks.

Interestingly, HDM challenged CD200R KO mice did not develop significant airway resistance compared to PBS control. (Figure 4.11 A & B). However, there was considerable variation in the magnitude of the response to methacholine in the CD200R KO mice. Both C57BL/6 WT and CD200R KO mice exhibited a similar degree of decreased lung compliance after HDM exposure, which was significantly different from PBS controls (Figure 4.11 C & D). There was no difference in total cell counts between HDM treated WT and CD200R KO mice (Figure 4. 12 A & B). Eosinophil and neutrophil counts also revealed that there were no significant differences in the induction of these cells in response to HDM between WT and CD200R deficient mice (Figure 4.12 C-F). Th2 lymphocytes in the lung were not induced to significant levels following HDM treatment in C57BL/6 mice and there were no difference in numbers compared to CD200R KO mice (Figure 4.13 A). Th17 and CD200+ lymphocytes numbers were also not different between HDM treated WT and CD200R KO mice (Figure 4.13 B & C). In contrast, numbers of T regulatory lymphocytes were reduced in HDM treated KO mice compared to WT (Figure 4.13 D)

Lung tissue Th2 cytokines were induced in both WT and CD200R KO mice following HDM exposure. Again, there were no significant differences in the levels of IL-4, IL-5, IL-13 or IL-33 between WT C57BL/6 and CD200R KO mice (Figure 4.14 A-D). In PBS KO mice there was a significant increase in lung tissue IL-33 compared to PBS WT (Figure 4.14 D). However, this did not appear to skew the response to HDM. These data show that CD200R deficient mice exhibit a similar AAD phenotype to WT mice.

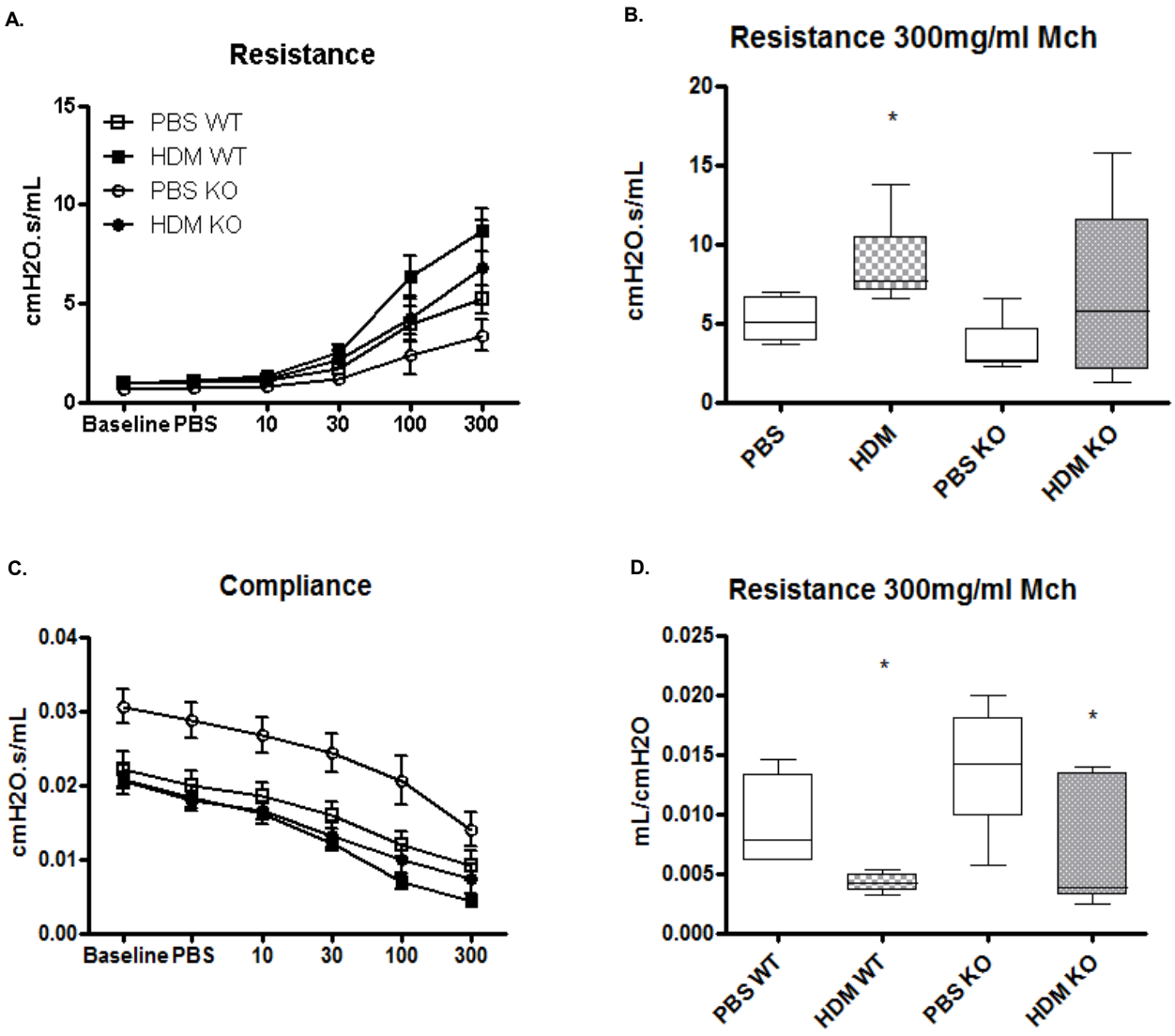


Figure 4.11 CD200R deficient mice did not exhibit exacerbated airway hyper-reactivity. AHR was measured following increasing doses of Methacholine (MCh) challenge. Dose response curve demonstrating Resistance (A) and Compliance (C) were measured 4 hours after HDM challenge. Mean levels of resistance (B) and (D) compliance at 300mg/ml MCh. n = 3-8 mice/group, bar represents median, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ relative to PBS control group by Mann Whitney test.

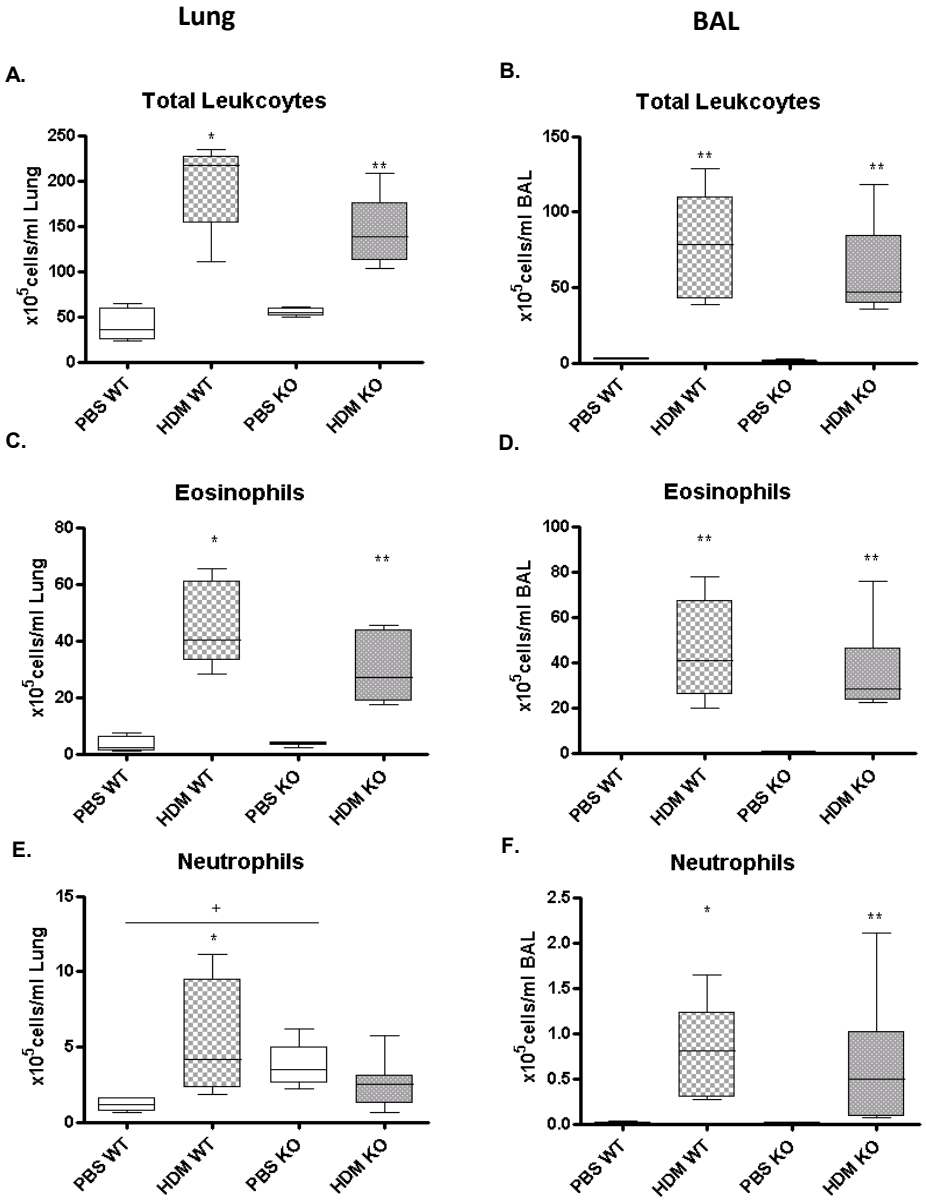


Figure 4.12 CD200R deficient mice did not exhibit exacerbated lung inflammation. Lungs were taken at 4 hours following 3 weeks of HDM challenge. Control mice received PBS. Total, eosinophil and neutrophil counts were quantified by flow cytometry. (A) Total lung tissue leukocytes (B) BAL leukocytes (C&D) Eosinophils (E&F) Neutrophils (n = 3-6 mice/group, bar represents median, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ and ** $p<0.01$ relative to PBS control group by Mann Whitney test. $^{+}$ $p<0.05$ WT group relative to KO group by Mann Whitney test

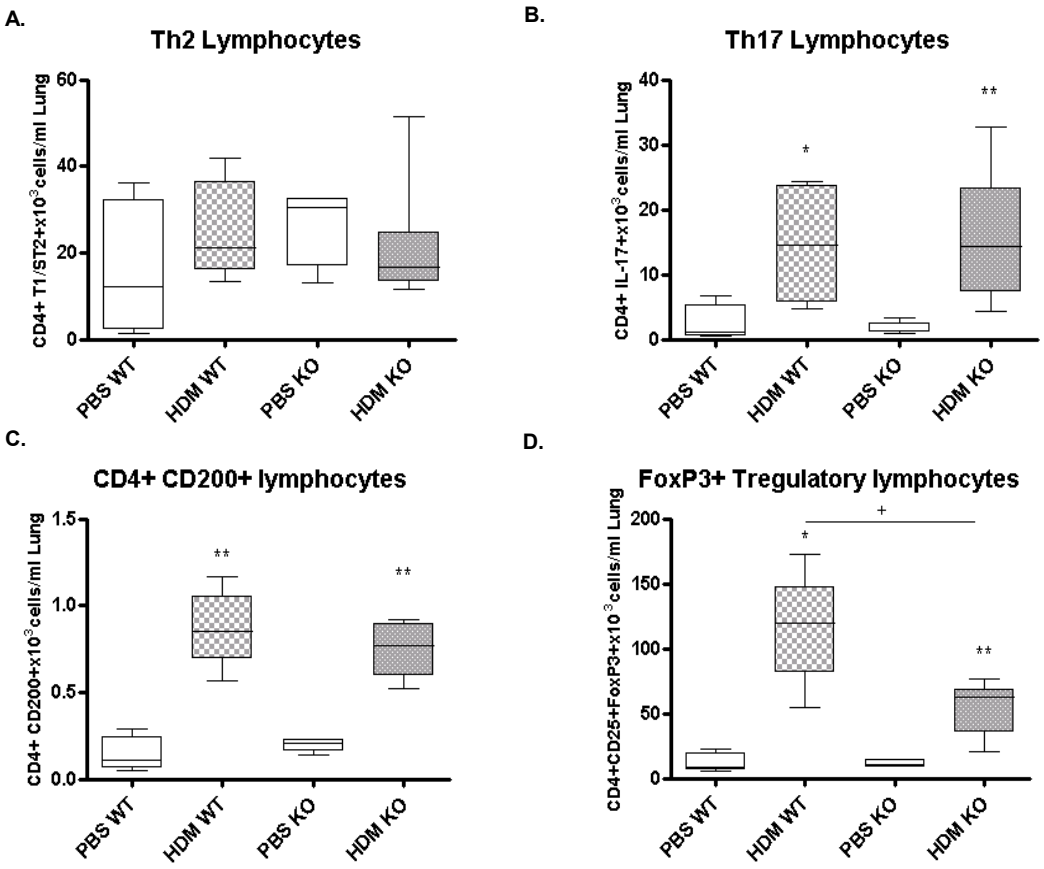


Figure 4.13 CD200R deficient mice exhibited lower levels of T regulatory lymphocytes.

Lungs were taken at 4 hours following 3 weeks of HDM challenge. Control mice received PBS. (A) CD4+ T1/ST2+ (B) CD4+ IL-17+ (C) CD4+ CD200+ and (D) CD4 CD25 FoxP3+ lymphocytes were determined by flow cytometry. n = 3-6 mice/group, bar represents median, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ and ** $p<0.01$ relative to PBS control group by Mann Whitney test. $^{+}$ $p<0.05$ WT group relative to KO group by Mann Whitney test

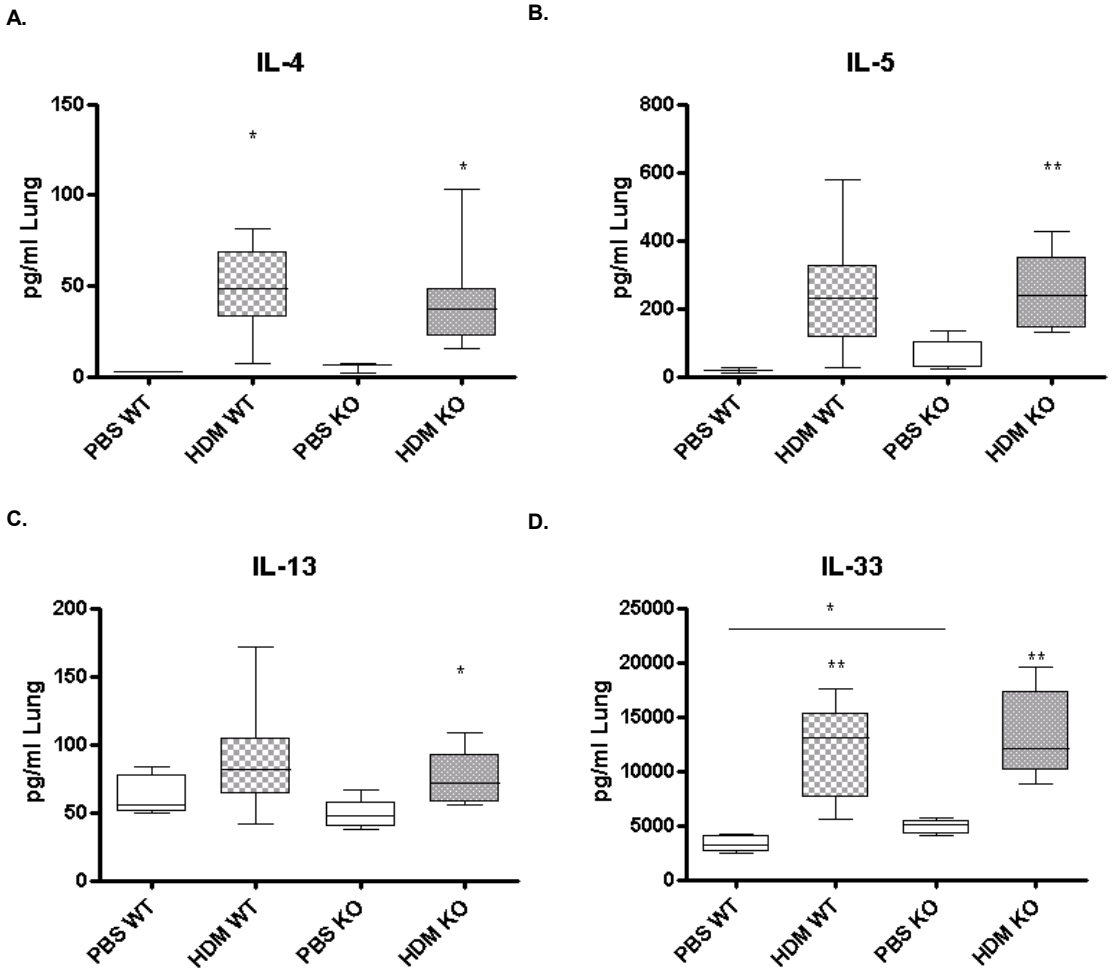


Figure 4.14 CD200R deficient mice did not exhibit elevated Th2 cytokines.

Lungs were taken at 4 hours following 3 weeks of HDM challenge. Control mice received PBS. Cytokines were quantified in the lung homogenate by ELISA. (A) IL-4, (B) IL-5 (C) IL-13 (D) IL-33. n = 3-6 mice/group, bar represents median, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ and ** $p<0.01$ relative to PBS control group by Mann Whitney test. $^{+}$ $p<0.05$ WT group relative to KO group by Mann Whitney test

4.4 Discussion

4.4.1 Eicosanoids during the resolution phase of HDM induced allergic airways disease

The omega 3 acids; Docosahexaenoic (DHA), Eicosapentaenoic acid (EPA) and their derivatives have been described as a family of specialised pro-resolving molecules (SPM's) that are important in the resolution of inflammation. The DHA and EPA derived members of the SPMs include Resolvin D1 (RvD1), Resolvin E1 (RvE1), Protectin D1 (PD-1) and Maresin (MaR1). Administration of exogenous RvD1, RvE1, PD-1 has been shown to resolve the features of allergic inflammation in OVA models of AAD (Levy, Kohli et al. 2007, Haworth, Cernadas et al. 2008, Rogerio, Haworth et al. 2012). We attempted to measure RvE1, PD-1 and MaR1 in our HDM model, however, these were not easily detectable in our lung tissue and no measurable increases above the limit of detection of the assay were recorded. It is interesting to note that despite RvD1, RvE1 and PD-1 being measurable in human asthmatics, only PD-1 has been reported to be measured in murine lung tissue (Levy, Kohli et al. 2007). MaR1 is a macrophages specific resolution lipid that is synthesised from DHA. It has been shown in a model of peritonitis to have beneficial actions on macrophages that may direct tissue homeostasis and resolution (Serhan, Yang et al. 2009). It is still to be investigated if exogenous administration of MaR1 during allergic airways disease would promote the resolution of the features of disease. Eicosanoids from arachidonic acids, the HETEs and Lipoxin's could be reliably measured in our lung tissue. Eicosanoids are traditionally considered early mediators of inflammation, however, it is becoming increasingly clear that they may also provide anti-inflammatory and pro-resolving cues (Serhan 2007, Gilroy 2010).

The pro-resolving lipid Lipxon A (4) LXA4 is derived from Arachidonic acid and can be synthesised by neutrophils, monocytes and macrophages via 3 different transcellular pathways by the enzymes 5-LOX, 15-LOX and COX2 pathways (Planaguma and Levy 2008). LXA4 has also been implicated in asthma and initially was shown to be decreased in the blood of severe asthmatics compared to mild/moderate sufferers. (Levy, Bonnans et al.

2005). It was later demonstrated that airway expression of LXA4, FPR2/ALX and the enzyme 15-LOX were decreased in severe asthmatics (Planaguma, Kazani et al. 2008). This group also demonstrated that asthmatic alveolar macrophages had impaired ability to synthesise LXA4 (Bhavsar et al, 2010). In experimental models of disease, analogues of LXA4 have been shown to promote resolution of allergic inflammation (Levy, Lukacs et al. 2007). LXA4 has also been described to interact with the newly discovered type 2 innate lymphoid cells (ILCs) which secrete IL-13. ILCs express FPR2/ALX and LXA4 was shown to reduced IL-13 production by these cells (Barnig, Cernadas et al. 2013). Furthermore, it has been described to inhibit eosinophil and neutrophils migration as well as inhibiting eotaxin and IL-5 secretion in a pleural model of allergic inflammation (Bandeira-Melo, Bozza et al. 2000). In our experimental model, we wanted to determine if LXA4 was measurable in the lung during the resolution phase. We found that LXA4 was not detectable in the lung from control mice but was induced following HDM exposure and remained detectable throughout the resolution phase. This expression during peak inflammation corroborates the theory that resolution pathways are initiated early during inflammation and can later act as counter regulatory pro-resolving mediators (Serhan and Savill 2005). Levels of LXA4 at day 7 were reduced compared to peak inflammation but remained significantly elevated and detectable during resolution compared to PBS controls, suggesting that LXA4 may be important in the resolution phase of HDM induced AAD. Prostaglandin E2 (PGE2) derived from cyclooxygenase (COX) activation has been shown to have dichotomous roles in allergic inflammation (Chung 2005). Classically it is known as have pro-inflammatory function and promote the influx of neutrophils, macrophages and monocytes (Kalinski 2012). Additionally, PGE2 is also a chemoattractant for mast cells which upon granulation can induce AHR (Weller et al, 2007). However, PGE2 has bronchodilatory effects and prevents allergen induced AHR. It has also been shown to inhibit eosinophils trafficking to the lung (Sturm, Schratl et al. 2008). PGE2 can counter regulate LTB4 mediated anti-microbial function of alveolar macrophages (Lee et al, 2009) further supporting a regulatory role for PGE2 in the lung. In our model, PGE2 was induced by HDM and remained at significantly elevated levels

during resolution. This suggests that after cessation of allergen challenge there is a continued up regulation of PGE2. It would be interesting to measure other mediators in this pathway. It would be interesting to investigate if the persistence of PGE2 is due to continual activation of upstream enzymes COX1/COX2 and PGE synthases or to determine if there is a reduction in 15-PGDH, the enzyme that degrades PGE2? Little is known about the role of PGE synthases and 15-PGDH in asthma. In colorectal cancer, the stimulation of tumour progression meditated by PGE2 has been attributed to suppressed levels of 15-PGDH (Backlund, Mann et al. 2005). COX1/2 has been implicated in aspirin induced asthma. The inhibition of COX by NSAIDs is meant to inhibit the production of CysLT and LTs that are detrimental to lung function, however, this also inhibits COX2 mediated PGE2 which contributes further to the loss of lung function (Bennett 2000). Further investigation into the resolution phase of PGE2 may provide clues as to which pathways might be relevant to target for future therapeutic intervention in HDM induced allergic disease.

12 and 15-HETE are products of the 12/15 lipoxygenase enzyme. The human homologue, 15LO, is the most abundantly up regulated gene in monocytes following IL-4 exposure (Chaitidis, O'Donnell et al. 2005). It is also induced by IL-13, suggesting an important function in Th2 inflammation. 12-HETE is up regulated during OVA induced allergic inflammation (Morgan et al, 2009) and mice lacking 12/15 LOX are protected from OVA induced allergic airways disease.(Hajek, Lindley et al. 2008). This suggests that 12-HETE may be pro-inflammatory. However, this study looked at peak inflammation time point only. In the present study, we found 12-HETE is elevated following HDM exposure but remains significantly elevated during the resolution phase. 12-HETE is expressed predominantly by monocytes and macrophages, which are known to express the enzyme 12/15 LOX, and we found that levels of 12-HETE correlated with macrophage and mononuclear cell count in the airway during resolution phase. Similar to LXA4 expression, 12-HETE induced during early inflammatory events may persist to ensure the resolution of inflammation and promote the return to homeostasis. Indeed, products of 12/15 LOX have also been described to activate the anti-inflammatory nuclear factor PPARγ, further supporting a role in resolution (Kunh &

O'Donnell, 2006). Activation of PPARγ has been shown to reduce AHR. (Honda, Marquillies et al. 2004). This suggests a potential pathway that is employed to promote resolution in our model.

These eicosanoid data shows that these lipids are induced as a result of allergen exposure and remain elevated during the resolution phase, suggesting a regulated interaction to promote resolution of allergic inflammation. The downstream effects of these lipids are still unclear and their potential activities during the onset and resolution phase require furthers investigation. This could be achieved by manipulating the enzymes involved in their in vivo generation (COXs & LOXs) and downstream receptors (ie PPARγ) that mediate their biological functions.

4.4.2 Targeting ChemR23 during resolution

We were unable to quantify SPMs directly in our model of HDM induced AAD and its resolution. To ascertain if there was a potential role for omega 3 fatty acids in directing resolution of inflammation we chose to target the ChemR23 receptor for the EPA derivative Resolvin E1 (RvE1) and chemerin. In our model we used C-15 - a peptide agonist for ChmR23. C-15 has been shown to mediate anti-inflammatory effects in the picogram range. Therefore, we used this as a tool to investigate the role of ChemR23 in our model. We did not see any effects of C-15 peptide treatment on any of the feature of HDM induced allergic airway disease. One possibility is that the dose of C-15 peptide was too low. We chose to administer 6.4pg of C-15 per mouse as it has been previously shown to be effective at this low dose in vivo using the zymosan peritonitis model (Cash, Hart et al. 2008). Previous studies reporting an effect of C-15 have either been in vitro assays or in more simplistic models of inflammation, such as the zymosan induced peritonitis (Luangsay, Wittamer et al. 2009). ChemR23 is expressed on macrophages and it may be that administration of the peptide via the i.v route did not enable C-15 peptide to accumulate at the sufficient concentration in the lung, our target organ. Thus, in a more complex established model of disease a larger dose of C-15 for longer may be required to see any therapeutic effect.

4.4.3 The role of CD200R during resolution

Chapter 3 demonstrated that the myeloid negative receptor, CD200R was up regulated on alveolar macrophages during the resolution phase of inflammation. This suggested a potential role in directing resolution of HDM induced inflammation. In an attempt to elucidate a role for CD200R in this process, HDM challenged mice received 3 i.p injections of OX-110, an antibody agonist for CD200R. Mice receiving CD200R agonist did not show any improvement in lung function, levels of HDM induced pulmonary inflammation or pathology. Conversely, there was an observed increase in total lung tissue leukocytes, specifically Th2 lymphocytes and the Th2 cytokine IL-4. However, there was also an increase in CD200+ lymphoyctes and FoxP3+ T regulatory cells in CD200R agonist treated mice exposed to HDM. These data confirm an earlier report that in vitro activation of CD200R on BMDCs promoted an induction of a FoxP3 + T regulatory population (Gorczynski, Lee et al. 2005) . In our model the increase in T regulatory lymphocytes was not sufficient to regulate the HDM induced inflammatory response. This increase in CD200+ lymphocytes suggests an up regulation of this homeostatic axis where there is the potential for an increase in CD200R activation. However, overall, the ligation of CD200R did not improve resolution. These data highlight the complex nature of immune regulation and suggest that there may be additional pathways involved.

To establish if CD200R itself was critical in meditating the allergic inflammation response; mice deficient in CD200R were employed. These mice were treated with 3 weeks of HDM. Interestingly, CD200R KO mice did not develop significant airway resistance. This is surprising because CD200R is suggested to negatively regulate pulmonary inflammation (Wissinger, Goulding et al. 2009). Therefore we hypothesised that absences of this negative regulatory would lead to enhanced AAD. It has previously been shown in a murine model of influenza, mice deficient in CD200R exhibited an exacerbated immune response, (Snelgrove, Goulding et al. 2008). However, total lung and BAL numbers were comparable between HDM treated WT and CD200R KO. Although differential analysis did not show any differences in leukocyte subsets, FACS analysis did reveal that there was a decrease

FoxP3+ T regulatory lymphocytes. This observation is in parallel with the increase observed following the activation of CD200R. Again, although there was a decrease in T regulatory lymphocytes this was not enough to exert a loss of regulation overall and there were no changes to the AAD phenotype. .

These two studies both show the opposite from the original hypothesis. It was expected that treatment with CD200R agonist would promote resolution; however, this was not the case. On the contrary, there was an increase in lung cell numbers. AHR data alluded to a possible increase in airway resistance although this was not significant. It has been described that CD200R activation can induced TGFb production (Holmannova, Kolackova et al. 2012). This would support the increase of T regulatory lymphocytes observed following CD200R activation, and the reduction of T regulatory cells in the CD200R KO mice as TGFb is required to induce naturally occurring T regulatory cells. However, in the context of asthma TGFb also has pro-fibrotic and remodelling role. It may be in the context of HDM induced inflammation TGFb may be exerting pathogenic effects that would counteract the regulatory effects of CD200R. CD200R3, a homologue of CD200R, which is exclusively expressed on mast cells and basophils, is known to be an activating receptor containing ITAM domains (Kojima et al, 2007). It is feasible that OX-110 agonist cross reacts with this receptor and therefore may be inducing mast cell activation and counter balances the negative regulation of the alveolar macrophage. In the CD200R KO study, mice treated with HDM for 3 weeks did not show any worsening of AAD, in fact, airway resistance was comparable with PBS controls.

This data shows that targeting CD200R did not promote resolution of AAD. However, as CD200R and its homologues are expressed on eosinophils, neutrophils, mast cells and basophils, which are all implicated in the pathogenesis of allergic inflammation, this also suggests a dual role for the CD200R-CD200 pathway in allergic inflammation which requires further investigation.

4.5 Conclusion

The concept of resolution as an active process has only recently been accepted with the discovery of specialised pro-resolving mediators. This is exemplified by deficiencies in these specialised pro-resolving mediators in asthmatics which are thought to contribute to the propagation of inflammation. Overall, the SPMs were not detectable in our samples. Targeting the receptor for Resolvin E1 did not promote resolution of inflammation. Additionally, activation of the CD200R did not result in any profound effects on the resolution of HDM induced AAD, indeed TH2 inflammation was worsened. These data suggest that more than one pathway is required to orchestrate the resolution of HDM induced inflammation. Additionally, it appears that some pro-resolving mediators have pleiotropic effects acting to initiate or enhance inflammation at early time points following allergen exposure and in established disease may act to limit inflammatory response. Given the complexity of the molecules involved in active resolution it may be that targeting an upstream cellular mediator(s) may provide more profound effects. In Chapter 3 it was shown that alveolar macrophages are up regulated during the resolution phase, suggesting an important role. In Chapter 5 the role of alveolar macrophages in resolution will be investigated.

Chapter 5.

Alveolar macrophages are required for the resolution of house dust mite induced allergic airway disease

5. 1 Introduction

In the previous chapter, it was demonstrated that targeting ChemR23 or CD200R pathways did not promote resolution of allergic airways disease. ChemR23 and CD200R are expressed by cells of the myeloid lineage and in particular CD200R expression on alveolar macrophages is known to have an important regulatory function. Since manipulation of specific pathways did not promote an overall phenotype, the next step was to target alveolar macrophages directly. This chapter aimed to investigate the role of alveolar macrophages during the resolution of HDM induced allergic airways disease.

Previous studies in animal models of asthma have shown that intra-tracheal administration of clodronate containing liposomes can effectively deplete alveolar macrophages from the airways (Van Rooijen and Sanders 1994). Alveolar macrophages phagocytose the clodronate containing liposomes and the ingested clodronate induces apoptosis resulting in the removal of alveolar macrophages. Liposomes do not cross epithelial or endothelial barriers thus the apoptotic effects induced by clodronate are restricted to local alveolar macrophages which reside in the alveolar spaces (Thepen, Van et al. 1989). Depletion of alveolar macrophages using clodronate encapsulated liposomes is a useful tool for investigating macrophage function. Previous studies have shown alveolar macrophages play a critical role in regulating the onset of allergic inflammation, (Thepen et al, 1992, Tang et al 2001, Careau & Bissonnette, 2004, Careau et al 2010, Bang et al 2011). It has also been shown that adoptive transfer of alveolar macrophages can down regulated the pulmonary immune response in OVA models of AAD (Careau & Bissonnette, 2004, Careau et al 2010, Bang et al 2011).

However the role of alveolar macrophages in a model of asthma initiated with an inhaled clinically relevant allergen, such as HDM has not previously been investigated. Therefore using clodronate macrophage depletion and adoptive transfer techniques, this chapter examines the role of alveolar macrophages in directing the resolution of HDM induced inflammation.

In separate experiments we have previously observed that depletion of alveolar macrophages prior to mucosal exposure to HDM allergen results is an exacerbation of airway disease (Kate L. Tomlinson, PhD thesis 2011; Mathie, Tomlinson et al manuscript in preparation). This chapter describes the role of alveolar macrophages during the resolution phase.

5.1.1 Aim

To determine if alveolar macrophages promote the resolution of HDM induced inflammation.

5.2 Methods

5.2.1 Alveolar macrophage depletion during resolution of HDM induced inflammation

Two protocols of HDM induced inflammation were used to investigate the effect of liposome encapsulated clodronate (ClodronateLiposomes.org, The Netherlands) depletion of alveolar macrophages during the resolution phase, (Figure 5.1). In the first protocol (A), using an acute HDM exposure regime, mice were challenged with 3 doses of HDM over a week, then received 50µl clodronate encapsulated liposome or PBS encapsulated liposomes as a control via intra-tracheal (i.t.) administration on day 1 after the final HDM challenge. Mice were then culled 6 days later. In the second protocol (B), mice received HDM 3 times a week for 3 weeks and received 50µl clodronate encapsulated liposome or PBS encapsulated liposome vehicle i.t. at 1 and 5 days post the final HDM challenge. Mice were culled 7 and 14 days later and the effect of macrophage depletion on AHR and inflammation was assessed.

5.2.2 Alveolar macrophage adoptive transfer during resolution of HDM induced AAD

Alveolar macrophages were retrieved from naïve donor mice via intra-tracheal broncho-alveolar lavaged with 3 x 1.0ml 1xHBSS+0.5mM EDTA, centrifuged and the cell pellet incubated with Vybrant DiD (1:100) (Invitrogen) for 15 mins, at 37^{o}C, washed and resuspended in PBS. Cells were immediately transferred i.t. in 50ul volume to HDM treated recipients. Recipients received 3 doses of HDM 3 times a week. 2x10^{5} alveolar macrophages were transferred directly into the airways at 1 day and 5 days after last HDM challenge. Mice were culled on day 7 following final HDM challenge where AHR and inflammation were assessed.

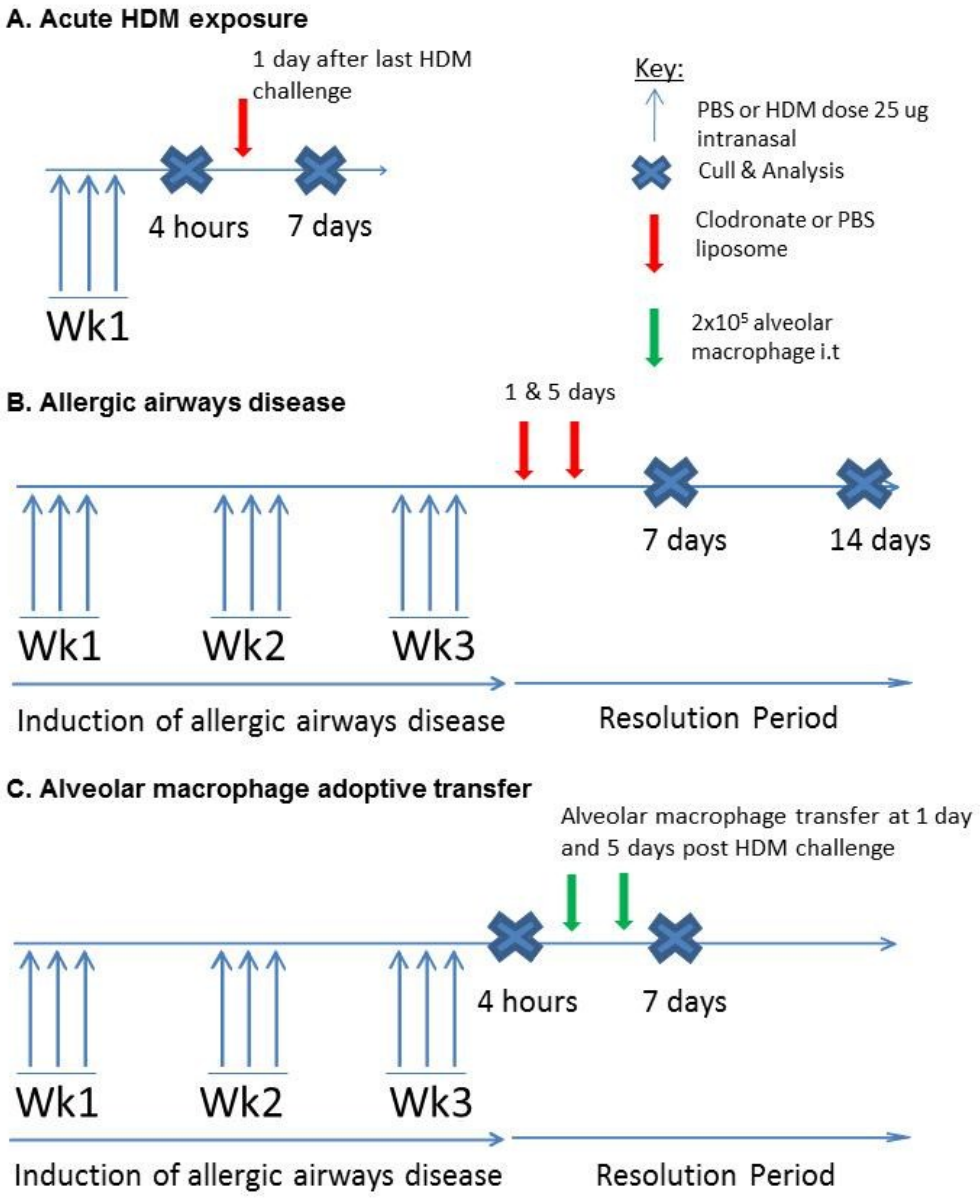


Figure 5.1 Experimental protocols for the depletion and adoptive transfer of alveolar macrophages during the resolution phase of HDM induced inflammation. (A) Acute HDM exposure protocol; Mice were exposed to HDM i.n. 3 times a week for 1 week. Control mice received i.n. PBS alone. Mice were culled at 4 hours and 7 days post final challenge. To assess the role of alveolar macrophages during resolution, 50µl clodronate liposomes were administered i.t. 1 day after last HDM challenge. Mice were culled 7 days after the final HDM challenge (B) Allergic protocol; Mice received HDM i.n. 3 times a week for 3 weeks to induce AAD. Control mice received i.n. PBS alone. Mice received 50µl clodronate liposome at days 1 and 5 after last HDM exposure. To assess the effect on resolution, mice were culled at 7 and 14 days following the last HDM challenge. (C) Adoptive transfer of alveolar macrophages during resolution of HDM induced allergic airways disease**.** Mice were exposed to intranasal HDM 3 times a week for 3 weeks. Control mice received i.n. PBS alone. Transfer of $2x10^5$ alveolar macrophages lavaged from naive BALB/c were labelled with Vybrant DiD and administered i.t. to recipient mice 1 day and 5 days after last HDM challenge. Mice were sacrificed at 4hrs and 7days after last HDM challenge.

5.3 Results

5.3.1 Clodronate treatment depletes alveolar macrophages in the airways up to 8 days post liposome administration

In this study alveolar macrophages were depleted after final exposure to HDM allergen to investigate their role during the resolution of inflammation. In protocol A, mice were exposed to 3 doses of HDM and culled 4 hours and 7 days later. We have previously shown that after 3 airway challenges with HDM, mice exhibit an inflammatory response but have not yet had time to develop a humoral IgE allergic immune response. In this protocol, the aim was to investigate the role of alveolar macrophages during the resolution of acute HDM exposure. Mice were culled 6 days after clodronate treatment and exhibited a significant reduction in the number of alveolar macrophages, (Figure 5.2 A). In protocol B, mice received clodronate liposome on day 1 and day 5 following 3 weeks of HDM exposure. 7 days after the final HDM challenge numbers of alveolar macrophages were significantly reduced in the clodronate treated groups (Figure 5.2 B & C). This reduction in numbers of alveolar macrophages was maintained throughout the 14 day time point studied.

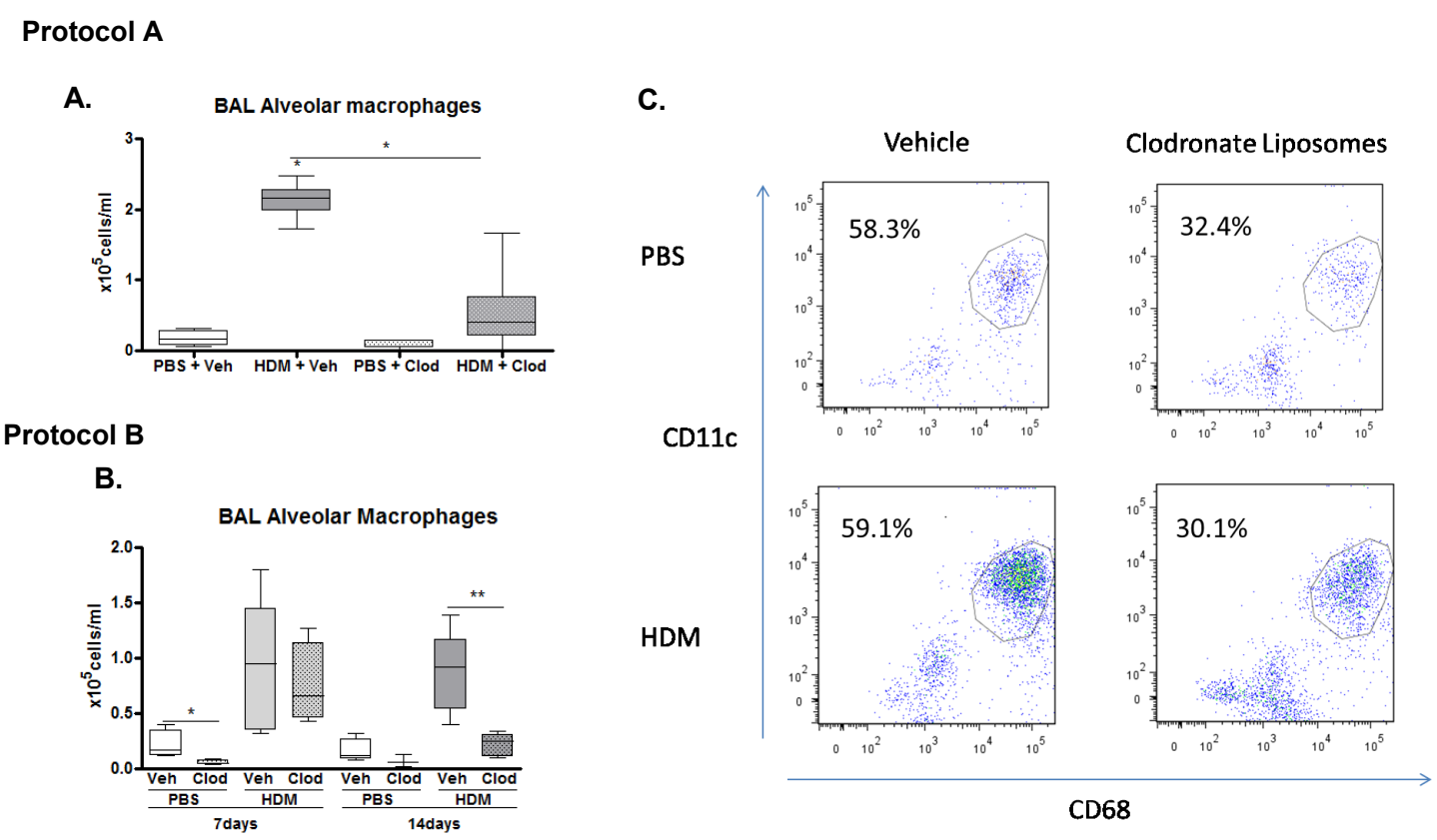


Figure 5.2 Clodronate encapsulated liposome treatment depletes alveolar macrophages. (A) Mice received 50µl clodronate liposome i.t. on day 1 after 1 week of HDM exposure. Mice were culled 6 days and alveolar macrophages in the BALF were analysed by FACS. (B&C) Mice received 50µl clodronate liposome i.t at days 1 and 5 after 3 weeks of HDM. Airways were lavaged at 7 days and 14 days after last HDM challenge. Levels of CD68+ CD11c+ macrophages in the airway were quantified by flow cytometry. n = 4-8 mice/group, plots depict the median and interquartile range and minimum and maximum values * $p<0.05$ ** $p<0.01$ and *** $p<0.001$ relative to PBS control group by Mann Whitney test

5.3.2 Clodronate liposome treatment delays resolution of HDM airway inflammation

In the acute HDM exposure protocol, mice exposed to short term HDM challenge exhibited a significant increase in total airway inflammation. The degree of inflammation was lower than that observed following 3 weeks of challenge and resolved to baseline by 7 days, but eosinophils and lympho-mononuclear cells remained significantly elevated. (Figure 5.3 B, C, E). This inflammatory infiltrate in the BAL predominantly composed of eosinophils and neutrophils, (Figures 5.3 C & D). A small but significant elevation in lymph-mononuclear levels was also observed, (Figure 5.3 E). However, there was no change in the numbers of macrophages, (Figure 5.3 F).

In contrast, treatment with liposome control induced an increase in inflammatory cell recruitment in mice previously exposed to HDM and there was no resolution of airway inflammation (Figure 5.3.B). There was a significant elevation in the numbers of neutrophils, lympho-mononuclear cells and macrophages compared to HDM challenged mice at 7 days. Interestingly, there was no effect on eosinophils (Figure 5.3 C). This effect of liposome administration was exacerbated in mice treated with liposomes containing clodronate. Total airway inflammation was significantly elevated in clodronate liposome treated mice compared to control liposomes (Figure 5.3 B). This was associated with enhanced levels of neutrophils and lympho-mononuclear cells, (Figure 5.3 D & E).

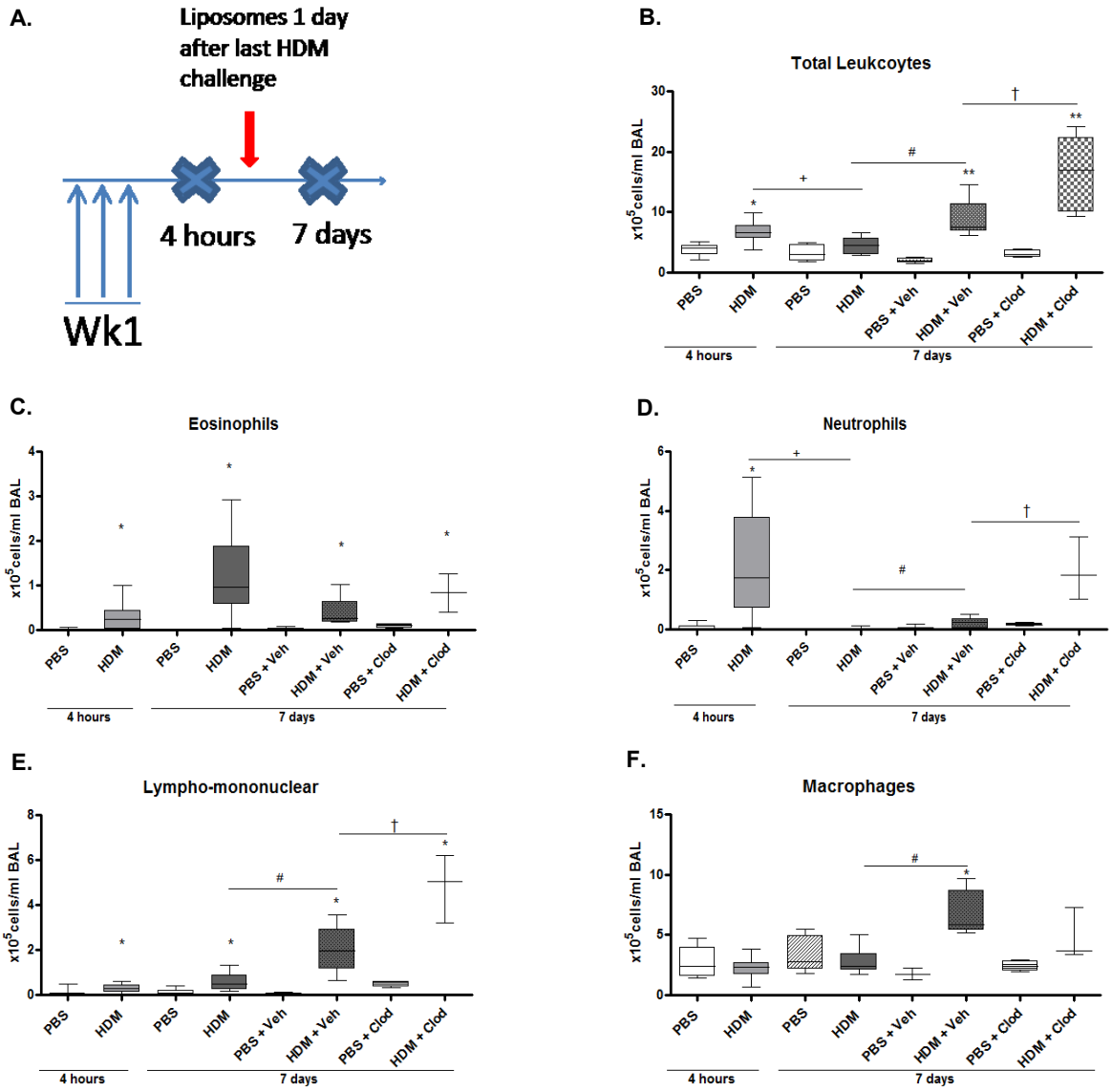


Figure 5.3. Airway inflammation after 3 doses of HDM and following alveolar macrophage depletion. BAL was taken at 4 hours and 7 days following 3 challenges of HDM. Control mice received PBS. A group of mice were treated with clodronate containing liposome 1 day after last challenge. Control mice received vehicle liposome containing PBS. (A) Acute HDM exposure protocol (B) Total cell numbers and differential cell counts (C) Eosinophils (D) Neutrophils, (E) Lympho-mononuclear & (F) macrophages were identified and quantified from WG stained cytospins. n = 3-8 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ relative to PBS control group by Mann Whitney test. $^{+}$ $p<0.05$ relative to HDM peak inflammation by Mann Whitney test. $^{\#}$ $p<0.05$ relative to HDM + vehicle liposomes compared to HDM 7days post final challenge by Mann Whitney test. † $p<0.05$ relative to HDM + clodronate liposomes compared to HDM + vehicle liposomes by Mann Whitney test.

5.3.3 Clodronate liposome treatment delays resolution of lung HDM inflammation

Administration of either vehicle or clodronate encapsulated liposomes did not result in an inflammatory response in PBS treated mice (Figure 5.4). In the lung tissue, challenge with 3 doses of HDM resulted in a modest, but significant increase in total inflammatory cells at 4 hours post final allergen challenge, (Figure 5.4 A). The composition of the infiltrate in the lung was predominantly eosinophils and neutrophils, with eosinophils remaining significantly elevated at 7 days, (Figure 5.4 B & C). Numbers of macrophages which are not altered at peak inflammation following 3 doses of HDM, were elevated during the resolution phase as seen previously in Chapter 3. Treatment with vehicle or clodronate containing liposomes did not alter total levels of lung tissue inflammation (Figure 5.4 A). However, neutrophils were significantly increased in HDM treated clodronate treated mice at 7 days (Figure 5.4 C).

These data show that treatment with clodronate liposomes reduces the number of alveolar macrophages resulting in altered resolution of acute HDM induced inflammation, with a specific delay in the clearance of neutrophils in the lung and BAL and airway lympho-mononuclear cells.

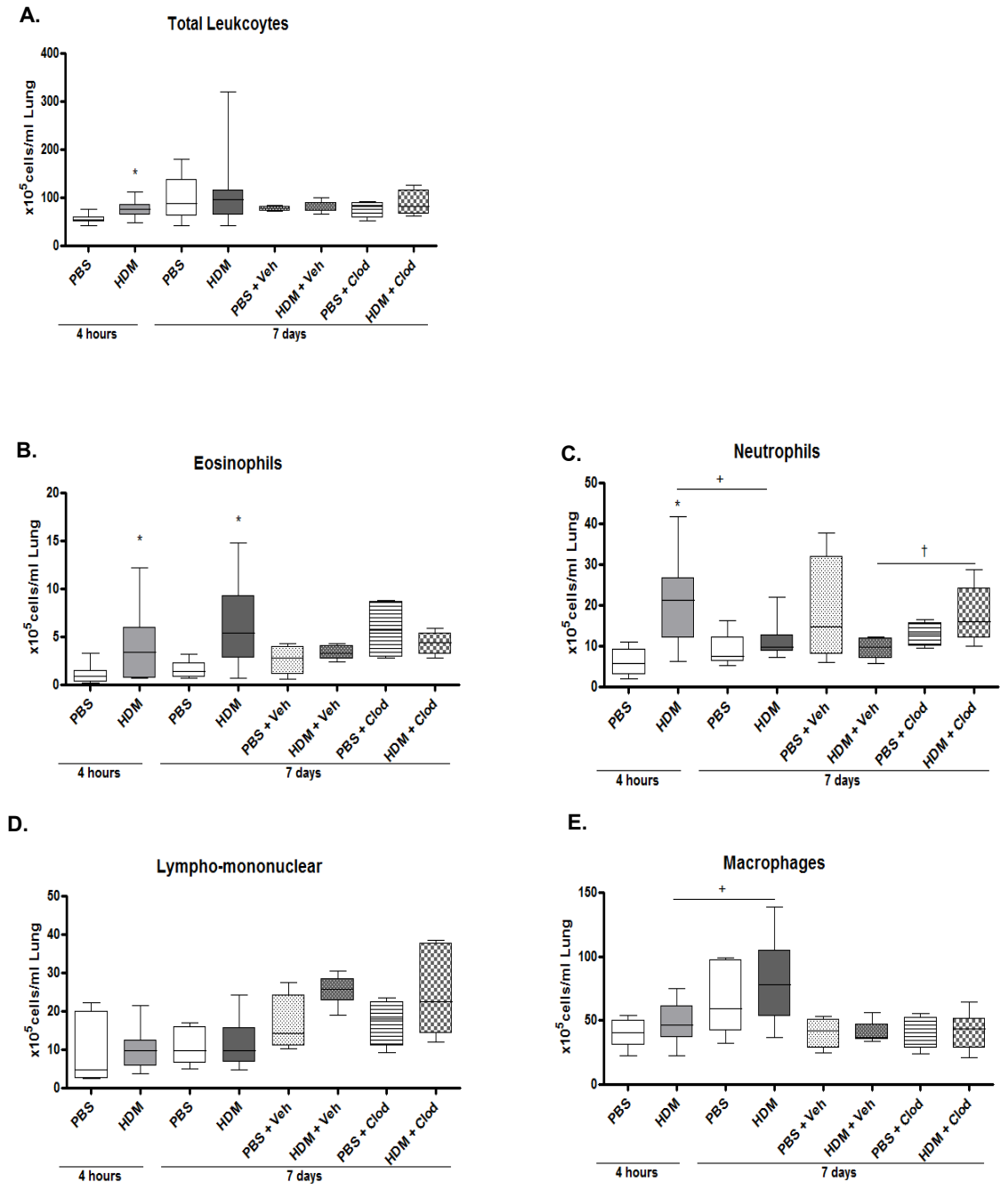


Figure 5.4 Lung tissue inflammation 3 doses of HDM and following alveolar macrophage depletion. Lung tissue was taken at 4 hours and 7 days following 3 challenges of HDM. Control mice received PBS. A group of mice were treated with clodronate containing liposome 1 day after last challenge. Control mice received vehicle liposome containing PBS. Total cell numbers (A) and differential cell counts (B) Eosinophils (C) Neutrophils, (D) Lympho-mononuclear & (E) Macrophages were identified and quantified using WG staining. n = 4-8 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ relative to PBS control group by Mann Whitney test. $^{+}$ $p<0.05$ relative to HDM peak inflammation by Mann Whitney test. † $p<0.05$ relative to HDM + clodronate liposomes compared to HDM + vehicle liposomes by Mann Whitney test.

5.3.4 Mice that received clodronate encapsulated liposome have elevated levels of Th2 lymphocytes at 7 days

A significant increase in pulmonary CD4+ T1/ST2+ Th2 cells was observed in mice challenged with 3 doses of HDM. Levels returned to baseline by 7 days after last challenge. However, treatment with clodronate liposomes delayed the clearance of Th2 lymphocytes at 7 days, (Figure 5.5 A). In the lung tissue, Th2 cytokines, IL-4 and IL-13 were induced in the lung after 3 challenges of HDM and resolved by 7 days, (Figure 5.5 B & D). IL-5 was not significantly induced by HDM and levels were not altered by vehicle liposome or clodronate treatment, (Figure 5.5 C). At 7 days treatment with vehicle liposome induced a change in the levels of IL-4 in PBS mice to levels comparable with levels in mice at peak inflammation. Clodronate containing liposomes did not induce any further changes in IL-4 (Figure 5.5B). Treatment with clodronate did not appear to alter lung tissue levels of IL-13 during resolution phase of HDM induced inflammation (Figure 5.5D).

We then investigated the role of alveolar macrophages in resolution of established AAD following 3 weeks of HDM challenge by treating mice with clodronate liposome.

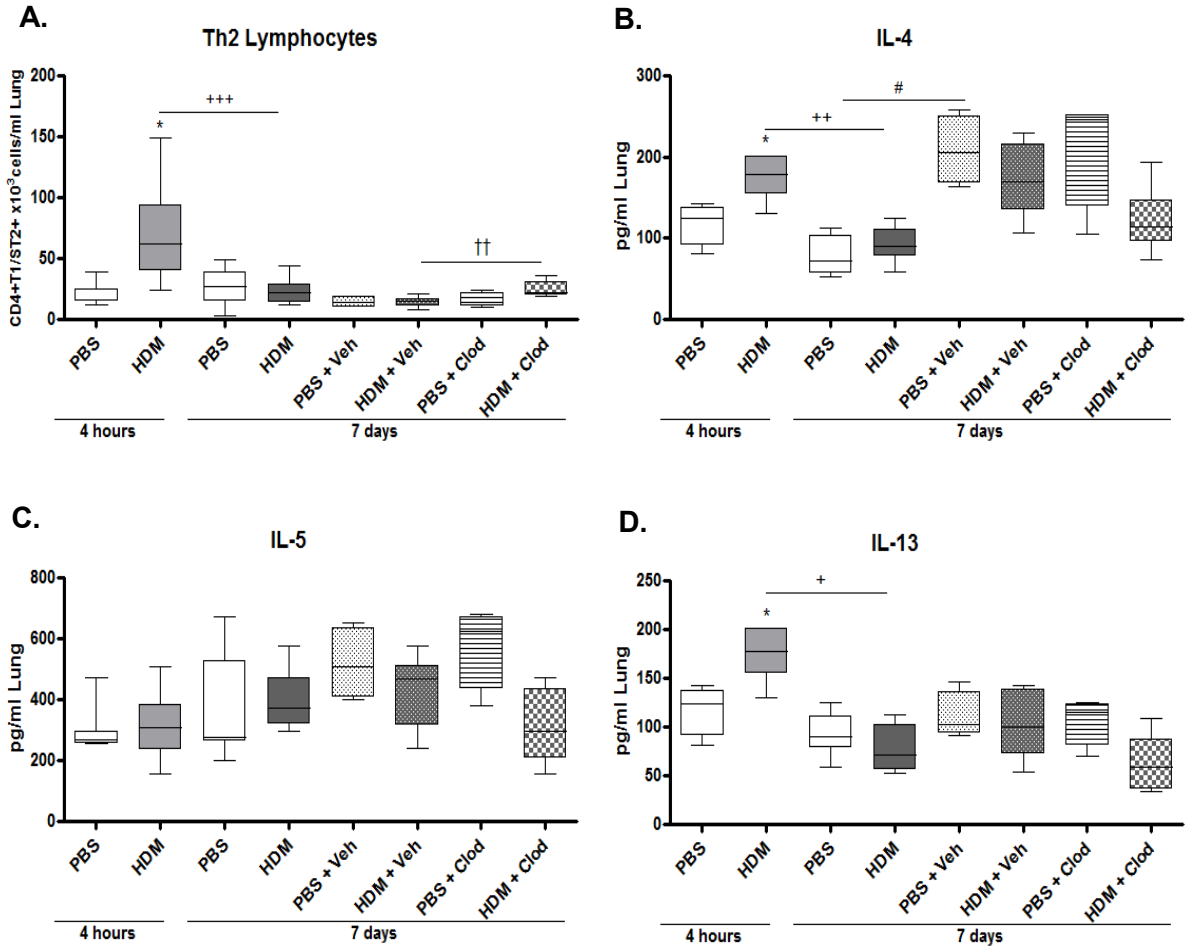


Figure 5.5 Th2 immunity after 1 week HDM and following alveolar macrophage depletion. Lungs were taken at 4 hours and 7 days following 1 week of HDM challenge. Control mice received PBS. A group of mice were treated with clodronate containing liposome 1 day after last challenge. Control mice received PBS containing liposome. (A) Levels of Th2 lymphocytes were quantified by flow cytometry. (B) IL-4, (C) IL-5, (D) IL-13. Cytokines were quantified by ELISA. n = 4-8 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ relative to PBS control group by Mann Whitney test. $^{+}$ $p<0.05$ $^{++}$ $p<0.01$ and $^{++}$ $p<0.001$ relative to HDM peak inflammation by Mann Whitney test. $^{\#}$ $p<0.05$ relative to HDM + vehicle liposomes compared to HDM 7days post final challenge by Mann Whitney test. † $p<0.05$ relative to HDM + clodronate liposomes compared to HDM + vehicle liposomes by Mann Whitney test.

5.3.5 Treatment with liposome clodronate did not alter airways resistance after 3 weeks exposure to HDM.

Airway resistance was elevated in HDM treated mice 7 days post final challenge, however, in these mice, which also received vehicle containing liposomes during the resolution phase, this was not significant. Treatment with clodronate containing liposomes did not significantly affect AHR (Figure 5.6).

5.3.6 Depletion of alveolar macrophages after 3 weeks of HDM exposure delays the resolution of neutrophils and Th2 lymphocytes

The role of alveolar macrophages in resolution of AAD following more prolonged HDM exposure was investigated. Mice receiving clodronate liposomes had significantly higher BAL cell numbers at 7 and 14 days compared to mice receiving vehicle. Similar to our observations after 1 week HDM exposure, removal of alveolar macrophages did not alter total lung tissue cell numbers but did result in an increase in the number of cells accumulating in the airway lumen (Figure 5.7 A & B). There were no significant changes to eosinophil numbers in either the airway or the lung, (Figure 5.7 C & D). However, neutrophils and lympho-mononuclear cells remained elevated above baseline 14 days post final HDM challenge in mice deficient in alveolar macrophages (Figure 5.7 E-H). Thus, depletion of alveolar macrophages delays the resolution of pulmonary inflammation, primarily because of a failure to clear neutrophils and lympho-mononuclear cells.

Further investigation into the lymphocyte populations was carried out using flow cytometry. Despite no increase in the total number of lymphocytes recovered from the lung tissue following clodronate treatment, (Figure 5.7 B) an increase in T1/ST2+ Th2 lymphocytes was observed 14 days post HDM challenge, (Figure 5.8 A). In contrast, IL-13+ Th2 lymphocytes were not modulated by alveolar macrophage depletion, (Figure 5.8 B) which correlates with AHR being unaltered in these mice. Analysis of CD4+ CD25+ FoxP3+ and inducible CD4+ IL-10+ T regulatory lymphocytes and γδ IL-17+ T lymphocytes showed that cell numbers were not significantly changed by alveolar macrophage depletion. This suggests that

alveolar macrophages do not directly influence these cell populations during the resolution phase, (Figures 5.8 C-E).

The data indicate that the reduction of alveolar macrophages by clodronate liposome treatment impairs the clearance of neutrophils from the airway. This highlights the fact that alveolar macrophages play a role in regulating inflammation in the airway during resolution of HDM induced AAD. In addition, alveolar macrophages can also influence tissue leukocytes as indicated by the delayed resolution of CD4+ T1/ST2+ lymphocytes and neutrophils.

5.3.7 Depletion of alveolar macrophages in the airway delayed the clearance of interstitial macrophages

Although there were no changes in differential counts of macrophages in the lung tissue, FACS analysis of macrophage subsets revealed that interstitial macrophage numbers remained significantly elevated in clodronate treated HDM mice (Figure 5.8 F). Thus it may be that alveolar macrophages in the airway lumen interact directly with the lung tissue resident macrophages and influence either cell recruitment or proliferation during resolution of AAD.

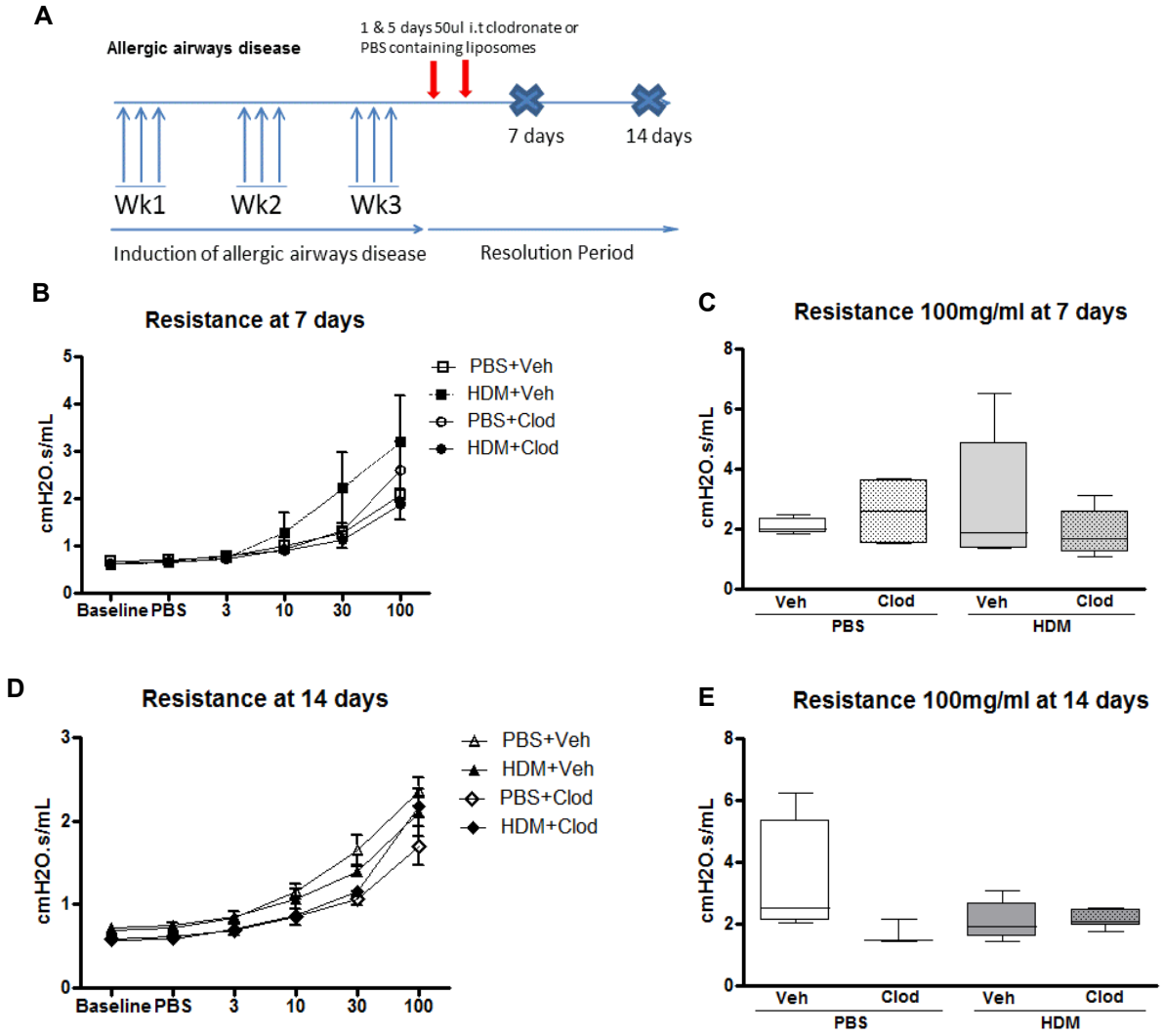


Figure 5.6 Airway hyper-reactivity following depletion of alveolar macrophages. AHR was measured by MCh challenge**.** Control mice received PBS. Mice were then treated with clodronate or vehicle containing liposomes. Control mice received PBS containing liposome. (A) Clodronate or PBS liposome administration protocol in allergic airways disease model (B) Changes in resistance and (C) resistance at 100mg/ml MCh at 7 days after final HDM challenge (D Changes in resistance and (E) resistance at 100mg/ml MCh at 7 days after final HDM challenge. n = 4-6 mice/group, plots depict the median and interquartile range and minimum and maximum values.

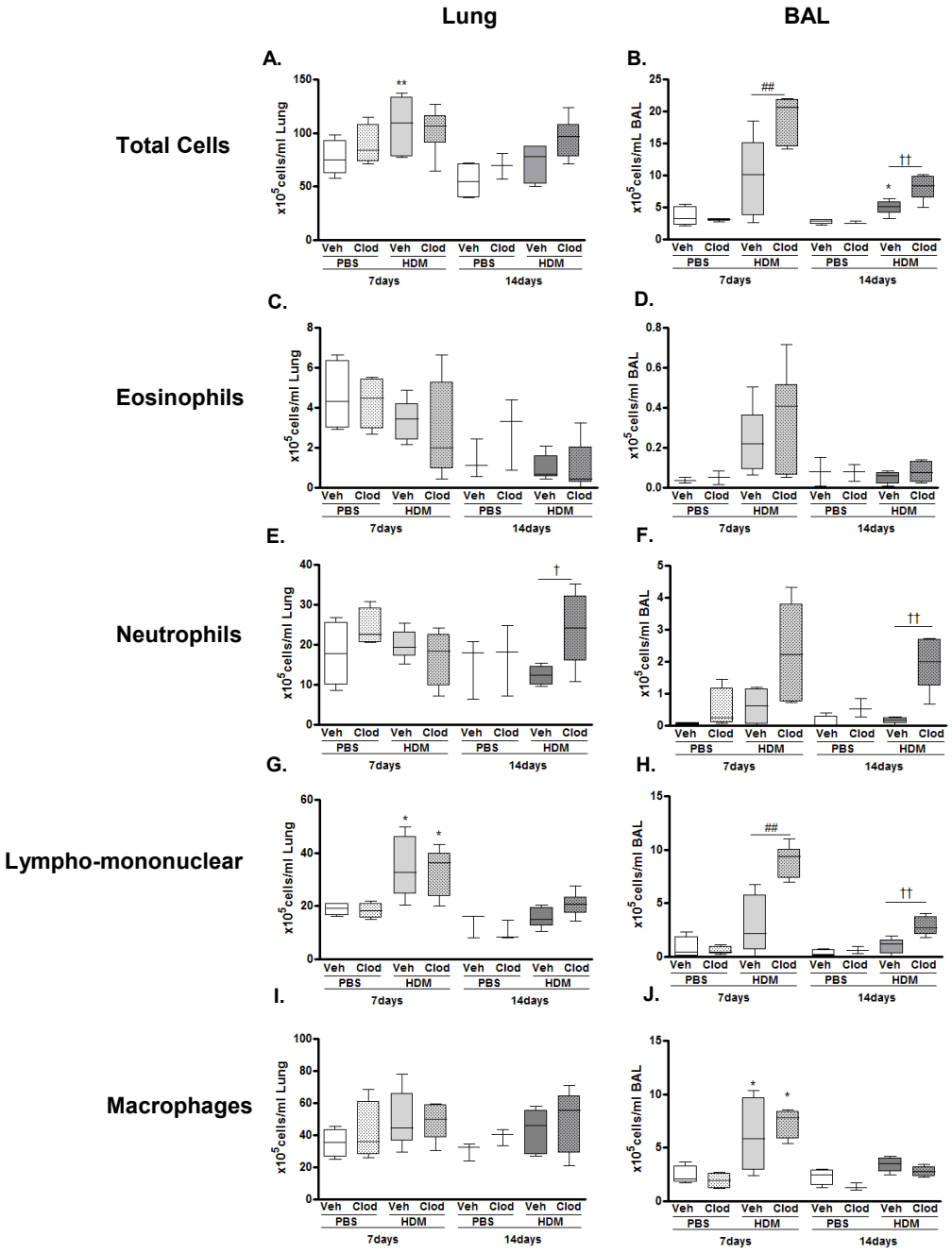


Figure 5.7 Pulmonary inflammation following clodronate treatment. Lung and BAL were acquired at 7 days and 14 days following 3 weeks of HDM challenge. Control mice received PBS. Mice were then treated with clodronate containing liposome. Control mice received vehicle PBS containing liposome. Total cell numbers (A&B) and differential cell counts: (C&D) Eosinophils (E&F) Neutrophils, (G&H) lympho-mononuclear and (I&J) macrophages were identified and quantified using WG staining. n = 3-8 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ relative to PBS control group by Mann Whitney test. $^{+}$ $p<0.05$ relative to PBS control group by Mann Whitney test. $^{\#}$ $p<0.05$ and $^{\#\#}$ $p<0.01$ relative to HDM + clodronate liposomes compared to HDM + vehicle liposomes at 7days post final challenge by Mann Whitney test. † $p<0.05$ and †† $p<0.05$ relative to HDM + clodronate liposomes compared to HDM + vehicle liposomes at 14 days by Mann Whitney test.

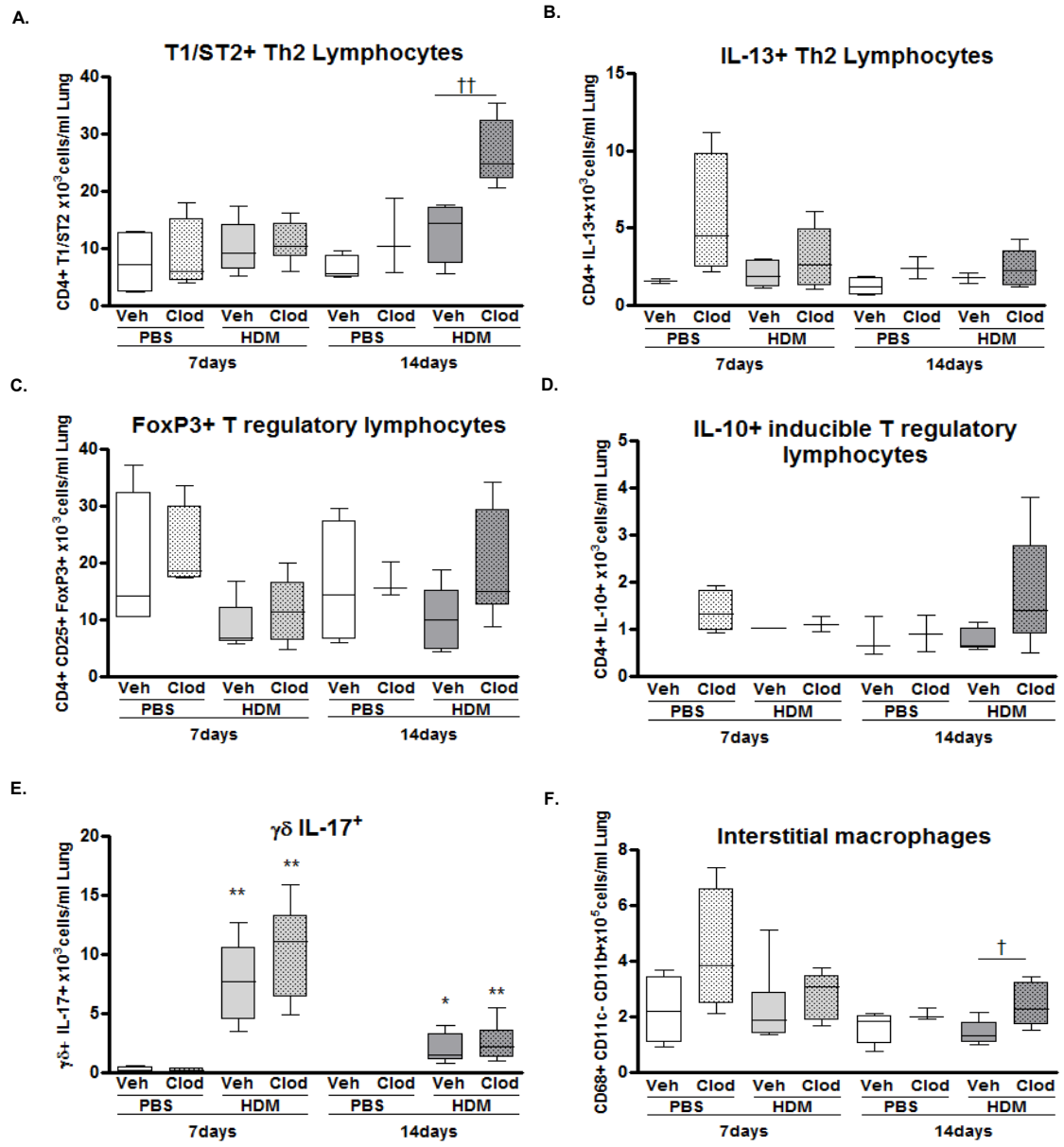


Figure 5.8 Lung tissue lymphocytes following alveolar macrophage depletion. Lungs were taken at 7 days and 14 days following 3 weeks of HDM challenge. Numbers of (A) CD4+ T1/ST2+ (B) CD4+ IL-13+ (C) CD4+ CD25+ FoxP3+ (D) CD4+ IL-10+ (E) γδ IL-17+ lymphocytes and (F) interstitial macrophages were quantified by flow cytometry. n = 2-6 mice/group, plots depict the median and interquartile range and minimum and maximum values. ** $p<0.01$ relative to PBS control group by Mann Whitney test. † $p<0.05$ and †† $p<0.05$ relative to HDM + clodronate liposomes compared to HDM + vehicle liposomes at 14 days by Mann Whitney test.

5.3.8 Lung tissue cytokines are not altered following macrophage depletion

In the lung tissue, levels of the Th2 cytokines IL-4, IL-5, IL-13 and the Th1 cytokine IFNγ were not altered by clodronate treatment. This is similar to that observed in the 1 week HDM model. The depletion of alveolar macrophages did not directly affect levels of either Th2 and Th1 cytokines, (Figures 5.9 A-D).

IL-33 in the lung remained elevated in the HDM challenged groups at 7 and 14 days, (Figure 5.9 E). At 7 days post final HDM challenge, clodronate treatment of mice had no significant effect on IL-33 levels (Figure 5.9 E). Treatment of mice with either vehicle PBS containing or clodronate containing liposomes affected the production of IL-33, even in non-allergic mice (Levels in Figure 3.7D compared to Figure 5.9E). In the clodronate treated PBS mice, levels of IL-33 were lower than the vehicle treated PBS mice, suggesting that liposomes themselves may affect IL-33 production (Figure 3.7D). Interestingly, at 14 days, mice treated with clodronate liposomes exhibited significantly lower levels of IL-33 compared to the vehicle group.

The Th2 cytokines IL-4, IL-5, IL-13 and IL-33 were not detectable in the BAL in any treatment groups by ELISA. However, following an increase in neutrophils numbers in the airway the neutrophil chemokine, KC was measured in the BAL after HDM and liposome treatment and levels were found to be significantly elevated at 14 days in HDM challenged mice that had received clodronate liposomes (Figure 5.9 F). This correlates with the increase in neutrophils levels observed in the lung and airways at this time point.

These results suggest that alveolar macrophages influence regulation of the neutrophil response to HDM. The reduction in IL-33 following clodronate treatment indicates either that alveolar macrophages are a source of IL-33 or that they are directly regulating IL-33 production in the lung.

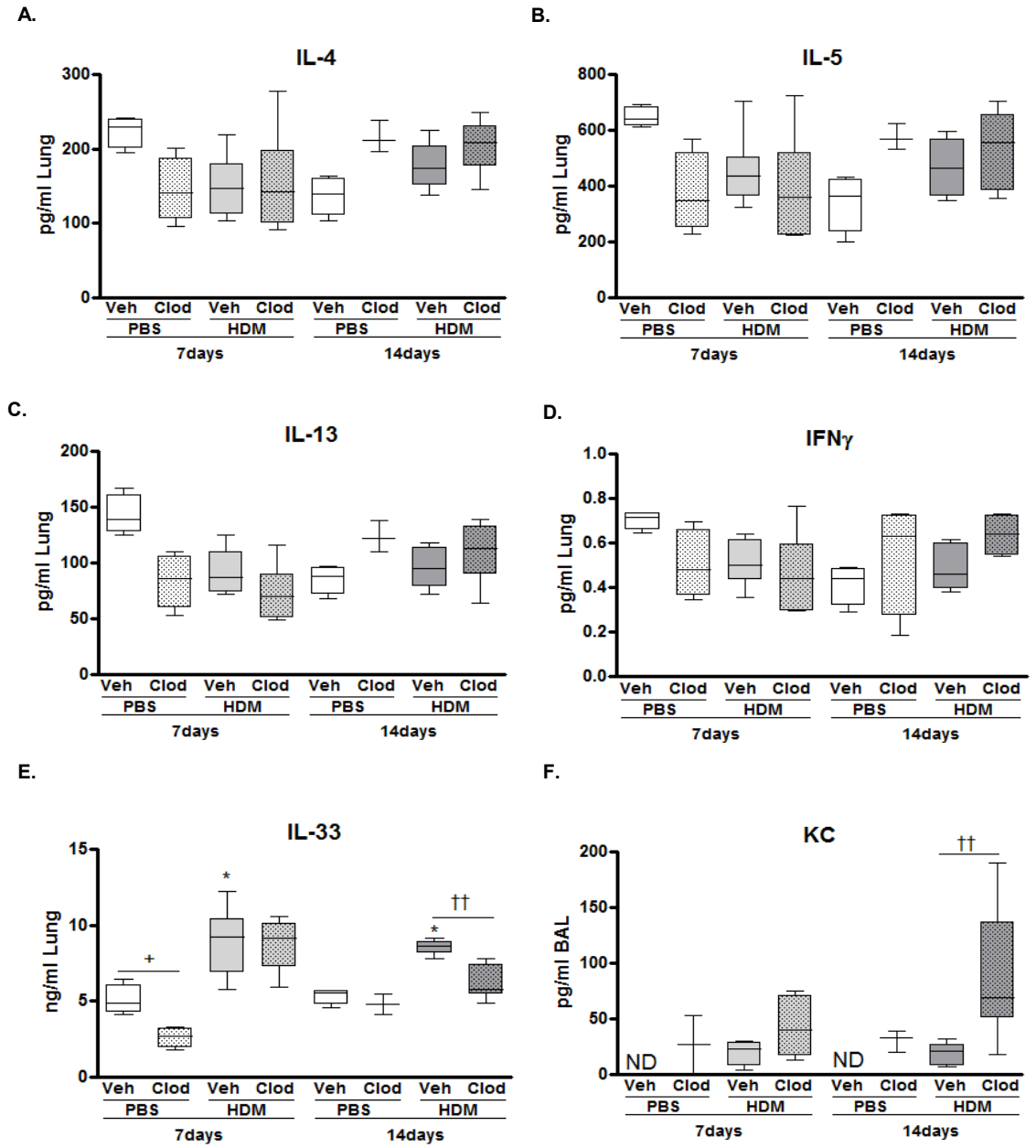


Figure 5.9 Lung tissue cytokines following alveolar macrophages depletion. Lungs and BAL were taken at 7 days and 13 days following 3 weeks of HDM challenge. (A) IL-4, (B) IL-5 (C) IL-13, (D) IL-33, (E) IFNγ. Cytokines were quantified in lung homogenate. (F) KC measured in BAL supernatant using ELISA. n = 4-8 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ relative to PBS control group by Mann Whitney test. $^{+}$ $p<0.05$ relative to PBS control group by Mann Whitney test. $^{+}$ $p<0.05$ relative to PBS + clodronate liposomes compared to PBS + vehicle liposomes at 7days post final challenge by Mann Whitney test. †† $p<0.05$ relative to HDM + clodronate liposomes compared to HDM + vehicle liposomes at 14 days by Mann Whitney test.

5.3.9 Adoptive transfer of naive alveolar macrophages does not ameliorate HDM induced allergic airway hyper-reactivity.

To elucidate further the role of alveolar macrophages during resolution, alveolar macrophages were adoptively transferred to HDM treated recipients. However, the transfer of naive alveolar macrophages did not significantly change airway resistance compared to mice at 7 days that did not receive alveolar macrophages, (Figure 5.10 A & B). This data suggests that alveolar macrophages do not mediate a direct effect on airway resistance at this time point.

5.3.10 Transfer of alveolar macrophages enhances clearance of leukocytes in the lung but not in the BAL.

Total lung tissue leukocytes recruited in response to HDM were significantly reduced at 7 days in mice that received alveolar macrophages, (Figure 5.11 A). In contrast, total cell numbers in the BAL did not change following alveolar macrophage transfer, (Figure 5.11 B). In both the lung and airway, eosinophils numbers remained unchanged following adoptive transfer of macrophages, (Figure 5.11 C & D). The transfer of alveolar macrophages did, however, significantly reduce the number of lung tissue neutrophils, lympho-mononuclear cells and macrophages, (Figures 5.11 E, G & I). Neither cell recruitment to or clearance from the airway lumen was significantly affected by macrophage transfer, (Figures 5.11 F, H & J).

Flow cytometric analysis of the cell populations in the lung confirmed the observation that lung tissue macrophages CD68+ CD11c -CD11b+ were reduced following i.t. administration of alveolar macrophages at 7 days. At this time point, baseline levels of interstitial macrophages were higher compared to that calculated for the 4 hour time point. However, the trend between the groups at this time point follow the differential cells counts, therefore, indicating that administration of alveolar macrophages into the airway lumen influences resolution of lung tissue inflammation.

5.3.11 Lung tissue Th2 cytokines are not altered following alveolar macrophage transfer.

Th2 lymphocytes induced by HDM remained significantly elevated 7 days after HDM challenge. The transfer of alveolar macrophages did not affect Th2 numbers, (Figure 5.12 A). Levels of IL-4, IL-5 IL-13 and IL-33 in the lung induced by HDM were not altered following macrophage transfer, (Figure 5.12 B-E) This suggests that alveolar macrophages do not directly influence Th2 cytokines at this time point.

5.3.12 Regulatory cytokines are not altered following alveolar macrophage transfer.

Alveolar macrophages are suggested to exert suppressive effects on pulmonary inflammation. Therefore, we measured levels of the regulatory cytokines in the lung following alveolar macrophage manipulation. IL-10 was increased in the lung 7 days following HDM challenge (Figure 5.2F). Lung tissue levels of TGFβ were not modulated by HDM exposure or after transfer of alveolar macrophages, (Figure 5.12 G). In contrast, the newly identified macrophage derived regulatory cytokine, IL-27 was significantly induced 4 hours after last HDM exposure with levels returning to baseline by 7 days (Figure 5.12 H). The adoptive transfer of alveolar macrophages did not influence levels of any of these regulatory cytokines during resolution of AAD

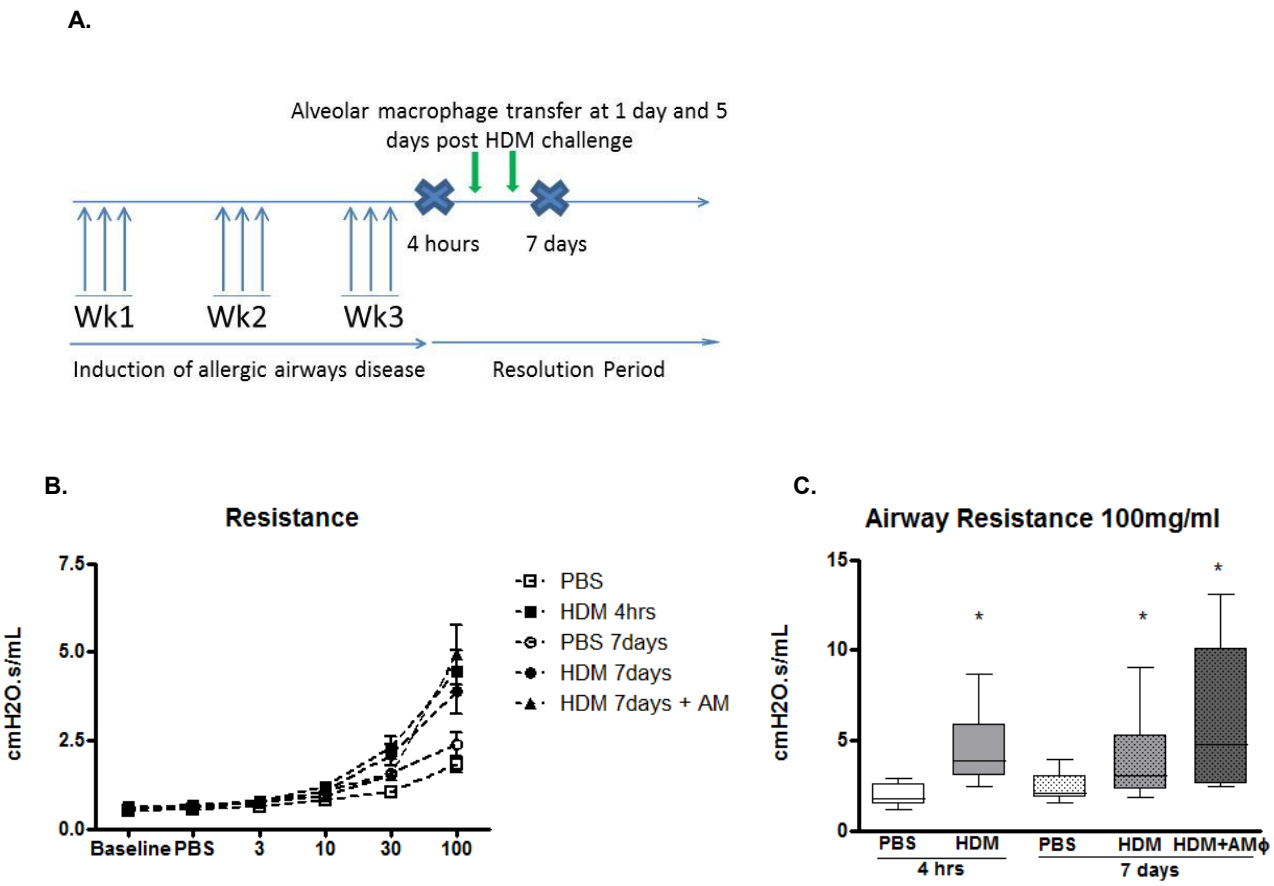


Figure 5.10 Airway hyper-reactivity following adoptive transfer of Alveolar macrophages. (A) Alveolar macrophage transfer protocol. AHR was measured by MCh challenge**.** Control mice received PBS. (B) Changes in resistance were measured, 4 hours and 7 days after HDM challenge and (C) levels of resistance at 100mg/ml MCh. n = 4-6 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ relative to PBS control group by Mann Whitney test.

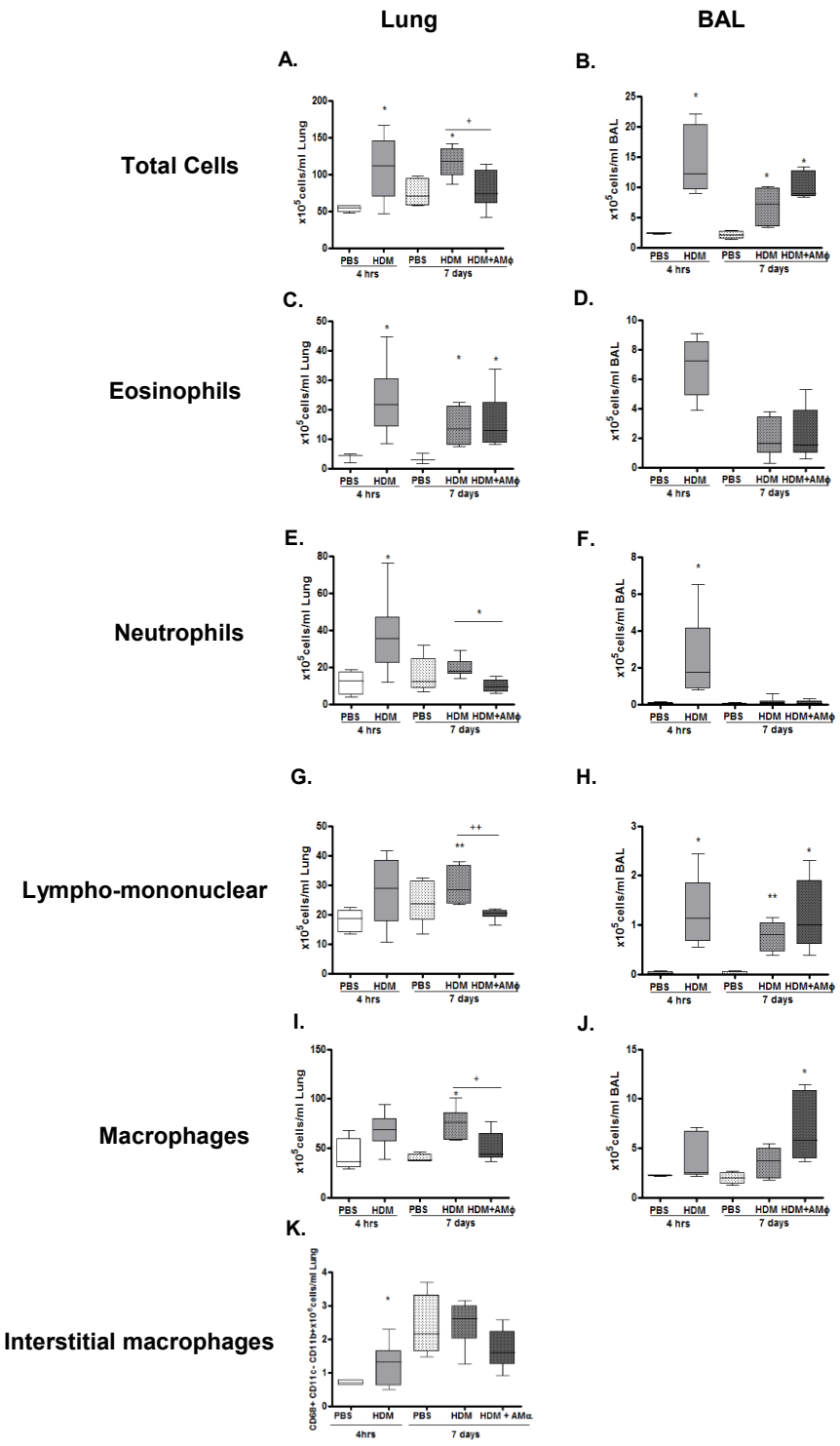


Figure 5.11 Pulmonary inflammation following adoptive transfer of Alveolar macrophages. Lung and BAL were acquired at 4 hours and 7 days following 3 weeks of HDM challenge. Total cell numbers (A&B) and differential cell counts: (C&D) eosinophils, (E&F) neutrophils, (G&H) lympho-mononuclear and (I&J) macrophages cells were identified and quantified using WG staining. (K) Interstitial macrophages determined by flow cytometry. = 4-8 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ and ** $p<0.01$ relative to PBS control group by Mann Whitney test. $^{+}$ $p<0.05$ and $^{++}$ $p<0.01$ relative to HDM compared to HDM + alveolar macrophage at 7 days post HDM challenge by Mann Whitney test.

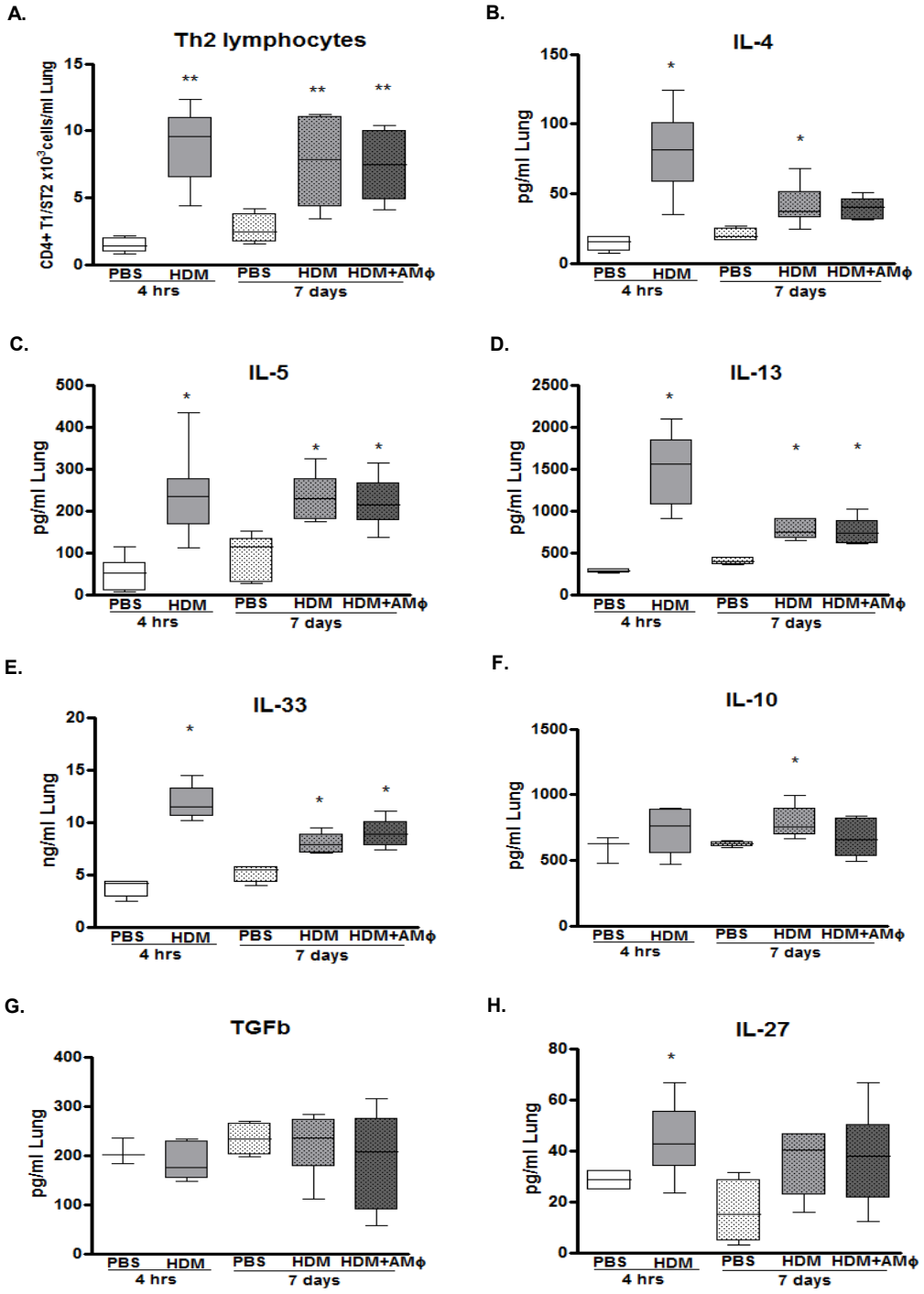


Figure 5.12 Th2 lymphocytes and lung tissue cytokines following alveolar macrophage transfer. Lungs were taken 4 hours and 7 days following 3 weeks of HDM challenge. (A) CD4+ T1/ST2+ lymphocytes quantified by flow cytometry. Cytokines were quantified in lung homogenate by ELISA. TGFβ was measured by bioassay. (B) IL-4, (C) IL-5 (D) IL-13, (E) IL-33, (F) IL-10 (G) TGFβ and (H) IL-27. n = 3-8 mice/group, plots depict the median and interquartile range and minimum and maximum values. * p<0.0 and ** p<0.01 relative to PBS control group by Mann Whitney test.

5.4 Discussion

The aim of this chapter was to investigate if alveolar macrophages play a role during the resolution of allergic airways disease. This chapter describes the depletion and adoptive transfer of alveolar macrophages during resolution of HDM induced inflammation.

5.4.1 Modulation of alveolar macrophages affects resolution of pulmonary inflammation

Alveolar macrophages were removed from the airway by administration of clodronate encapsulated liposomes to mice following exposure to HDM extract in order to determine the role of alveolar macrophages in mediating resolution of allergen induced AAD. Administration of clodronate containing liposomes i.t. efficiently reduced the levels of alveolar macrophages up to 14 days post HDM challenge. Reduction of alveolar macrophages in non-allergen exposed mice had no effect on lung resident cell populations and did not result in the recruitment of inflammatory cells from the blood or bone marrow.

An acute allergen exposure protocol was used to investigate the effect of alveolar macrophage depletion on HDM induced inflammation during the sensitisation stage, prior to established disease. As few as 3 doses of HDM induced significantly elevated levels of airway inflammatory cells composed of eosinophils, neutrophils and lymphocytes. Pulmonary inflammation returned to baseline within 7 days. However, depletion of alveolar macrophages delayed the resolution of neutrophils and lymphocytes, suggesting that alveolar macrophages promote the clearance of these cells from the lung.

Clodronate depletion of alveolar macrophages also resulted in a delay in the resolution of airway inflammation in mice with established AAD. Similarly, the reduction in alveolar macrophages specifically affected the retention of neutrophils and CD4+ T1/ST2+ lymphocytes within the lung tissue demonstrating that alveolar macrophages play an active role in the removal of these cells. It is conceivable that in an allergic setting, reducing the alveolar macrophage population provides a signal for KC to recruit neutrophils, which like

macrophages are a phagocytic cell type that could potentially be acting in a surveillance capacity to protect the lung from harmful inhaled stimuli.

In a complimentary study naïve alveolar macrophages were adoptively transferred into mice with established allergic airways disease. It has been shown that adoptive transfer of naïve alveolar macrophages but not sensitised alveolar macrophages can counter regulate OVA induced allergic inflammation (Careau, Proulx et al. 2006, Bang, Chun et al. 2011). This supports the theory that alveolar macrophages may alter their regulatory capacity after exposure to antigen (Wissinger, Goulding et al. 2009). Indeed, it has been demonstrated that allergen sensitisation induces a pro-inflammatory phenotype in alveolar macrophages with expression of IL-1β, IL-6 and TNFα (Careau, Proulx et al 2006, Bang, Chun et al 2011).

In previous studies, alveolar macrophages were transferred prior to allergen challenge whereas in our study we adoptively transferred alveolar macrophages during the resolution phase at the height of inflammation. This is the first study to look at the role of alveolar macrophages on the resolution of allergen induced inflammation. Additionally, in our model we investigated the effect of macrophage transfer on the endogenous levels of inflammation because in previous studies alveolar macrophages have been adoptively transferred to recipient mice with AAD only after clodronate liposome treatment which exacerbates the disease phenotype. Interestingly, the addition of alveolar macrophages at peak inflammation decreased the numbers of lung tissue leukocytes but did not alter total airway cell counts during resolution. Lung tissue neutrophil numbers and T1/ST2+ lymphocytes were reduced following alveolar macrophage transfer. In contrast, eosinophils were unaffected. These data in conjunction with our observations where we employed an opposite strategy to deplete alveolar macrophages indicate that alveolar macrophages have direct effects on the resolution pathway of specific leukocyte subsets. Adoptive transfer of alveolar macrophages has also been shown to inhibit myeloid dendritic cell allergen capture and accumulation in the lymph nodes in response to OVA in sensitised mice (Bedoret, Wallemacq et al. 2009).

Thus targeting alveolar macrophages locally in the airway appears to have long reaching effects in the peripheral organs

Clodronate liposomes reduced the numbers of alveolar macrophages, however, the results also demonstrated that vehicle containing liposomes also mediated some effects. This was particularly observed in the acute protocol where vehicle resulted in increased airway neutrophila. This method of clodronate depletion is fairly crude and likely results in retention of dead macrophages in the airway spaces, so alternative strategies to manipulate macrophages would be beneficial. Another strategy to target alveolar macrophages would be to utilise LysM(cre). Activation of cre recombinase in these mice can deplete alveolar and tissue macrophages (Nieuwenhuizen, Kirstein et al. 2012). These mice could be crossed with mice that have cytokine floxed genes to enable investigation into the effects of macrophage derived cytokines. However, it should be noted that the LysM(cre) transgene also targets neutrophils. The ideal model would be a CD68 (cre) that could be crossed with floxed genes, such as IL-33, to target macrophage specific cytokine secretion.

We have also shown that removal of alveolar macrophages by clodronate liposomes in established AAD resulted in a decrease in tissue IL-33 levels suggesting that either alveolar macrophages are a source IL-33 or that they regulate IL-33 production in the lung. However, the adoptive transfer of alveolar macrophages did not modulate IL-33. It has recently been reported in an OVA model of allergic inflammation, that alveolar macrophages express the receptor T1/ST2 and can produce IL-33. However, in this report depletion of alveolar macrophages resulted in improved airway inflammation and remodelling and the authors suggest that IL-33+ alveolar macrophages are promoters of allergic disease (Mizutani, Nabe et al. 2013). Interestingly, these results both contradict and support our findings where we show depletion of alveolar macrophages is detrimental to AAD. Moreover, in murine models of OVA and fungal induced allergic airway inflammation, IL-33 citrine reporter mice revealed that IL-33 production was restricted to type 2 pneumocytes of the alveolar epithelium rather than alveolar macrophages (Hardman, Panova et al. 2013). IL-33 is generally considered to

be pro-inflammatory, the role Mizutani and colleagues describe for IL-33 correlates with a recent publication from our group where we demonstrate in our murine HDM model that IL-33 drives remodelling and in paediatric patients that IL-33 is not modulated by steroids (Saglani, Lui et al. 2013). It is still to be demonstrated whether IL-33 from alveolar macrophages are critical in human disease. In future, it would be of interest to determine whether alveolar macrophages could express IL-33 in our model and whether IL-33+ macrophages contribute to the pathology of AAD. It is interesting to note that alveolar macrophages also express T1/ST2, suggesting that there may be an autocrine effect of IL-33 in the lung.

5.4.2 Modulation of alveolar macrophages did not affect AHR

Interestingly, adoptive transfer or depletion of alveolar macrophages after allergen exposure did not alter AHR or levels of Th2 cytokines. Previous studies utilising an OVA model of AAD in rats have shown that alveolar macrophage depletion increases AHR (Careau and Bissonnette 2004, Bang, Chun et al. 2011) (Careau, Turmel et al. 2010). However, as previously discussed, in these models, macrophages were depleted prior to or during allergen challenge, as opposed to during established disease in our study. Alveolar macrophages have been shown to have a role controlling sensitisation to allergen, and this likely accounts for the increased AHR observed when macrophages are depleted during this phase. In order to sensitise mice to OVA animals are administered OVA, in conjunction with an alum adjuvant administered i.p. followed by airway challenge to induce asthma like pathology. In contrast, intranasal administration of HDM induces sensitisation and development of disease via mucosal sensitisation of the airways (Johnson, Wiley et al. 2004, Gregory, Causton et al. 2009). Thus, the HDM model mimics the likely route in which alveolar macrophages encounter allergen in man. The role of alveolar macrophages in HDM driven AAD is not well described and despite a recent paper characterising the M1 and M2 phenotype of macrophages by immunohistochemical analysis in three models of HDM induced inflammation, their functional role was not assessed (Draijer, Robbe et al. 2013).

5.4.3 Macrophage plasticity

These data illustrate that alveolar macrophages play a potential role in the resolution of the mucosal allergic immune response. However, it is interesting that the depletion of alveolar macrophages after established disease was not more dramatic and appears to only affect specific pathways involving neutrophils and T1/ST2+ lymphocytes. It is possible that other leukocytes, such as T regulatory lymphocytes, which we have shown are elevated following HDM challenge, also play a role in resolution of AAD and may directly regulate other pro-inflammatory pathways. It may be that HDM impairs the regulatory capacity of alveolar macrophages. In non-eosinophilic asthmatic patients, it has been shown that alveolar macrophages had impaired ability to carry out efferocytosis of granulocytes and apoptotic leukocytes (Simpson, Gibson et al. 2013). This is corroborated by a report where alveolar macrophages from OVA sensitised mice were shown to have a lower phagocytic ability and exhibited a more pro-inflammatory response to ex vivo TLR stimulation compared to steady state macrophages (Naessens, Vander Beken et al. 2012). Moreover, a recent paper demonstrated that the pulmonary response to *S. pneumonia* infection was exacerbated in mice sensitised to HDM (Habibzay, Saldana et al. 2012). Again, this was shown to be a result of altered TLR signalling, where TLR signalling inhibitors were elevated resulting in reduced neutrophil recruitment. Thus, alveolar macrophages from sensitised and challenge animals have an altered phenotype compared to naïve mice and have reduced regulatory function. By increasing our understanding of the role of macrophages in resolution of inflammation it may be possible manipulate specific pathways during resolution and restore homeostatic function.

However, characterising macrophages during resolution has not been clear-cut. Macrophages are known for their plasticity and their ability to switch phenotype allows them to the respond accordingly to changes in the local environment. Characterisation studies and transcriptome analysis comparing pro-inflammatory and resolution phase macrophages have also shown that resolution macrophages may be difficult to categorize. Using a model of

resolving peritonitis it was revealed that macrophages from the resolution phase exhibited a "hybrid" of M1/M2 markers (Bystrom, Evans et al. 2008). Further analysis using affymetrix based mRNA transcriptome analysis confirmed that resolution macrophages expressed genes associated with M1 macrophages for functions such as, proliferation, MHCII for antigen presentation and CCL5 and CXCL13 chemokines for lymphocyte recruitment. Resolution phase macrophages also express genes associated with resolution and wound healing; ALOX15 which is required for the enzymatic production of lipoxins, Timd4 a gene required for the recognition of apoptotic cells and the anti-inflammatory growth factor, TGFβ2 (Bystrom, Evans et al. 2008, Stables, Shah et al. 2011). Macrophages are designed to respond to an array of stimuli and their plasticity in vivo and the difficulty in maintaining a stable phenotype ex vivo is a confounding factor in studying determining their precise role in different disease settings.

Of particular interest in this resolution study, there was the reduction in lung tissue macrophages following alveolar macrophage transfer. As mentioned above, the converse was observed in the alveolar macrophage depletion studies: when alveolar macrophages were removed the number of interstitial macrophages increased. The removal of alveolar macrophages resulted in a delay in the resolution of interstitial macrophages. These macrophages reside in the tissue and their role is unclear. We found that an increase in interstitial macrophages correlated with worsened inflammation suggesting a pro-inflammatory role in the HDM model. It has previously been suggested that these tissue macrophage are regulatory and in an OVA model were shown to regulate inflammation via secretion of IL-10 detected by IHC (Bedoret, Wallemacq et al. 2009, Toussaint, Fievez et al. 2012). Intracellular cytokine staining for macrophages by FACS was attempted in this study, however, consistent staining was not achieved so the results were not convincing. Bederot et al, isolated alveolar macrophages and interstitial macrophages from the lung and stimulated them with LPS in vitro to measure cytokine secretion in cell culture supernatant.

Future studies could utilise this method with HDM as a stimulant to measure IL-10, TGFβ and other regulatory cytokine production from the subsets at peak disease and resolution.

The relationship between alveolar macrophages and interstitials are not understood and further investigation into the role of IMs in the allergic lung would be valuable. Alveolar macrophages are known for their regulatory function and their interaction with interstitial macrophages would present an exciting mechanism that may be critical for pulmonary regulation. We show that alveolar macrophages have distinct suppressive properties which act rapidly to regulate the local microenvironment. Toussaint et al (2012) alluded to homeostatic function for IL-10+ interstitial macrophages. A future experiment would be to isolate these interstitial macrophages after alveolar macrophage transfer and examine the key cytokines that are being produced by interstitial macrophages following alveolar macrophage transfer.

5.5 Conclusion

While the depletion of alveolar macrophages delayed resolution of HDM induced inflammation, the transfer of extra alveolar macrophages enhanced resolution of some facets of allergic inflammation. These studies clearly highlight the importance of alveolar macrophages during the resolution phase of HDM induced inflammation. However, it is unclear precisely how these cells mediate resolution. The next chapter will investigate mediators that may be important in directing resolution.

Chapter 6.

The Annexin A1 - FPR axis regulates the onset of allergic airways disease

6. 1 Introduction

In Chapter 3, the pro-resolving lipid Lipoxin A (4) (LXA4) was found to be induced in the lung following HDM exposure and remained elevated during the resolution phase. LXA4 signals through ALX/FPR2 and antagonists of this receptor have been shown to delay resolution of inflammation (Dufton and Perretti 2010). FPR2 also mediates anti-inflammatory effects of the pro-resolving peptide Annexin A1. Annexin A1 and LXA4 have both been shown to be up regulated in colitis patients suggesting this pro-resolving circuit and the receptor FPR2 are critical for mediating resolution at mucosal surfaces (Vong, Ferraz et al. 2012). In the context of allergic airways disease Annexin A1 KO mice were shown to have elevated AHR using an OVA model of AAD (Ng, Wong et al. 2011). This paper described that naïve Annexin A1 deficient mice had spontaneous AHR that predisposes them to develop an exacerbated response to allergen. The authors proposed that this may be a susceptibility factor for the development of asthma. However, the expression and role of Annexin A1 and FPR2 in the pulmonary allergic inflammatory environment, induced by clinically relevant allergens at the mucosal surface, particularly in models of asthma is not well described. This chapter addresses the function of the Annexin A1:FPR2 axis in the HDM model of allergic airways disease.

6.1.1 Aims

To investigate the role of ANXA1 in the pathogenesis of HDM induced allergic airways disease using an Annexin A1 KO mouse

To determine the role of ANXA1 and FPR2 during the challenge phase of HDM induced allergic airways disease using pharmacological manipulation

6.2 Methods

6.2.1 Annexin A1 KO mice

ANXA1 knock-out (KO) mice were obtained from Charles River. ANXA1 gene function is abrogated by insertion of a LacZ gene construct under the Annexin promoter (Hannon, Croxtall et al. 2003). Wild type (WT) BALB/c and ANXA1 KO mice were exposed to 25ug HDM 3 times a week for 3 weeks. WT and KO control mice were treated with PBS. Mice were sacrificed at 4 hours after last challenge and the effect of ANXA1 deficiency on airway hyper-reactivity and inflammation was assessed (Figure 6.1).

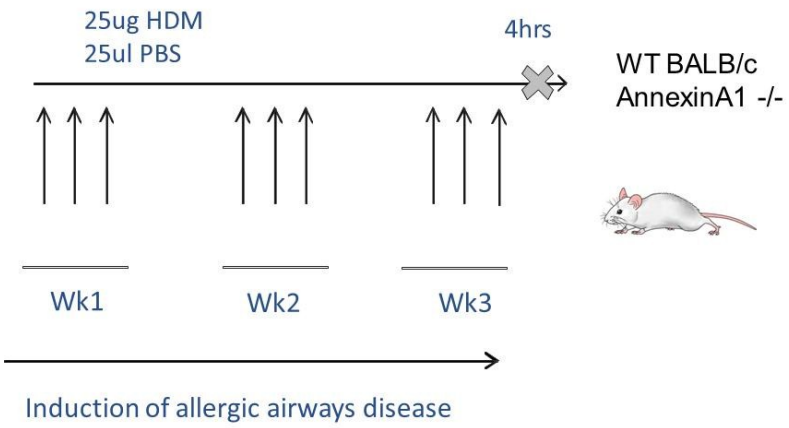


Figure 6.1 Experimental protocol for the induction of HDM induced allergic airways disease in BALB/c wild type and Annexin A1 knock-out mice. Adult WT and ANXA1 KO mice aged 6-8 weeks were exposed to intranasal (i.n) HDM 3 times a week for 3 weeks and culled at 4 hours after last allergen challenge. Control mice received i.n PBS alone.

6.2.2 Therapeutic treatment with the FPR2 antagonist WRW4 and the Annexin A1 mimetic ANXA2-50

To complement studies utilising Annexin A1 KO mice, WT mice were also treated with a FPR2 antagonist. In parallell, an Annexin A1 mimetic was used to determine the effect of over expression of Annexin A1 during allergen challenge. Female BALB/c WT mice were challenged with 25ug HDM 3 times a week for 3 weeks. In the final week, HDM treated mice received daily intranasal administration of either 5ug of the FPR2 antagonist WRW4, 5ug of the Annexin A1 mimetic ANXA2-50 or PBS as a vehicle control (Veh). Control mice received PBS + WRW4, PBS + ANXA2-50 or PBS + Veh. Lung function, BAL and lung tissue harvest was carried out 4 hours after last HDM challenge (Figure 6.2).

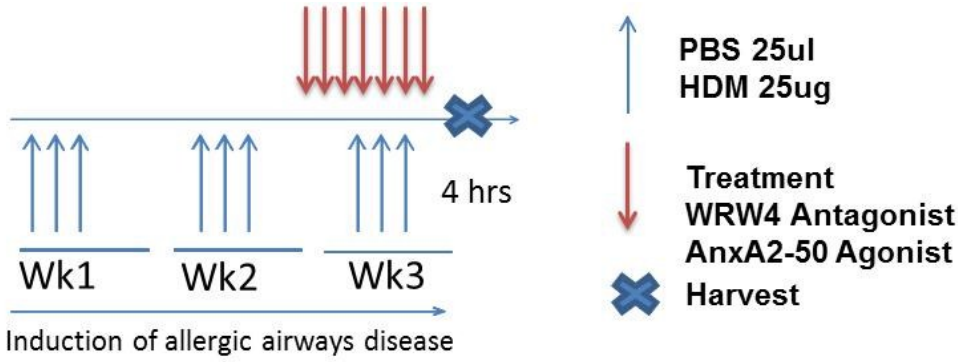


Figure 6.2 Experimental protocol for the therapeutic administration of WRW4 and AnxA2-50 in HDM challenged BALB/c wild type mice. Adult mice aged 6-8 weeks were exposed to intranasal (i.n) HDM 3 times a week for 3 weeks and culled at 4 hours after last allergen challenge. Control mice received i.n PBS. In the final week, HDM and PBS treated groups received either Vehicle, 5ug of WRW4 or AnxA2-50 i.n.

6.3 Results

6.3.1 Annexin A1 is expressed in alveolar macrophages, epithelial cells and neutrophils after HDM exposure and during resolution

Pulmonary expression of Annexin A1 was determined by immunofluorescence staining of lung tissue sections from wild type BALB/c mice. In PBS treated control mice Annexin A1 positive cells were restricted to macrophages in the parenchyma and a small number of cells in the vicinity of the airways, (Figure, 6.3 A & B). After HDM exposure, there was an increase in the number of Annexin A1 positive cells in the peribronchial cellular infiltrate and parenchyma. Expression appeared restricted to mononuclear cells, with less intense expression in the airway epithelial and cells of the conducting airway, (Figure 6.3 C & D). 7 days after the last allergen challenge there was a significant reduction in the number of Annexin A1 positive cells, (Figure 6.3 E & F). However, expression on macrophages in the parenchyma remained higher than in PBS mice, (Figure 6.3 F). Expression of Annexin A1 was also quantified by RT-PCR, (Figure 6.3 H). Levels of mRNA transcripts in the lung tissue demonstrated an 8 fold increase at peak inflammation compared to PBS controls. Although, there was a significant reduction in expression at 7 days, levels remained 2 fold higher than in non-allergic controls.

6.3.2 FPR2 is expressed in alveolar macrophages, epithelium after HDM exposure and during resolution

The same sections were stained for localisation of FPR2 expression in the lung. In PBS mice, FPR2 positive cells were less frequent compared to Annexin A1. Expression was restricted to macrophages and at lower intensity on the epithelium (Figure 6.4 A & B). Following HDM challenge FPR2 expression was increased in epithelium and there was an increase in the intensity and number of alveolar macrophages (Figure 6.4 C & D). There was also clear staining of small mononuclear cells in the lower airway with close proximity to alveolar macrophages. At day 7 the intensity of the staining in the airway looked comparable to peak inflammation with the number of positive cells in the parenchyma remaining higher

compared to PBS (Figure 6.4 E & F). mRNA analysis revealed that FPR2 increased to 6 fold higher and almost 2 fold higher at 7days. FPR2 was expression in Annexin a1 KO mice was lower compared to WT mice (Figure 6.4G). This staining demonstrated that FPR2 is regulated by HDM exposure.

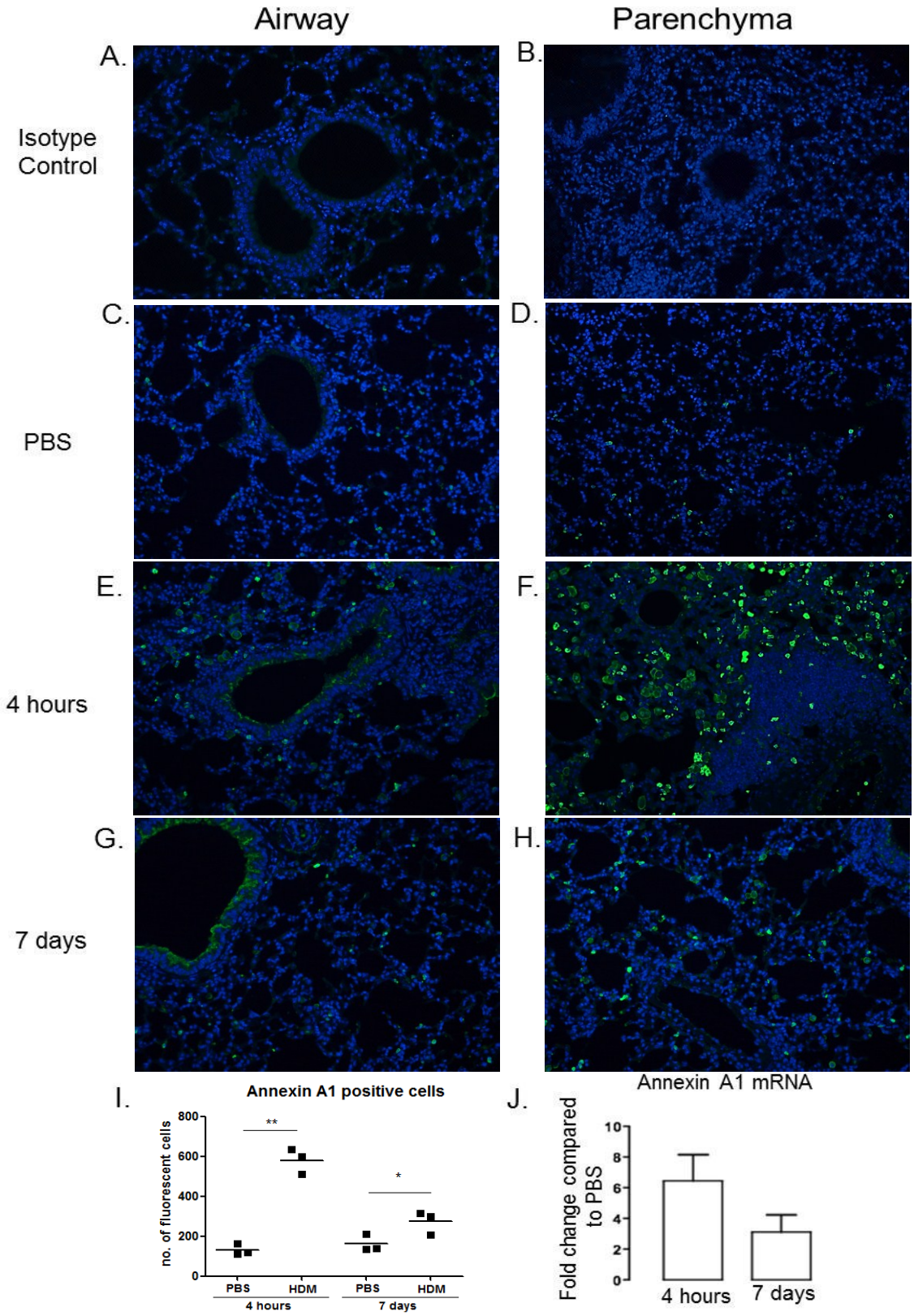


Figure 6.3 Annexin A1 expression in the airways and lung tissue. WT mice were challenged with HDM for 3 weeks and lung tissue taken at 4 hours and 7 days after last HDM challenge. Lung tissue was fixed and mounted sections were stained for localisation of Annexin A1 in the airways (A, C, E & G) and parenchyma (B, D F & H) in PBS and HDM treated mice. (I) Quantification of Annexin A1 positive cells calculated from total counts in 3 fields of airways and 3 fields of parenchyma IHC localisation. (J) mRNA expression of Annexin A1 in lung homogenate.

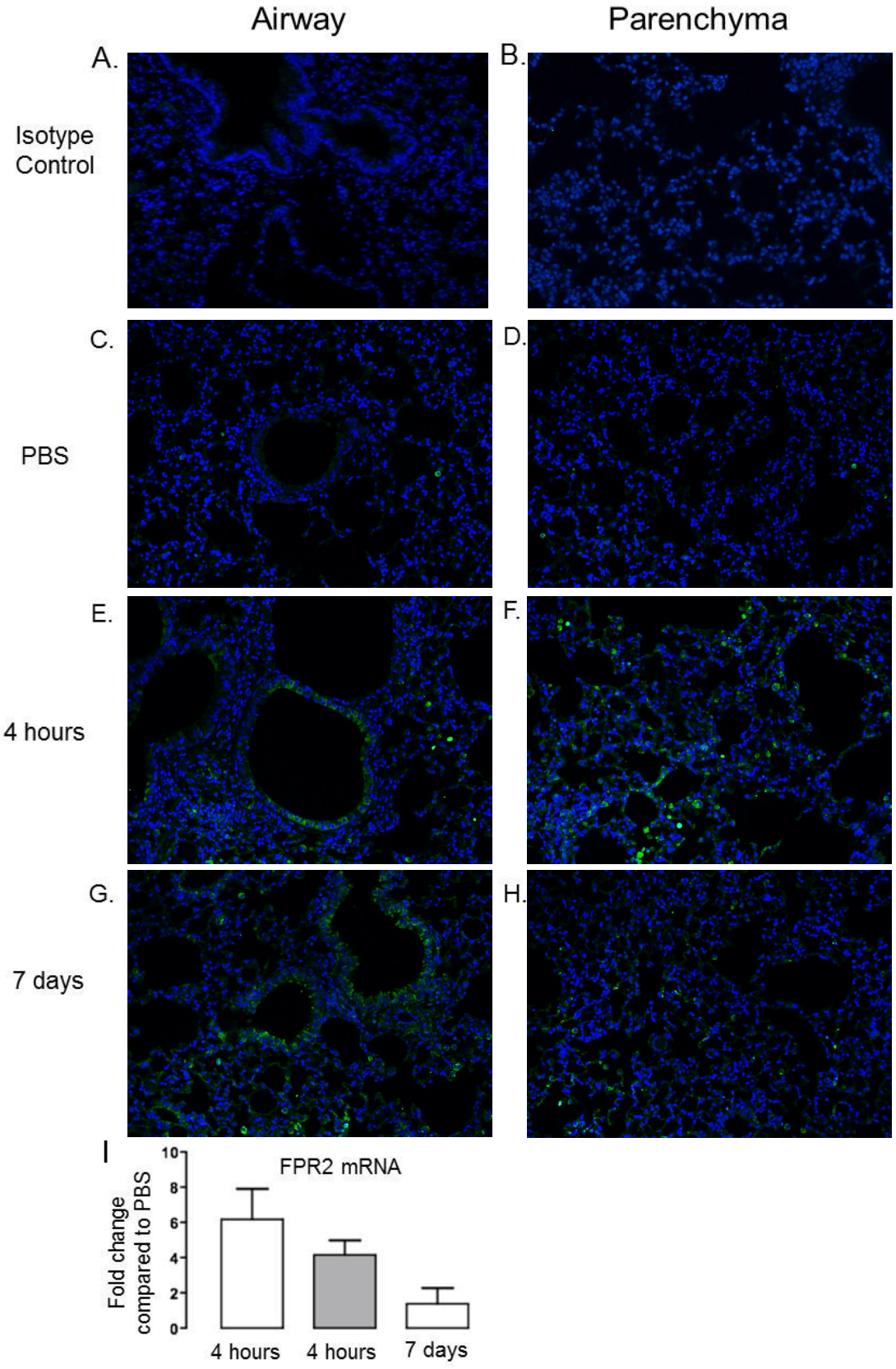


Figure 6.4 FPR2 expression in the airways and lung tissue. WT mice challenged with HDM for 3 weeks and lung tissue taken at 4 hours and 7 days after last HDM challenge. Lung tissue was fixed and mounted sections were stained for localisation of FPR2. (A, C, E) Airway sections and (B,D & F) parenchyma in PBS and HDM treated mice (G) mRNA expression of FPR2 in WT lung homogenate (white bars) and in Annexin A1 KO lung (grey bars).

6.3.3 Annexin A1 deficient mice exhibit elevated airway hyper-reactivity in response to HDM challenge

The functional role of Annexin A1 in the development of allergic inflammation was investigated using Annexin A1 KO mice. Mice deficient in Annexin A1 and challenged with HDM for 3 weeks showed exacerbated airway hyper-reactivity compared to WT controls. Airway resistance was significantly higher (Figure 6.5 A & B) while lung compliance was significantly reduced in Annexin A1 KO mice compared to HDM treated WT controls, (Figure 6.5 C & D). There appeared to be slightly higher resistance and lower compliance in non-allergic KO mice compared to WT controls but the effect was not significant.

6.3.4 Annexin A1 deficient mice have an enhanced allergic inflammatory response to HDM challenge

Total cell counts in the lung tissue and BAL were similar between WT and Annexin A1 KO mice, (Figure 6.6 A & B). In the lung tissue, Annexin A1 KO mice had higher eosinophils numbers compared to WT mice, (Figure 6.6 C). In the airway, Annexin A1 KO mice exhibited significantly more pronounced eosinophilia, (Figure 6.6 D). In contrast, there were no significant differences observed in neutrophil counts between WT and Annexin A1 KO mice, (Figure, 6.6 E & F). Flow cytometric analysis revealed that Th2 lymphocytes were significantly higher in the airway but not in the lung tissue of Annexin A1 KO compared to WT mice (Figure 6.6 G & H).

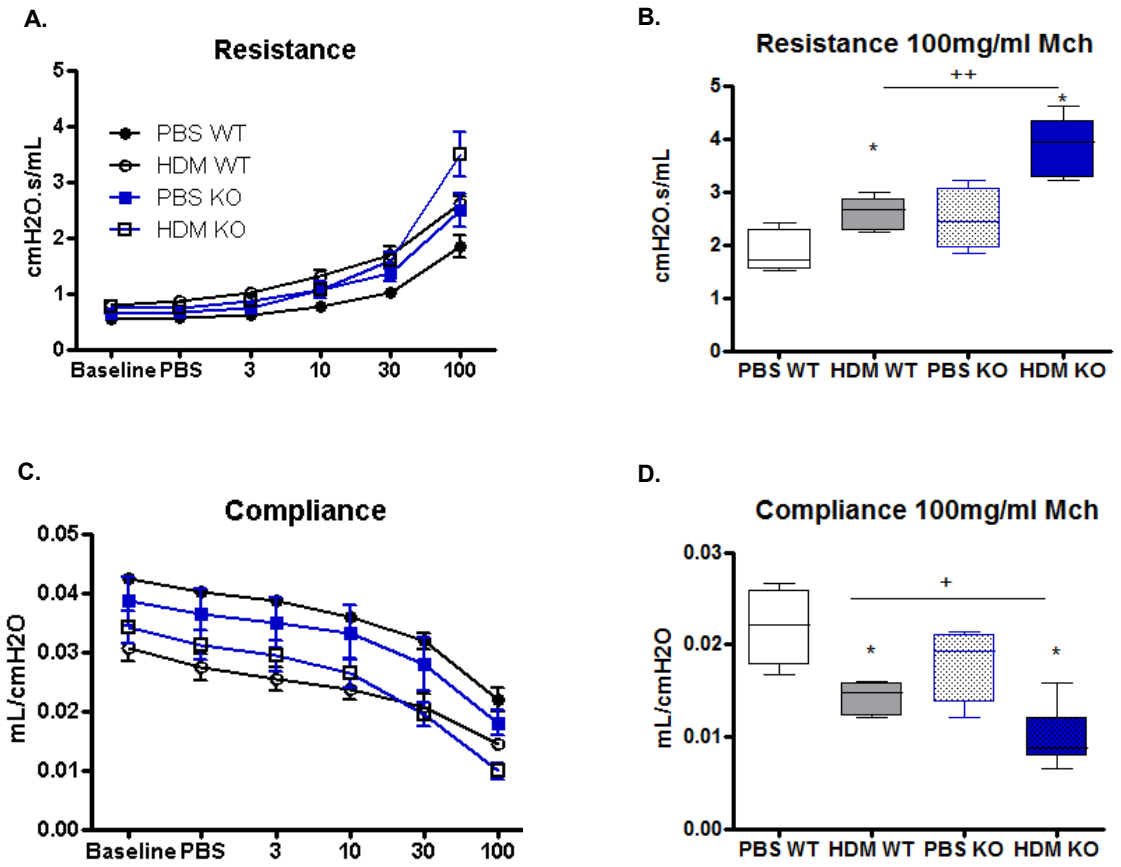


Figure 6.5 Annexin A1 deficient mice have exacerbated airway hyper-reactivity. (A) Airway resistance and (C) lung compliance in BALB/c WT and ANXA1 KO mice were measured in response to increasing doses of MCh. Measurements were made 4 hours after last HDM challenge. Mean levels of (B) resistance and (D) compliance at 100mg/ml MCh. n= 4-6 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ relative to PBS control group. $^{+}$ $p<0.05$ and $^{++}$ $p<0.01$ comparing HDM treated WT and KO group by Mann Whitney test.

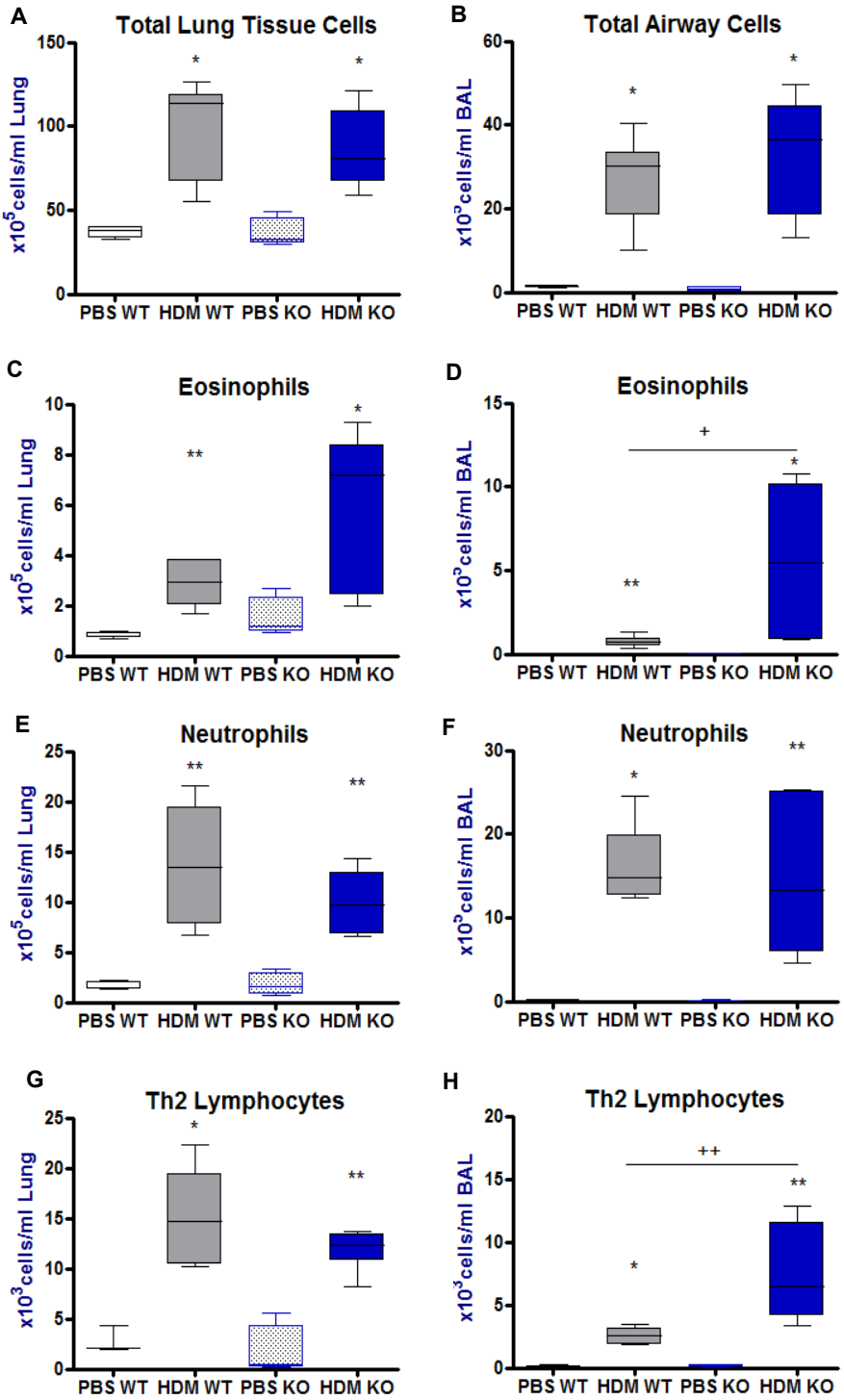


Figure 6.6 Eosinophils and Th2 lymphocytes are elevated in Annexin A1 KO mice Lung tissue and BAL was acquired at 4 hours after last HDM challenge. (A) Total lung tissue and (B) BAL cell counts (C&D) eosinophils (E&F) neutrophils (G&H) CD4+ T1/ST2+ lymphocytes were quantified by flow cytometry. n = 4-6 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ and ** $p<0.01$ relative to PBS control group. $^{+}$ $p<0.05$ and $^{++}$ $p<0.01$ comparing HDM treated WT and KO group by Mann Whitney test

6.3.5 Annexin A1 deficient mice have decreased levels of IL-17 secreting innate lymphoid cells

Innate lymphoid cells have been described to express FPR2, therefore we determined whether numbers of ILCs in our model would be affected by a deficiency in Annexin A1. ILCs are defined as Lin-CD45+ICOS+ and were quantified by flow cytometry. ILCs in the lung were significantly induced following HDM exposure (Figure 6.7A). There was a reduction in total ILCs in Annexin A1 deficient mice, however, this was not found to be statistically significant (Figure 6.7 A). Similarly, IL-33 responsive, T1/ST2+ ILCs were induced by HDM but were not significantly different in Annexin A1 KO mice, (Figure 6.7 B). Although there was a slight increase in IL-13+ ILCs (ILC2s) in HDM treated Annexin A1 KO mice compared to HDM treated WT mice this was not significantly different (Figure 6.7 C). Interestingly, the numbers of IL-17+ ILCS (ILC3s) were shown to be significantly decreased in HDM treated Annexin A1 KO mice compared to WT, (Figure 6.7D). This may suggest a regulatory role for IL-17+ ILCs in this model and their generation may be Annexin A1 dependent.

6.3.6 Annexin A1 deficient mice have decreased levels of interstitial macrophages

Immunohistochemical staining suggested that in the lung, macrophages were the predominant cell type expressing Annexin A1. Therefore, the numbers of both alveolar (AM) and interstitial macrophages (IM) were determined by flow cytometry. As previously described, numbers of AMs were not affected by HDM treatment (Figure 3.9). Numbers of AMs were not found to be significantly different in Annexin A1 KO mice compared to WT, (Figure 6.8 A.) However, IMs measured in the lung tissue were shown to be reduced in HDM treated Annexin A1 deficient mice compared to HDM treated WT mice, (Figure 6.8 B). This suggests that Annexin A1 may be important in regulating the tissue macrophage population.

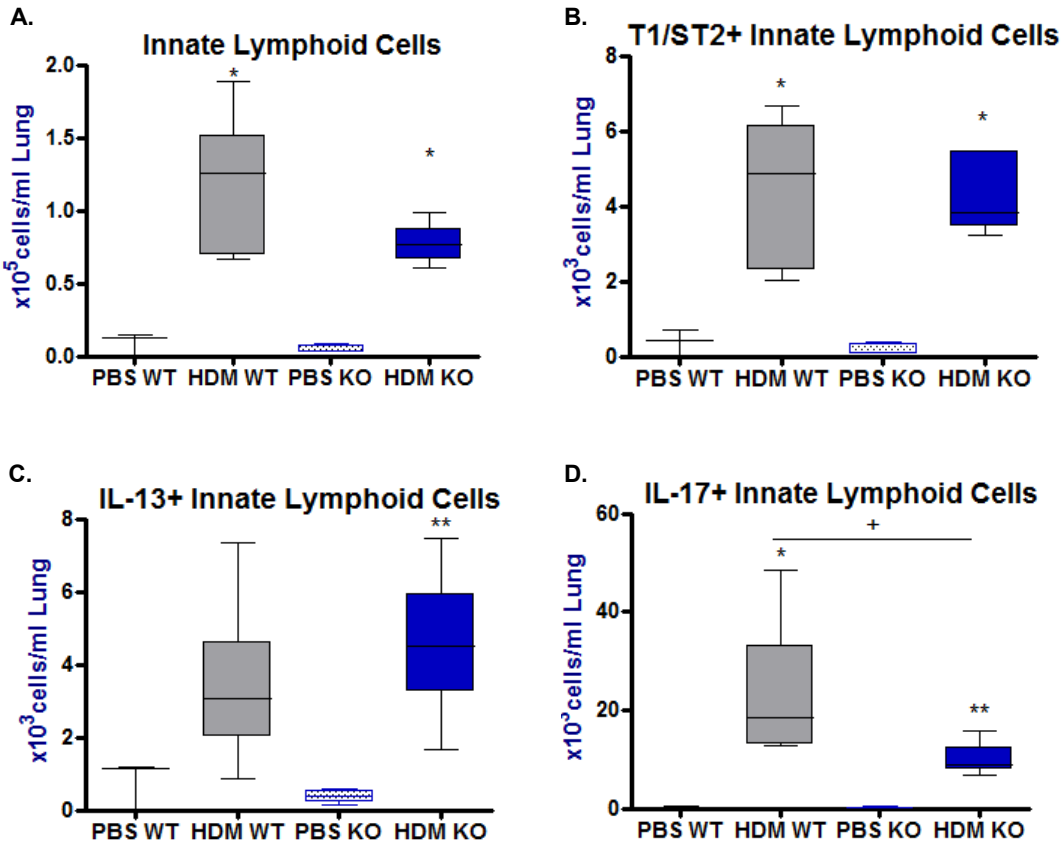


Figure 6.7 Innate lymphoid cells in WT and Annexin A1 KO mice Lung tissue was acquired at 4 hours after last HDM challenge. (A) Total ILCs (B) T1/ST2+ ILCs (C) IL-13+ ILCs (D) IL-17+ ILCs were quantified by flow cytometry. n = 3-6 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ and ** $p<0.01$ relative to PBS control group. $^{+}$ $p<0.05$ comparing HDM treated WT and KO group by Mann Whitney test

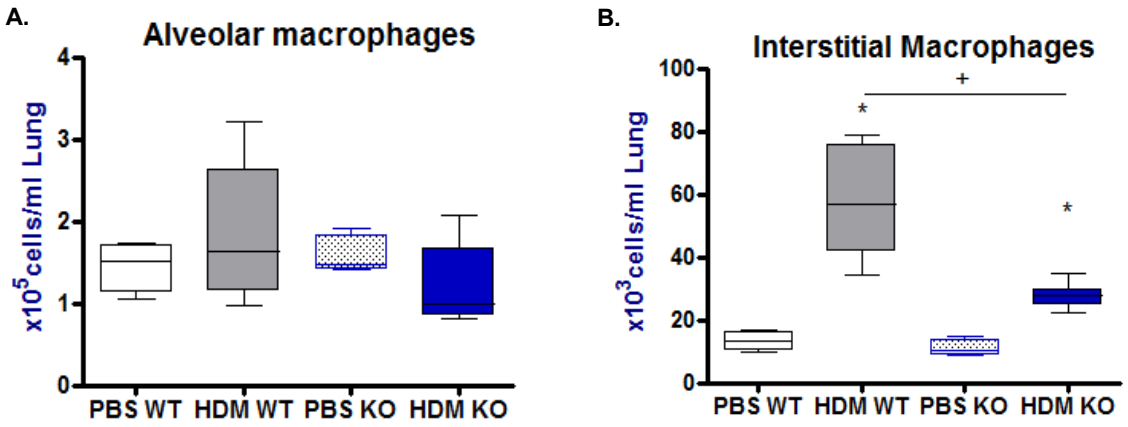


Figure 6.8 Interstitial macrophages are reduced in Annexin A1 KO mice Lung tissue was acquired at 4 hours after last HDM challenge. (A) Total CD68+ CD11c+ alveolar macrophages (B) CD68+ CD11c- CD11b+ interstitial macrophages were quantified by flow cytometry. n = 4-6 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ relative to PBS control group. $^{+}$ $p<0.05$ comparing HDM treated WT and KO group by Mann Whitney test

6.3.7 Annexin A1 deficient mice have elevated levels of Th2 cytokines in response to HDM

Measurements of lung tissue cytokines following HDM exposure showed an increase in the Th2 immune response in Annexin A1 KO mice compared to HDM treated WT. IL-4 was elevated in the lung tissue of Annexin A1 KO compared to WT, however, this did not reach significance, (Figure 6.9 A). Interestingly, the elevated eosinophils and AHR observed in the HDM treated Annexin A1 deficient mice was supported by significantly increased levels of both the eosinophil survival factor IL-5 and IL-13, which has a role in initiating and maintaining AHR (Figure 6.9 B & C). In contrast, levels of IL-33 were comparable between WT and ANXA1 KO mice following HDM exposure. (Figure 6.9 D). These data indicated that mice deficient in ANXA1 present a skewed Th2 immune response following HDM challenge.

6.3.8 Regulatory cytokines are elevated in Annexin A1 KO mice

To determine if there were any effects of Annexin A1 deficiency on endogenous regulatory cytokines lung tissue levels of IL-10 and IL-27 were measured. In PBS treated mice the classic anti-inflammatory cytokine IL-10 was found to be higher in Annexin A1 KO compared to WT mice, (Figure 6.9 E). Levels of IL-10 following HDM challenge were also elevated in the KO mice compared to WT mice. A newly described regulatory cytokine, IL-27 was also quantified in the lung tissue. IL-27 is a member of the IL-12 cytokine family and has been described to down regulate Th2 lymphocyte polarisation (Yoshimoto, Yoshimoto et al. 2007). Following HDM exposure, there was a significant increase in IL-27 levels in both WT and Annexin A1 KO mice. The allergen induced increase in pulmonary IL-27 was significantly greater in the Annexin A1 KO mice compared to WT (Figure 6.9F). These results show that there is a shift in baseline levels of IL-10 in the ANXA1 KO mice and in response to allergen challenge the Annexin A1 KO mice produced higher levels of these regulatory cytokines.

6.3.9 Chemokines are unaltered in Annexin A1 deficient mice following HDM challenge

To determine if the exaggerated levels of inflammation exhibited in Annexin A1 KO mice was associated with increased chemokines in the lung, levels of key chemokines associated with the allergic response, were quantified by ELISA. At baseline, there were no significant differences between WT and KO mice in any of the chemokines measured. Allergen challenge induced pulmonary production of KC/CXCL1, TARC/CCL17, MCP1/CCL2, MDC/CCL22, Eotaxin1/CCL11 & Eotaxin2/CCL24 in both WT and KO mice, (Figure 6.10 A-F) and there was no difference in the magnitude of the response between the two groups.

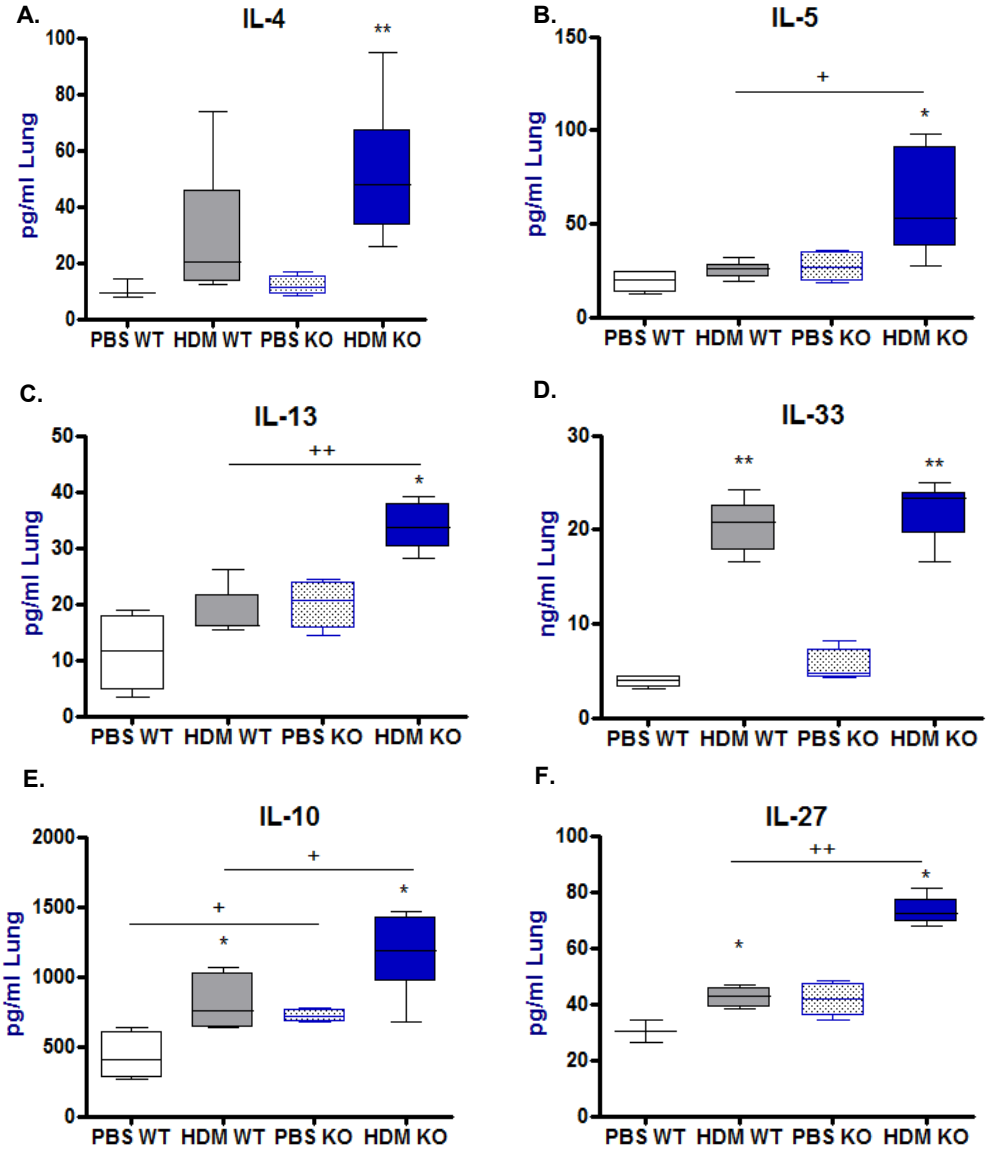


Figure 6.9 Lung tissue cytokines are elevated in Annexin A1 KO mice. Lungs were taken at 4 hours following 3 weeks of HDM challenge. Cytokines were quantified in lung homogenate by ELISA. (A) IL-4 (B) IL-5 (C) IL-13 (D) IL-33 (E) IL-10 (F) and (G) IL-27. n = 4-8 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ and ** $p<0.01$ relative to PBS control group. $^{+}$ $p<0.05$ and $^{++}$ $p<0.01$ comparing HDM treated WT and KO group by Mann Whitney test

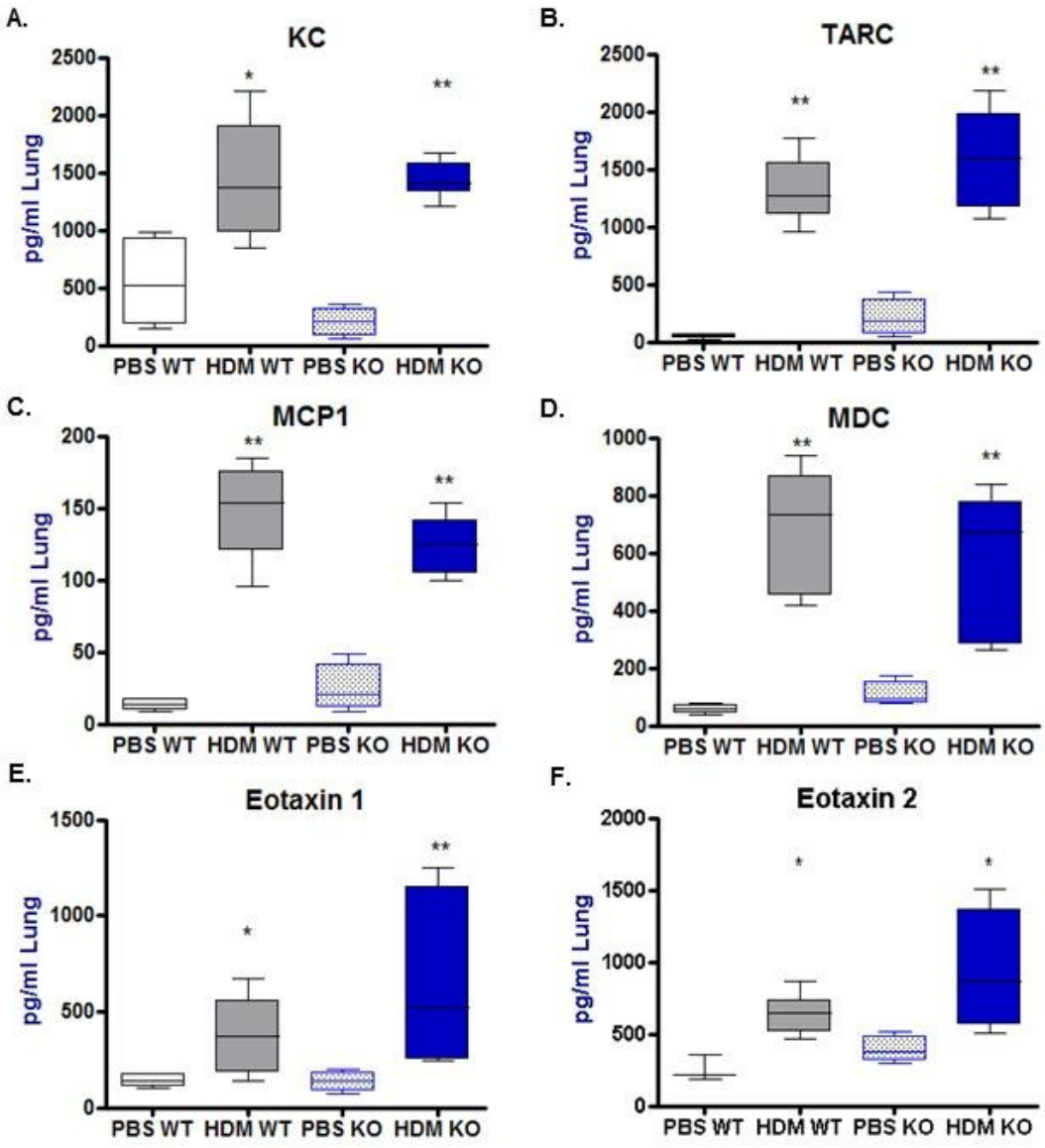


Figure 6.10 Lung tissue chemokines are unaltered in Annexin A1 KO. Lungs were taken at 4 hours following 3 weeks of HDM challenge in WT and Annexin A1 KO mice. Control mice received PBS. Chemokines were quantified in lung homogenate by ELISA. (A) KC/CXCL1 (B) TARC/CCL17 (C) MCP1/CCL2 (D) MDC/CCL22 (E) Eotaxin1/CXCL11 and (F) Eotaxin 2/CCL24. n = 3-8 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ and ** $p<0.01$ relative to PBS control group by Mann Whitney test.

6.3.10 Eicosanoid production in the airway and lung tissue

Annexin A1 is an inhibitor of the enzyme Phospholipase A2 (PLA2), which hydrolyzes membrane phospholipids to release Arachidonic Acid (AA). Thus eicosanoids synthesised from AA were measured in Annexin A1 deficient mice following HDM exposure. In collaboration with Val O'Donnell at Cardiff University, lipids derived from the arachidonic acid pathway, via both cyclooxygenase (COX) and lipoxygense (LOX) enzymatic pathways were measured in lung by LS/MS/MS. In the lung, PGD2 & PGE2 were induced following HDM exposure, (Figure 6.11 A & B). At baseline, PGE2 levels were significantly higher in PBS KO compared to PBS treated WT mice. Following HDM exposure, the increase in pulmonary PGE2, but not PGD2, in the KO mice was significantly greater than in WT mice (Figure 6.11 A & B). LTB4 was not modulated following HDM in WT mice, however, significantly increased levels were induced in Annexin A1 KO mice (Figure, 6.11 C). LXA4 was only detectable at very low levels in the lung in this study compared to values obtained from the study in Chapter 3. There was little induction of LXA4 following HDM but at baseline there was some indication of increased levels in the KO compared to WT mice (Figure, 6.11 D). LOX derived lipids also include the family of hydroxyeicosatrienoic acids (HETES). In Chapter 3, HDM exposure in WT BALB/c induced a significant increase in 5, 8, 11, 12 and 15-HETE (Figure 4.2 C-G). However, in this study, HDM exposure resulted in an increase in only 11 and 15-HETE in WT mice. In Annexin A1 deficient mice, enhanced pulmonary production of 8, 11, 12 and 15-HETE in response to HDM was observed compared to WT treated mice, (Figure 6.11 F-I)

These data show that lipids from both the COX and LOX pathways are rapidly induced following HDM challenge. The deletion of ANXA1 has specific effects on these lipid pathways with key mediators increased in the lung.

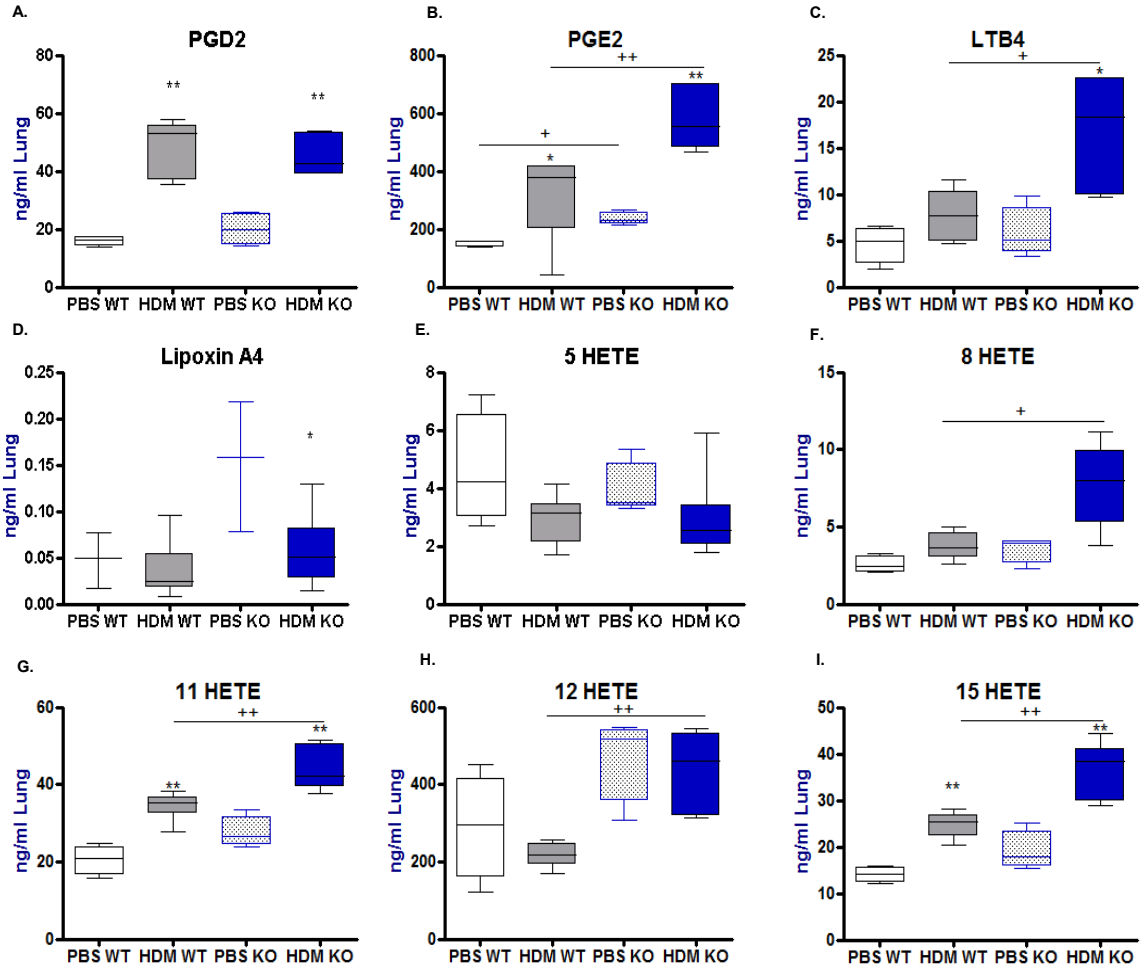


Figure 6.11 Eiconasoids induced in lung tissue are elevated in Annexin A1 KO. Lung was acquired at 4 hours after HDM challenge in WT and KO mice. Levels of (A) PGD2, (B) PGE2, (C) LTB4, (D) LXA4, (E) 5-HETE, (F) 8-HETE, (G) 11-HETE, (H) 12-HETE, (I) 15-HETE were extracted and quantified by LS/MS/MS n = 4-6 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ and ** $p<0.01$ relative to PBS control group. $^{+}$ $p<0.05$ and $^{++}$ $p<0.01$ comparing HDM treated WT and KO group by Mann Whitney test

6.3.11 Bone marrow derived macrophages from Annexin A1 KO mice express higher levels of cytokines and chemokines compared to WT mice.

To determine if macrophage specific expression of Annexin A1 was important to macrophage function, bone marrow from WT and KO mice were cultured for 10 days with either GM-CSF or M-CSF to derive macrophages of either M1 or M2 phenotype, respectively. M1 macrophages have a pro-inflammatory phenotype and are characterised by iNOS, secreting of IFNγ and IL-12. M2 macrophages are characterised by Arg1 and induced by IL-4 and IL-13. These bone marrow derived macrophages (BMDMs) were then exposed to HDM or LPS and the supernatant harvested at 4 hours, 24 hours and 40 hours after stimulation for quantification of secreted chemokines and regulatory cytokines. This method was employed to give an indication of macrophage derived cytokine and chemokine production that may occur in lung tissue following HDM exposure.

BMDMs responded to LPS challenge by secreting the regulating cytokines IL-10 and IL-27 within 24 hours (Figure 6.12 A-D). Levels of the cytokines were further increased after 48 hours. M1 like macrophages deficient in Annexin A1 produced elevated levels of IL-10 and IL-27 when stimulated with LPS compared to WT (Figure 6.12 A & C). M2 macrophages also responded to LPS by secreting IL-10 and IL-27 (Figure 6.12 B & D) and at higher concentrations than M1 macrophages. However, unlike M1 macrophages, in M2 macrophages Annexin A1 deficiency did not modulate secretion of these regulatory cytokines.

Basal secretion of the chemokines MDC/CCL22 and TARC/CCL17 was measured in both M1 and M2 macrophage cultures (Figure 6.12 E-G). M1 macrophages produced predominantly MDC/CCL22 whereas M2 macrophages preferentially synthesised higher levels of TARC/CCL17 (above the limit of detection) (Figure 6.12 E-G). MDC/CCL22 secretions by M1 macrophages was not modulated in response to HDM or LPS but levels of this cytokine were consistently higher in cultures derived from Annexin A1 deficient mice compared to WT. M2 macrophage cultures which had lower levels of MDC/CCL22 secretion

responded to LPS by increasing MDC/CCL22. Levels of MDC/CCL22 peaked at 48 hours in the WT mice and earlier, 24 hours, in the KO mice. TARC/CCL17 secreted by M1 macrophages was not regulated by LPS or HDM. Levels of TARC/CCL17 from M2 like macrophages were above the limit of detection. These studies show that macrophages deficient in Annexin A1 have higher chemotactic potential suggesting that Annexin A1 may work to limit Th2 cellular recruitment.

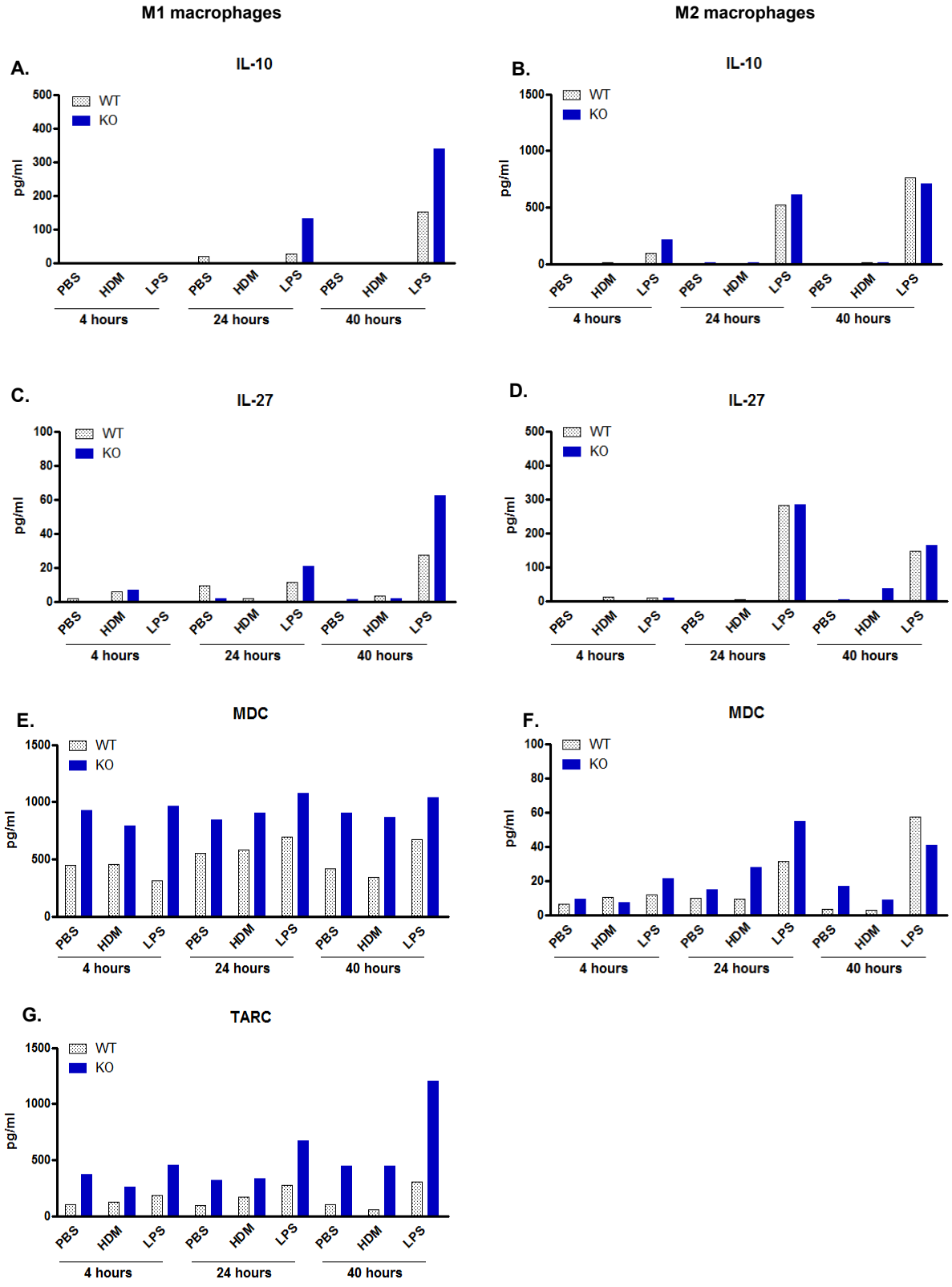


Figure 6.12 Cytokine and chemokine production of bone marrow derived macrophages. M1 and M2 BMDMs were cultured with either HDM or LPS. Supernatant was collected after 4 hours, 24 hours and 40 hours after incubation. Measurements of cytokines and chemokines were carried out by ELISA. n = 2 mice/group, bar represents mean.

6.3.12 Pharmacological manipulation of FPR2 receptor significantly modulated AHR

BALB/c mice treated with HDM for 3 weeks received the FPR2 receptor antagonist WRW4 or the ANXA1 mimetic, AnxA2-50 daily for the final week of challenge. Treatment with WRW4 or AnxA2-50 did not show any baseline changes in lung function parameters in PBS exposed mice. HDM exposed mice treated with the WRW4 antagonist exhibited a significant increase in airway resistance. Conversely, a reduction in airway resistance was observed in HDM exposed mice with the agonist AnxA2-50, (Figure 6.13 A & B). Neither treatment with antagonist or agonist affected lung compliance (Figure 6.13 C & D). This study suggests that Annexin A1 acting via FPR2 receptor is important in regulating allergen induced airway resistance.

6.3.13 Therapeutic treatment with AnxA2-50 improves lung inflammation

Administration of WRW4 or AnxA2-50 did not affect total cell counts in the airway lumen and levels of cell recruitment were similar between all HDM treated groups, (Figure 6.14 A). However, lung tissue leukocytes were significantly altered following therapeutic intervention, (Figure 6.14 B). Total cell counts were significantly elevated in mice which received WRW4, the FPR2 receptor antagonist. In contrast, a significant reduction in pulmonary inflammation was observed in mice which received the agonist AnxA2-50. Differential cell counts in the BAL and lung showed a trend for an increase in neutrophils and Th2 lymphocytes following antagonist treatment and a reduction after agonist treatment, however, these did not reach statistical significance, (Figure, 6.14 C-H).

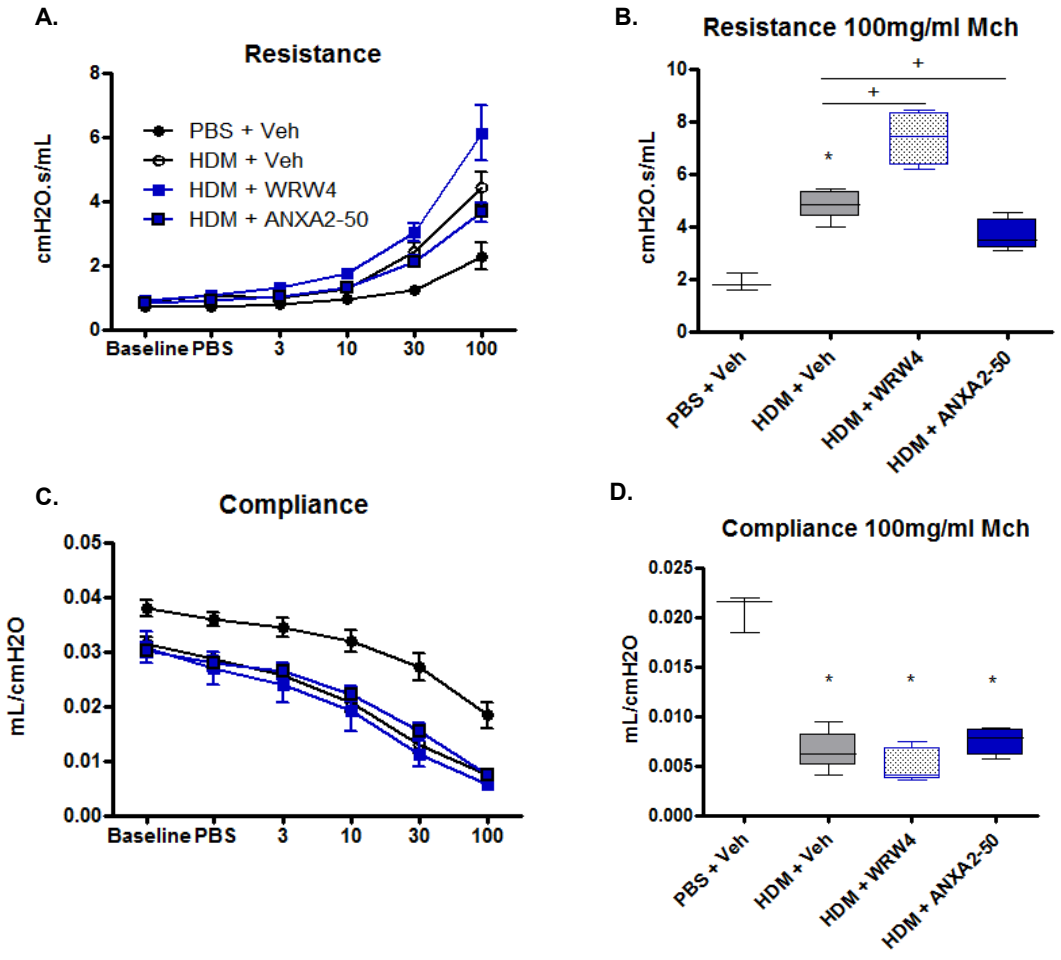


Figure 6.13 Treatment with WRW4 antagonist and ANXA2-50 alters airway resistance in HDM challenge mice. (A) Airway resistance and (C) lung compliance in HDM treated BALB/c WT mice were measured in response to increasing doses of MCh, Measurement were made 4 hours after last HDM challenge. Control mice received PBS. Mean levels of (B) resistance and (D) compliance at 100mg/ml MCh. n = 3-6 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ relative to PBS + Veh control group. + $p<0.05$ comparing treatment with HDM + Veh control group by Mann Whitney test.

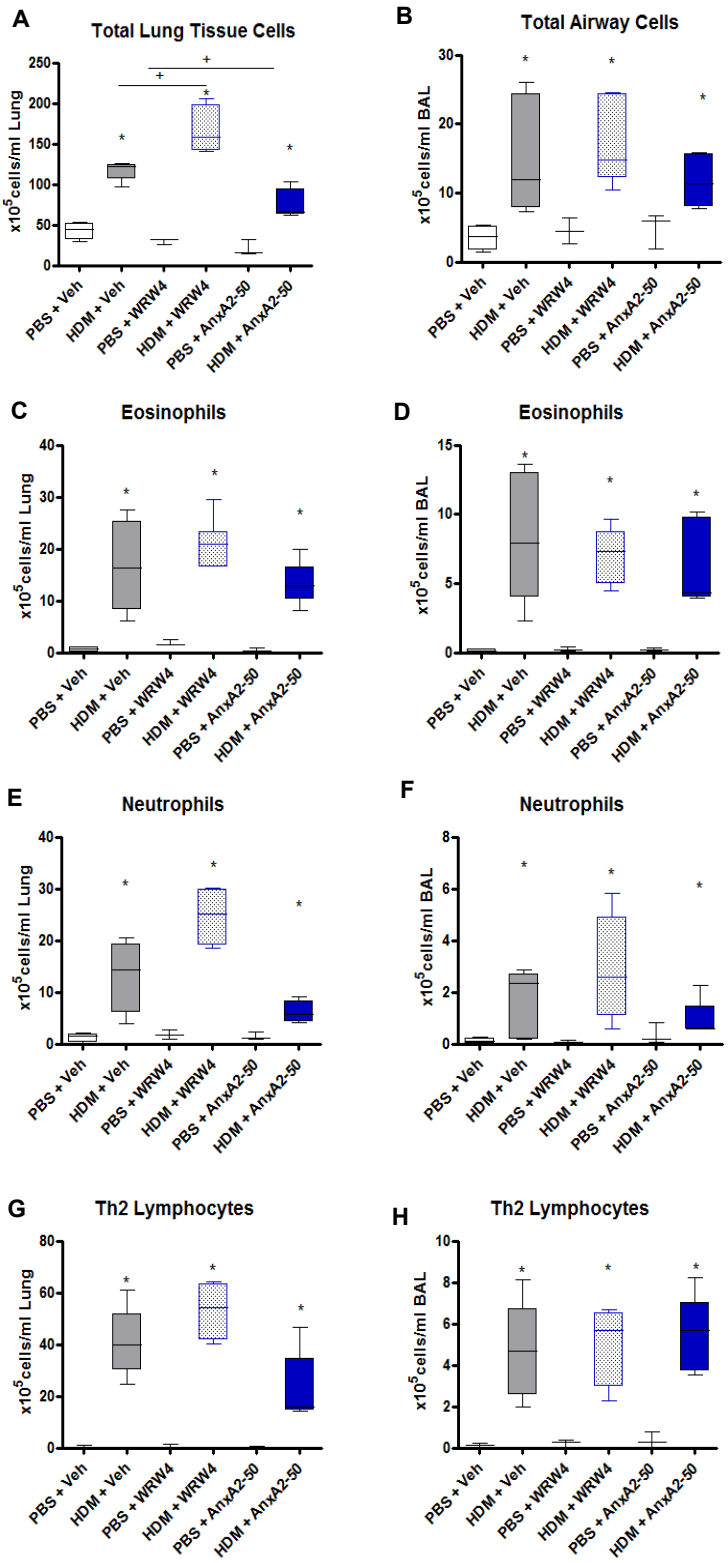


Figure 6.14 Lung tissue leukocytes are decreased following treatment with ANXA2-50.BAL and lung tissue were acquired at 4 hours after last HDM challenge. (A) Total lung cells (B) total airway cells (C) lung eosinophils (D) BAL eosinophils (E) lung neutrophils (F) BAL neutrophils (G) airway CD4+ T1/ST2+ lymphocytes and (H) CD4+ T1/ST2+ lymphocytes were quantified by flow cytometry. n = 3-6 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ relative to PBS + Veh control group. + $p<0.05$ comparing treatment with HDM + Veh control group by Mann Whitney test.

6.3.14 Pharmacological manipulation with AnxA2-50 promotes a reduction in innate lymphoid cells

Numbers of innate lymphoid cells were quantified by flow cytometry following WRW4 and AnxA2-50 administration. Total numbers of Lin-CD45+ICOS+ ILC cells in the lung following HDM remained unchanged in WRW4 treated group (Figure 6.15 A).However, ILCs were significantly reduced in the AnxA2-50 treatment group (Figure 6.15 A) although they did remain significantly higher compared to PBS control. It was also observed that there was no specific reduction in either the IL-13+ (ILC2) or IL-17+ (ILC3) subpopulations (Figure 6.15 B & C). This suggests that ILCs may be specifically regulated by Annexin A1. Further investigation of other ILC populations is required.

6.3.15 Pharmacological manipulation with either WRW4 or AnxA2-50 did not affect alveolar and interstitial macrophage populations.

Alveolar and interstitial macrophage populations measured in the lung showed no significant changes n numbers following pharmacological intervention with either WRW4 and AnxA2-50, (Figure 6.16 A & B).

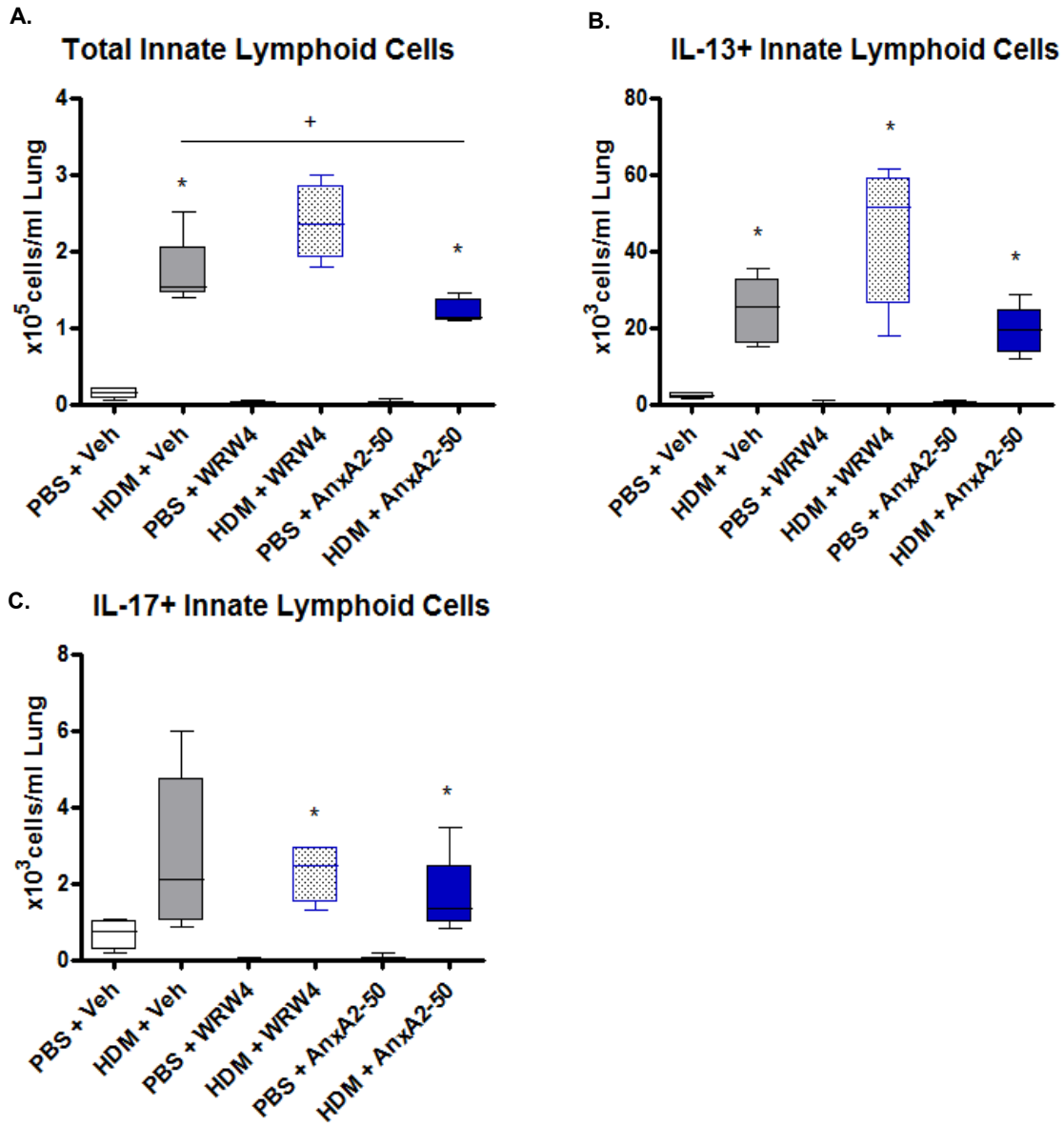


Figure 6.15 Innate lymphoid cells were significantly decreased following AnxA2-50 treatment. Lung tissue was acquired 4 hours after last HDM challenge. (A) Total ILCs (B) IL-13+ ILCs (C) IL-17+ ILCs were quantified by flow cytometry. n = 4-6 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ relative to PBS + Veh control group. + $p<0.05$ comparing treatment with HDM + Veh control group by Mann Whitney test.

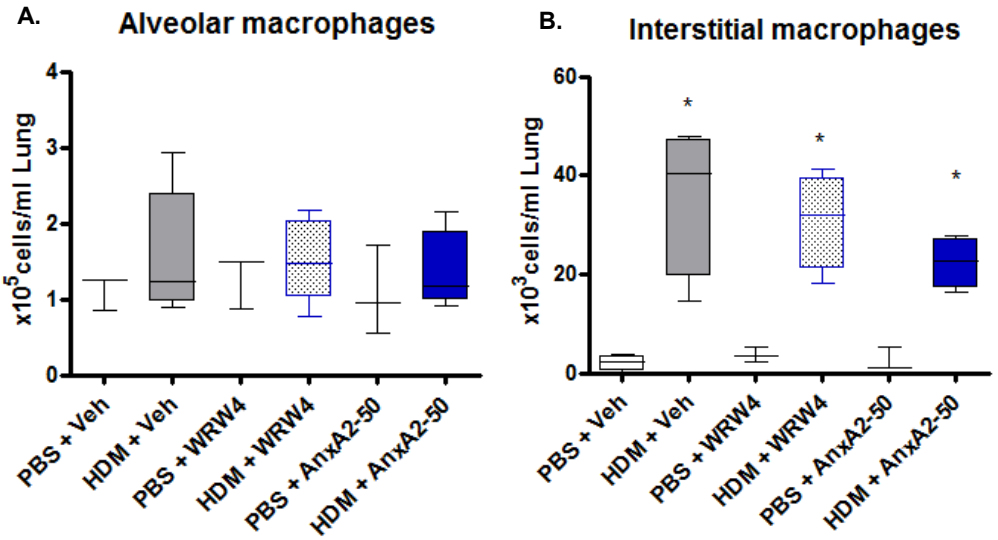


Figure 6.16 Alveolar macrophages and interstitial macrophages were not affected following WRW4 and AnxA2-50 treatment Lung tissue was acquired at 4 hours after last HDM challenge. (A) Total CD68+ CD11c+ alveolar macrophages (B) CD68+ CD11c- CD11b+ interstitial macrophages were quantified by flow cytometry.n = 3-6 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ relative to PBS + Veh control group by Mann Whitney test

6.3.16 Pharmacological manipulation with FPR2 antagonist WRW4 and agonist AnxA2-50 influences lung tissue Th2 cytokine production.

Measurement of lung tissue cytokines in HDM treated mice which received the Annexin A1 mimetic AnxA2-50 reveal a significant decrease in IL-4 and IL-5 compared to HDM + Veh control group, (Figure 6.17 A & B). In fact levels of these Th2 cytokines were reduced to baseline. However, there were no significant effect on lung tissue IL-13 and IL-33 following agonist administration, (Figure 6.17 C & D). Treatment of mice with the FPR2 antagonist resulted in corresponding elevation of IL-4, IL-13 and IL-33 levels in response to HDM, (Figure 6.17 A, C & D). This correlated with increased airway resistance in these mice. IL-5 levels were unaffected following treatment with antagonist, (Figure 6.17 B). Lung tissue levels of IL-10 were unaltered following WRW4 administration. However, there was a significant reduction to baseline levels following AnxA2-50 treatment. This data suggest that therapeutic intervention at the ANXA1: FPR2 axis can directly influence pulmonary Th2 immunity and consolidate the role for Annexin A1 as resolution mediator of Th2 inflammation.

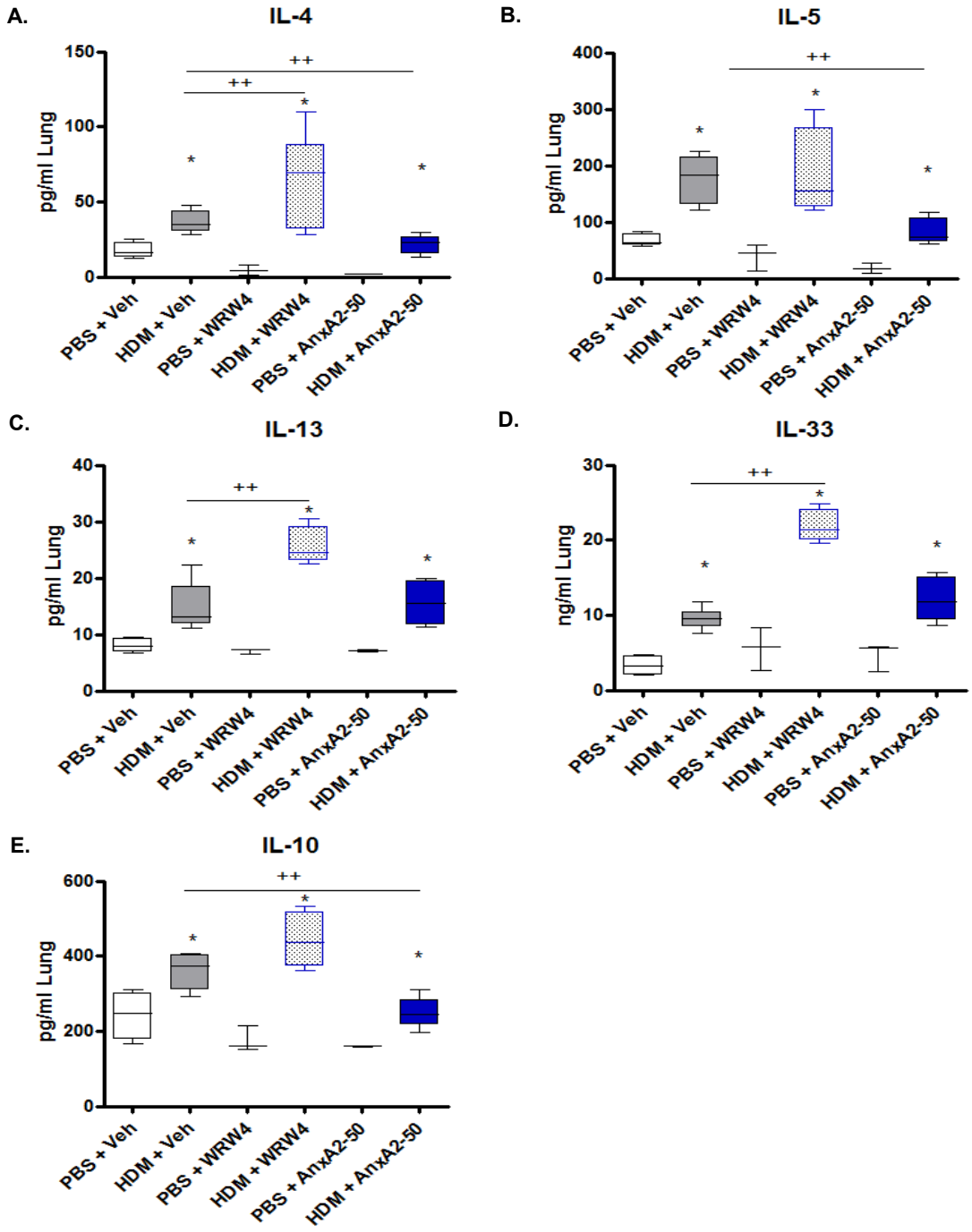


Figure 6.17 Lung tissue cytokines following treatment with WRW4 and ANXA2-50
Lung tissue was acquired at 4 hours after last HDM challenge. Cytokines were quantified in lung homogenate by ELISA (A) IL-4 (B) IL-5 (C) IL-13 (D) IL-33 and (E) IL-10. n = 3-6 mice/group, plots depict the median and interquartile range and minimum and maximum values. * $p<0.05$ relative to PBS + Veh control group. + $p<0.05$ and ++ $p<0.05$ comparing treatment with HDM + Veh control group by Mann Whitney test.

6.4 Discussion

The results in this chapter demonstrate that the Annexin A1-FPR2 axis is required to control airway hyper-reactivity and allergic inflammation. Annexin A1 is a potent anti-inflammatory protein with pro-resolving functions.

At baseline, Annexin A1 was found to be expressed predominantly by alveolar macrophages, with lower expression in airway epithelium. Following HDM exposure, there was an increase in the number Annexin A1 positive cells. This remained above baseline 7 days after last exposure to HDM with increased expression specific to alveolar macrophages. mRNA levels of Annexin A1 also demonstrated an 8 fold induction following HDM that remained increased 7 days after last allergen challenge. The Annexin A1 receptor FPR2 also displayed a similar profile. The functional role of Annexin A1 in allergic airways inflammation was investigated. Annexin A1 deficient mice sensitised and challenged with HDM for 3 weeks exhibited exacerbated airway hyper-responsiveness, eosinophilia and an increase the numbers of Th2 lymphocytes. This aggravated pathology was supported by a significant induction of Th2 cytokines in the Annexin KO mice. These data demonstrate that Annexin A1 plays an important role in regulating HDM induced disease. To determine if Annexin A1 deficient macrophages had an intrinsic phenotypic change, bone marrow derived macrophages from WT and Annexin A1 KO were stimulated with HDM. Following stimulation, Annexin A1 deficient macrophages secreted larger amounts of MDC/CCL22 and TARC/CCL17 compared to WT macrophages. This suggests a mechanism by which Annexin A1 deficient mice exhibit worse disease. These results reveal that Annexin A1 may have a role in regulating the recruitment of Th2 lymphocytes. It was also observed that a deficiency in Annexin A1 promoted an increase in the regulatory cytokines IL-10 and IL-27, both in the lung tissue in vivo and in vitro from bone marrow derived M1 like macrophages following HDM and LPS stimulation. This may seem counter intuitive as the knockouts exhibited a hyper inflammatory phenotype. It has also been reported that Annexin A1 can mediate its anti-inflammatory function via induction of IL-10 and mice deficient in IL-10 do

not respond to Annexin A1 or LXA4 induced resolution (Souza, Fagundes et al. 2007). However, the results here do suggest that there may be an enhancement of these anti-inflammatory pathways in an attempt to restrain enhanced Th2 inflammation and to compensate for the disruption of the Annexin A1 gene. Baseline increases in IL-10 in PBS mice demonstrated an underlying alteration during resting homeostasis in AnnexinA1 KO mice which may predispose to an exacerbated allergic inflammatory phenotype. Moreover it was found that that IL-17+ ILCs were significantly lower in HDM treated Annexin A1 KO mice compared to WT. There is nothing known about IL-17+ ILCs in the lung but it is speculated that IL-17 may have a regulatory function in AAD (Murdoch and Lloyd 2009). Therefore, perhaps the exacerbated pathology observed in Annexin A1 deficient mice is related to the IL-17+ ILC population.

Interestingly, the effect of ANXA1 deficiency was not global. Levels of total leukocytes in the airway and lung were not changed but the effects were restricted to AHR, Th2 lymphocytes, eosinophils and IL-13. A link between Annexin A1 and AHR has been described previously (Ng, Wong et al. 2011). This paper showed that unsensitised Annexin A1 deficient mice had higher basal levels of IgE and responded higher to methacholine challenge and showed enhance AHR following OVA induced AAD. They proposed that this may be a susceptibility factor for asthmatics. Whilst we saw exacerbated disease in the KO we did not see any basal levels differences in AHR. This may be due to the differences between lung function methods utilised. This paper measured changes in conscious and unconscious mice using chamber plethysmographs. These are enclosed chambers where changes in air pressure are transformed into resistance and compliance values. This is different from our method where we used forced manoeuvres via a ventilator. These R&C values are calculated through difference equations and therefore not directly comparable with each other. This may be why we do not see a significant change at baseline.

To confirm the role of Annexin A1 in AHR and Th2 inflammation we used a therapeutic intervention in HDM challenged BALB/c mice that received either FPR2 antagonist (WRW4)

or an Annexin A1 mimetic (AnxA2-50) during the last week of HDM exposure. Treatment with WRW4 reflected the results seen in HDM treated Annexin A1 KO mice, with an increase in AHR and Th2 pathology. Conversely, the administration of AnxA2-50 reduced lung function. Although there was a trend for a decrease in lung tissue eosinophils and Th2 lymphocytes following AnxA2-50 treatment, this was not significant. However, levels of ILCs in the lung tissue were reduced in mice receiving AnxA2-50 treatment. This reduction was not attributed to either IL-13 or IL-17 secreting populations. Barnig and colleagues demonstrated FPR2 expression by ILCs and postulated that LXA4 could directly interact and down regulate ILCs secreting IL-13. It is interesting to note that in this present study the FPR2 antagonist WRW4 did not have an overall effect on ILC cell numbers. Although ILCs have a role in promoting Th2 inflammation, it is now emerging that a subpopulation of ILCs may have a regulatory role at mucosal surfaces (Hepworth, Monticelli et al. 2013). Further analysis of this population is required.

Annexin A1 is highly expressed by neutrophils making up to 4% of their total cytosol products (Perretti and Flower 2004). Therefore it was interesting to note that in Annexin A1 KO mice there were no differences in neutrophil numbers. During models of peritonitis and ischaemic perfusion Annexin A1 has been shown to have direct effects on neutrophil recruitment by inhibiting adhesion and transmigration and promoting neutrophil apoptosis (Perretti and D'Acquisto 2009). Neutrophils are a component of the HDM inflammatory response so it was surprising not to see an effect of Annexin A1 deficiency in this compartment of the immune response. However, in the therapeutic protocols there appeared to be an effect on neutrophil numbers following WRW4 antagonist and AnxA2-50 administration. Although the effects were not significant, there was a clear trend towards an increase after WRW4 treatment suggesting that blocking Annexin A1 signalling prolonged neutrophil survival or recruitment to the lung tissue. Conversely, the decrease in neutrophil numbers observed in mice receiving AnxA2-50 alludes to a promotion of neutrophil apoptosis. It has been suggested that neutrophils can regulate Annexin A1 in the human

lung by the production of elastase which cleaves the Annexin A1 peptide. (Smith, Tetley et al. 1990). Although there were no changes to neutrophils, there was an increase in the leukotriene LTB4. This correlates with the heightened AHR observed in the Annexin A1 KO mice and neutrophils have been shown to produce LTB4. It could be speculated that enhanced LTB4 observed in the KO mice could partly be due to neutrophils, that lack AnnexinA1 and therefore loss of PLA2 regulation, secreting higher levels of LTB4. Studies to isolate neutrophils and measure lipid production would confirm how neutrophils contribute to LTB4 production. It was also observed that in HDM treated mice there was an elevation of lipids in Annexin A1 deficient mice. This included PGE2, 8-, 11-, 12- and 15-HETE. Annexin A1 is known to regulate PLA2 production, therefore this increase in eiconasoids indicates a loss in the regulation of lipid synthesis and thereby contributing to the increase in the magnitude of inflammation observed in Annexin A1 KO mice. As mentioned in previous chapters, the precise role of the HETEs in allergic airways disease is still to be elucidated. 12- and 15- HETE are induced following IL-4 and IL-13 secretion, suggesting a pro-inflammatory role in allergic disease. However, 12 and 15-HETE are also activators of the anti-inflammatory transcription factor PPARg, therefore, they may have a resolution function.

Although significant changes in distinct leukocyte subpopulations were not evident, there was a clear effect on lung tissue cytokines. Th2 cytokines IL-4, IL-13 and IL-33 were increased following WRW4 treatment which reflected the exacerbated airway resistance. Treatment of mice with WRW4 and AnxA2-50 did not always demonstrate opposing effects. Treatment with the agonist promoted a decrease in IL-4 and IL-5 but not IL-13 or IL-33. WRW4 also inhibits effects of other ligands of FPR2, such as LXA4 and RvD1. Therefore, comparing the difference in effects between WRW4 and AnxA2-50 can help delineate the different regulatory roles that Annexin A1, LXA4 and RvD1 may have in controlling allergic inflammation. The results suggest that Annexin A1 may have a role in regulating the Th2 response to HDM whereas LXA4 and/or RvD1 may mediate effects of more innate pathways

involved in the response to HDM, such as IL-33. Future studies would treat HDM allergic mice with LXA4 and RvD1 to confirm the observations with WRW4 treatment.

Annexin A1 promotes the recruitment of monocytes. This was associated with increases in CD11b expression. In our HDM model, CD11b+ CD68+ CD11c- interstitial macrophages in Annexin A1 deficient mice were decreased compared to WT. Interestingly, levels of this population were unaffected by therapeutic treatment with WRW4 and AnxA2-50. This suggests that global KO of Annexin A1 may have altered pulmonary homeostasis. Annexin A1 levels have not been investigated in allergic individuals or patients with asthma.

Annexin A1 was highly expressed in macrophages, which have shown to be critical in regulating HDM disease and the expression of Annexin A1 was increased in response to HDM. Macrophages derived from Annexin A1 KO mice had enhanced chemokine production in response to HDM and LPS. It is still to be determined whether the enhanced Th2 inflammation and AHR observed in vivo in Annexin A1 KO mice exposed to HDM are mediated by Annexin A1 from alveolar macrophages. Annexin A1 is also expressed by epithelial cells and the epithelium is a critical regulator of the pulmonary immune response so it cannot be ruled out that secretion by the epithelium may contribute to homeostasis governed by this pathway. Future studies would employ transgenic mice that have specific depletion of Annexin A1 in macrophages and/or airway epithelial cells. For example crossing CD68 (cre) or CCSP (cre) mice with either Annexin A1 (flox) or FPR2 (flox) mice resulting in macrophage or epithelial specific KO of these mediators to determine the contribution of each pulmonary compartment to pathology.

These results show that the Annexin A1:FPR2 axis is an important regulator of mucosal HDM induced allergic inflammation. The role of Annexin A1 in controlling other types of lung inflammation has also been investigated experimentally. Annexin A1 KO mice displayed an increase in inflammation and exacerbated indices of fibrosis in a model of bleomycin induced fibrosis (Damazo, Sampaio et al. 2011). This was reversed by administration of the Annexin

A1 mimetic, Ac-26. In another study LPS induced inflammation could also be regulated by ANXA1 administration. Thus, this peptide and pathway presents an attractive target for treatment of lung inflammation and the results in this chapter demonstrate an important role in the pulmonary mucosa.

In human disease, it is has been shown that FPR2 is expressed at lower levels on peripheral blood eosinophils and neutrophils, from severe asthmatics. (Planaguma, Kazani et al. 2008). FPR2 expression was not changed in monocytes. However, in myeloid cell populations it may be that the expression of FPR2 ligands, such as LXA4 and Annexin A1, is more important. Indeed, it was recently described in allergic individuals that LXA4 may exert its effects on ILCs and NK cells which express FPR2. These innate cells have been described to be critical in the generation of allergic inflammation. (Barnig, Cernadas et al. 2013). However, it has not yet been elucidated if pulmonary monocytes and or macrophages are the direct source of LXA4 or Annexin A1 in humans.

6.5 Conclusion

Annexin A1 is highly expressed by alveolar macrophages. Following HDM challenge, Annexin A1 is upregulated and remains increased during resolution. Deficiency in Annexin A1 promotes exacerbated AHR and Th2 inflammation. Stimulation of BMDM with LPS suggests that AnnexinA1 regulates chemokine production. Blocking FPR2 with a pharmacological antagonist had a detrimental effect on the development of AAD. Collectively, these data suggests that the Annexin A1:FPR2 pathway is important and may provide a novel therapeutic target for the treatment of asthma.

Chapter 7. Discussion

7.1 Summary of findings and impact of project

The working hypothesis for this study was that resolution of HDM induced allergic disease is an active process controlled by specific pro-resolving cells and molecules. There are few models of inflammatory resolution particularly in the lung thus the first aim was to establish a model of resolution and identify pro-resolving mediators. In order to address this, a murine model of HDM induced allergic airways disease which mimics the features of human disease was employed (Gregory, Causton et al. 2009). HDM allergen was administered via repeated intra nasal challenge which promotes mucosal sensitisation pulmonary inflammation, mucus production, airway smooth muscle proliferation, extracellular matrix deposition and AHR.

Disease parameters were analysed at different time points after the final allergen exposure. In parallel, mediators of pulmonary regulation and inflammatory resolution were quantified. This is the first time a model of resolution of inflammation has been fully characterised in the lung. Importantly, in the current study manipulation of resolution pathways were initiated after the onset of disease as would occur in asthmatic patients who present at the clinic. Following cessation of allergen challenge airway hyper-reactivity, allergic inflammation and Th2 immunity resolved to baseline levels. During this resolution phase, specific pro-resolution mediators of both the humoral and cellular arms of the immune response were up-regulated. During the resolution phase both total and allergen specific IgA in the lung and BAL were up regulated. Immunoglobulin A is present in abundance in the lung and is important in host defence at mucosal surfaces (Wines and Hogarth 2006) indicating that IgA is present to mediate host defence after cessation of allergen challenge. It is unknown how long HDM persists at mucosal surfaces, but IgA could be sequestering lingering allergen thus preventing further damage. The increase in IgA could also be an indicator of altered homeostasis after resolution of inflammation. Measuring IgA at a longer period of time after cessation of allergen challenge would indicate if IgA elevation persists or returns to basal levels. FcαRI is the major activating IgA receptor and expressed on myeloid cells(Wines and Hogarth 2006). Expression of the receptor on dendritic cells functions by capturing IgA

complexes for antigen presentation. It has been shown that IgA primed DCs have a lower capacity to induce effector T cells and preferentially induce proliferation of T regulatory lymphocytes and in vivo administration of IgA-DCs could prevent the development of experimentally induced EAE and Type1 diabetes (Diana, Moura et al. 2013) Therefore, it could be in our model that resolution phase IgA activates DCs that promotes an expansion of T regulatory lymphocytes that in turn could mediate resolution. Investigations into DCs populations in the lymphatics during resolution would give an indication if this pathway is critical to the return to homeostasis after allergen challenge.

Alveolar macrophages perform a sentinel role in the airway and their homeostatic function was increased during resolution, demonstrated by an increase in the regulatory receptor CD200R. It was also shown that rechallenge with allergen in the resolved lung promoted a rapid allergen recall response, restoring the allergic airways disease phenotype to a degree of severity seen at peak disease. This further strengthens the idea that despite the loss of clinical features the resting status of the lung is altered. Thus, pulmonary homeostasis is altered after the resolution of inflammation induced by allergen exposure as has previously been reported post viral infection in mice (Hussell and Cavanagh 2009). It has also been shown that prior HDM exposure results in a deficient protective response to subsequent infection with the bacterium *s.pneumonia* (Habibzay, Saldana et al. 2012). It is likely that homeostatic changes in the lung after mucosal allergic injury play a role in susceptibility to viral exacerbations. Viral infection frequently results in exacerbation of asthmatic symptoms in patients and hospitalisation is a considerable economic burden on the NHS. The identification of mediators that promote resolution of inflammation in the current study in response to allergen are likely to also play a role in other inflammatory settings and possibly also other tissues.

Of particular interest in this project were alveolar macrophages. Concomitant with waning of disease the number of alveolar macrophages increased. Depletion of alveolar

macrophages after disease was established delayed the resolution of inflammation. Conversely, enhanced resolution of inflammation was observed when alveolar macrophages were transferred into diseased recipients, demonstrating that following allergen exposure alveolar macrophages are able to direct resolution programs to promote homeostasis. In these studies we focused on exploring the role of alveolar macrophages based on their anatomical location and did not investigate their activated phenotype. Macrophages can adopt a variety of phenotypes based on their environment and based on in vitro analysis can be roughly classified in to M1, M2 and M2-like subsets. M1 macrophages are known as classically activated macrophages and induced by IFNγ and TNFα and M2 are induced by IL-4 and IL-13. The M2-like subset encompasses a broad variety of phenotypes that are not associated with the classical induction through IFNγ and TNFα, but are generally classified to express wound healing and repair genes . (Gordon and Taylor 2005, Mosser and Edwards 2008) In the allergic lung macrophages of both M1 and M2 phenotypes has been described (Boorsma, Draijer et al. 2013, Draijer, Robbe et al. 2013). This mix of macrophage phenotype could reflecting the heterogeneity of asthma and also the variety of stimuli comprised in the inhaled environment. In our model it would be interesting to phenotype our macrophages during the resolution phase. Isolation of both alveolar and interstitial macrophages and using affymetrix analysis would give an indication to how alveolar macrophages are altered after allergen. The characterisation of resolution macrophages in a model of peritonitis has been described and resolution phase macrophages demonstrated a hybrid phenotype of both M1 and M2 properties (Stables, Shah et al. 2011). In our HDM model it would be particularly interesting to characterise our lung isolated macrophages for wound healing genes. Macrophages are known to secrete, TGFβ, fibronectin, thus it may be that chronically activated macrophage in the lung express excessive levels of these genes and contribute to the remodelling seen in asthmatics. It may be that in chronic asthmatics remodelling is due to an aberrant over production of resolution and repair mechanisms.

A novel observation was that manipulating numbers of alveolar macrophages directly affected the interstitial macrophage population. The role of interstitial macrophages in asthma is relatively under investigated. They are distinct from alveolar macrophages and they reside between in the alveolar epithelium and vascular endothelium. Compared to alveolar macrophages they are less efficient at phagocytising but better a stimulating T-cell proliferation (Franke-Ullmann, Pfortner et al. 1996). They have been found to express IL-10 and thought to alter DC response to LPS (Bedoret, Wallemacq et al. 2009). In our HDM model, the role of interstitial macrophages has yet to be identified. Exactly how alveolar macrophages inversely regulate interstitial macrophage numbers remains to be elucidated but this could represent a novel homeostatic pathway in the lung.

There was an induction of pulmonary pro-resolution mediators in response to mucosal allergen exposure. LXA4 increased concomitant with the decline of features of AAD. Analogues of LXA4 have been shown to regulate OVA induced AHR (Levy, Lukacs et al. 2007). However, this is the first time that LXA4 has been shown to be induced in a HDM model of disease where sensitisation occurs at the mucosal surface. Importantly, it is demonstrated the LXA4 is temporally associated with resolution. The receptor for LXA4, FPR2, was also shown to be increased in the lung tissue during resolution. Annexin A1, another ligand for FPR2 receptor, was increased during peak disease and remained elevate during resolution. We demonstrated that Annexin A1 was important for regulating the features of HDM induced AAD and in Annexin A1 KO mice AHR and Th2 inflammation were heightened. Conversely, pharmacological manipulation with a FPR2 antagonist promoted exacerbated AHR and increased inflammation demonstrating an important role in mediating resolution (Dufton and Perretti 2010). Furthermore, exogenous administration of Annexin A1 mimetic, Anx2-50 reduced lung tissue levels and improved lung function. Mimetics for Annexin A1 have been shown to modulate inflammation in bleomycin induce fibrosis and LPS induced inflammation (da Cunha,

Oliani et al. 2012, Tae, Park et al. 2012). Our novel findings demonstrate that Annexin A1 targeting FPR2 can modulate airway inflammation after established disease induced by a clinically relevant allergen. Moreover, Anx2-50 agonist down regulated specific pathogenic pathways without compromising the protective innate immune response. This holds an exciting prospect for therapeutic intervention and this was particularly interesting as this agonist was administered intranasally. This is a route that would be favoured by human patients. These results offered promising therapeutic potential for this pathway.

Alveolar macrophages have been shown to be producers of LXA4, Annexin A1 and 12 & 15-HETE. Alveoar macrophages express FPR2 and PPARγ, the downstream receptor and nuclear factor, for LXA4, Annexin A1 and 12-and 15-HETE, respectively. It may be that removal of alveolar macrophages during the resolution phase prevents alveolar macrophage derived lipid production and activation of FPR2 and PPARγ mediated regulation. An interesting study would be to treat mice with clodronate depleted macrophage with either LXA4 and Annexin A1 in an attempt to restore resolution. This would elucidate if alveolar macrophages are a critical source of these pro-resolution lipids. 12- and 15-HETE were found to be increased following HDM exposure and increased during the resolution phase. These lipids are products of the 12/15 LOX enzyme. This enzyme is highly expressed in monocytes, macrophages and is one of the mose highly induced genes by IL-4 and shown to be critical in Th2 inflammation (Kuhn and O'Donnell 2006). However, their role in inflammation is not entirely clear. An animal model of OVA induced AAD, demonstrated that 12/15 LOX KO mice had reduced lung function suggesting a pro-inflammatory function for these lipids. (Andersson, Claesson et al. 2008). It could be that at different stages of disease 12-and-15 HETE may play different roles. In a model of TLR receptor dependent peritonitis, 12-HETEs were rapidly cleared during the peak phase of inflammation but reappeared during resolution.(Morgan, Dioszeghy et al. 2009). One of the downstream targets of 12-HETE is the nuclear factor

PPARγ which is highly expressed in macrophages and has potent anti-inflammatory function (Kuhn and O'Donnell 2006). It may be that these lipids could act via PPARγ to contribute to the resolution observed in our model. Targeting 12/15 LOX pharmacologically after established disease would elucidate if 12 and 15-HETE have a role in mediating AHR in established disease.

7.2 Future work

7.2.1 Investigating resolution mediators in a chronic model of allergic airways disease

In this project we focused on resolution of pro-inflammatory mediators and AHR which are associated with peak inflammation in the HDM model. It would be of interest to treat mice with an allergen for a longer period of time to determine whether manipulating pro-resolution mediators and cells could also impact on indices of airway remodelling. It has previously been shown that prolonged continuous exposure to HDM results in accumulation of extracellular matrix proteins and increase in airway smooth muscle mass and neovascularisation. Cessation of allergen challenge is associated with resolution of inflammation, however, elevated AHR to methacholine persists. Current asthma therapies target Th2 cytokines and pulmonary inflammation but structural changes are not modulated. It would be profoundly interesting to determine if manipulating pro-resolution pathways might also affect lung function changes which would signify a significant clinical breakthrough in the treatment of asthma.

7.2.2 To elucidate the role of interstitial macrophages in resolution of HDM inflammation

The transfer and depletion of alveolar macrophages highlighted a critical role in not only mediating local airway inflammation but far reaching effects on the lung tissue inflammation and serum immunoglobulin response to allergen. An exciting observation was the depletion of alveolar macrophages resulted in an increase in lung interstitial numbers and the transfer of alveolar macrophages induced a reduction in interstitial

macrophages. It is evident from these studies that the alveolar macrophage population have a direct influence on the tissue interstitial macrophage population. It is unclear if interstitial macrophages are beneficial or detrimental to the pulmonary inflammatory response. A depletion study targeting interstitial macrophage subset would help elucidate their role. It may be possible to deplete interstitial macrophages via i.v administration of clodronate (Jenkins, Ruckerl et al. 2011). Additionally it may be possible to sort these macrophage subsets by flow cytometry and investigate their gene expression by RT-PCR in order to determine whether they display an M1 or M2 phenotype. RT-PCR would also tell us if they expressed pro-inflammatory cytokines, and resolution receptors. Ultimately to define these cells with a unique transcription factor would allow us better identification. These cells are understudied and their role as innate modulators of lung tissue is far from being understood.

7.2.3 To establish if Annexin A1 from alveolar macrophages is responsible for pro-resolution phenotype

Deficiency in Annexin A1 exacerbated Th2 inflammation and airway hyper-reactivity. Additionally, the therapeutic administration of an Annexin A1 mimetic improved HDM induced lung function and lung tissue Th2 cytokine levels, however the key cellular source of Annexin A1 mediated resolution has not been determined. Alveolar macrophages are an abundant source, as are neutrophils and the airway epithelium. Future studies would employ transgenic mice that have specific depletion of Annexin A1 in macrophages neutrophils and/or airway epithelial cells. For example crossing CD68 (cre), Ly6C (cre) or CCSP (cre) mice with either Annexin A1 (flox) mice resulting in macrophage, neutrophil or epithelial specific KO of these mediators to determine the contribution of each pulmonary compartment to pathology.

7.2.4 To investigate the role of Annexin A1 and FPR2 in human disease

There is comparatively little in the literature regarding alveolar macrophages in asthmatics. FPR2 expression of peripheral blood monocytes and ILCs has been reported (Planaguma, Kazani et al. 2008, Barnig, Cernadas et al. 2013), however, its expression in the lung tissue is not fully described. Additionally, expression of Annexin A1 in the human lung has not been not been investigated. Levels of Annexin A1 could be measured in BAL by ELISA. The cell pellet from the BAL could be analysed by flow cytometry for FPR2 co-localisation with macrophage and lymphocyte markers to determine AnnexinA1 and LXA4 responsive populations. Cell pellets could also be analysed by RT-PCR to quantify mRNA of FPR2 and Annexin A1. Lung tissue biopsies stained by IHC would localise Annexin A1 and FPR2 in the asthmatic lung. In the Annexin A1 KO studies it was evident that a deficiency in Annexin A1 altered the eicosanoid production. In these human samples it would be interesting to identify if the human homologues of LXA4 and HETEs were dysregulated in the asthmatic lung and associated with Annexin A1 and FPR2 expression. Asthmatics are susceptible to secondary bacterial infections and viral exacerbations so it would be interesting to determine expression of Annexin A1, FPR2 and eicosanoids in these individuals and determine their contribution to in viral and bacterial co infections.

7.3 Final conclusion

In summary, this project describes a model of resolution following mucosal allergen induced disease and identifies resolution pathways and mediators which are activated following allergen exposure. Clinical trials targeting pro-inflammatory chemokines and cytokines implicated in asthma pathogenesis have not been universally successful and highlight that asthma is a heterogeneous disease. It is likely that stratifying therapy accordingly to biomarkers and asthma phenotype will benefit subgroups of patients who can be identified as most likely to respond to specific therapies. However, targeting resolution pathways rather than specific pro-inflammatory mediators may offer a more all-encompassing

therapeutic option. Manipulation of the Annexin A1:FPR2 axis influenced resolution and novel therapeutics designed to enhance Annexin A1 signalling pathway are an exciting avenue for further research for novel therapeutic interventions in patients who may not fit the classical asthma phenotype and do not respond to current therapies

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Appendix I.

Cytokines and Lymphocyte variability

Cytokine absolute values Figures 3.7, 3.15, 4.6 and 4.10.

IL-4 and IL-5 ELISAs were performed using paired antibodies (BD biosciences).

IL-4

Optical density was determined using a spectrophotometer at a wavelength of 450nm (A^{450}). Absolute values were calculated from the standard curve using linear regression analysis. In Figure 3.7 optical density values were plotted to determine the relative levels of IL-4 between peak inflammation and during the resolution time course. This was due to the standard curve not working and did not have more sample to repeat the ELISA is this study. Subsequently baseline levels of IL-4 (in PBS control mice) were determined to be between 5-20pg/ml in the lung (Figures, 3.15 and 4.6). Following HDM exposure IL-4 increased to ~200pg/ml, (Figures 3.15 and 4.10) and correspond with those we have previously reported in BALBc mice (Gregory et al, 2013). There was an exception in Figure 4.6 where IL-4 at peak inflammation averaged at 50pg/ml. These samples had undergone a second freeze thaw cycle as the first ELISA assay had failed. The IL-4 protein is known to be sensitive to temperature change and it may be that this extra freeze thaw cycle meant some of the IL-4 had degraded by the time of the second analysis and resulted in lower levels being detected in the treatment groups. In Figure 4.10 higher baseline IL-4 levels (~ 100pg/ml) were reported. These groups of mice also demonstrated higher baseline levels of IL-5. This was around the time that there was pinworm infection in the CBS animal facility which account for the Th2 skew.

Overall, HDM exposure induces a 2-3 fold increase in IL-4 levels in the lung at peak inflammation and despite the higher baseline in mice from Figure 4.10, these mice also displayed a similar fold increase.

Levels of IL-4 in Figure 4.13 were measured in C57BL/6 mice. These mice have a skewed Th1 immunity and are resistant to experimentally induced Th2 disease (Voogth & Vanoirbeek, 2010). Therefore, the lowers levels of induced IL-4 measured in C57BL/6 mice compared with BALBc (~50pg/ml vs 150pg/ml) mice reflect the strain differences in the response to repeated intranasal HDM exposure, Figures 3.17, 3.15, 4.6 and 4.10.

IL-5

Levels of IL-5 in Figure 3.7 are shown at a much higher magnitude compared to values reported in Figures 3.15, 4.6 and 4.10. In this study (Figure 3.7) samples were placed directly on the plate without dilution with carrier protein. The lung is a complex matrix containing many other proteins and requires a carrier protein, such as BSA in the assay diluent. Thus the absolute values in this assay may represent a less diffuse IL-5 and resulting in elevated levels. It should be noted, however, that the 2-fold increase in IL-5, at peak inflammation and during resolution, is comparable with the trends in the previous observations.

IL-13

IL-13 values reported in Figures 3.15, 4.6 & 4.10 were measured by ELISA using a Ready-Set-Go! ® paired antibody kit (catalogue number 88-7137-76, ebioscience). In contrast, IL-13 measurements shown in Figure 3.7 were quantified using IL-13 Quantikine™ pre-coated ELISA plates (catalogue number MC1300CB, R&D Systems) which reported lower levels of IL-13 following HDM (25-85pg/ml vs 130-180pg/ml). Compared to the PBS controls, there was a similar 3-4 fold increase observed.

Lymphocytes numbers

CD4 lymphocyte absolute values Figures 3.6, 3.14, 4.5 and 4.9.

T lymphocytes subsets were determined by FACS gating strategies outlined in the Materials and Methods chapter. Numbers of lymphocytes in Figures 3.6 and 4.9 were identified by CD4 expression. In Figures 3.14, & 4.5 lymphocyte populations were determined by CD3 and CD4.

Whilst each of these strategies gave comparable results when repeated, there were some differences between these strategies.

The addition of CD3 reduced the magnitude of T1/ST2+ lymphocytes from $x10^5$ to $x10^3$ cells/ml. This suggests that the CD4 lymphocyte only strategy (Figures 3.6 and 4.9) may include CD4+ CD3- populations accounting for the increase in these lymphocytes. CD4 and T1/ST2 can be expressed by monocytes and macrophages. However, the lymphocyte gate should exclude larger myeloid cells based on FSC vs SSC gating. During the course of the PhD programme the FACS acquisition changed from a FACS Aria™ analyser to a LSR-Fortessa™. The Fortessa has higher number of lasers with increased sensitivity. This allows for an increase in the number of channels and antibodies used, such as the addition of CD3. The Fortessa gives a higher resolution for FSC and SSC. This with the addition of CD3 produced a more discrete and defined population that would exclude larger cells and non-CD3 leukocytes. We continued to refine the gating strategy during the course of my PhD studies.

IL-10+ lymphocytes numbers were consistent using the CD4 only strategy (Figures 3.6 and 4.9), $1x10^5$cells/ml cells in PBS and increasing to $2x10^5$ cells/ml following HDM. However, there were different values between IL-10+ lymphocytes in Figures 3.14 and 4.5. Endogenous IL-10 has been shown to be difficult to detect with commercially available antibodies. We have attempted IL-10 staining in paraffin embedded lung sections with

unsuccessful results. Similarly, tests comparing ELISA kits and paired antibodies for detection of IL-10 have shown inconsistent measurements for IL-10 and it appears this variability is reflected in absolute numbers from flow cytometric analysis for the detection of IL-10 production.

Despite these differences in absolute numbers of T1/ST2 and IL-10+ lymphocytes, trends and fold increases following HDM treatment and during the resolution phase were highly reproducible for both strategies.

Appendix II.

Abstracts relating to PhD thesis

Resolution of House dust mite induced allergic airways disease

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The debilitating symptoms of impaired lung function and mucus secretion experienced by asthma patients can persist for several days following allergen exacerbation. The purpose of this study was to characterise the resolution of allergic pulmonary inflammation in mice following 3 weeks of intranasal challenge using house dust mite (HDM). Disease parameters were measured at 4 hours, 7 days and 13 days following cessation of allergen exposure. Control mice received PBS. Airway hyperreactivity (AHR) was sustained at 7 days post challenge compared to controls, returning to baseline by 13 days [Resistance at 100mg/ml = 5.73 & 3.20, vs. 1.93, P<, P, 0.05]. This was accompanied by persistent levels of Th2 lymphocytes [2.63 x 10^5cells/ml and 1.32 x10^5cells/ml vs. 0.79 x 10^5cells/ml, P < 0.05] and eosinophils [18.9 x 10^5 cells/ml and 7.16 x 10^5 cells/ml, vs. 1.8 x 10^5 cells/ml , P< 0.001], returning towards baseline by 13 days. Th2 lymphocyte numbers did not correlate with the epithelial derived cytokine, IL-33, which returned to baseline by 7days. The molecule CD200R is expressed on myeloid cells and has been implicated in maintaining pulmonary immune homeostasis. CD200R was shown to be expressed on eosinophils and alveolar macrophages. The proportion of CD200R positive eosinophils reduced 4 hours post challenge and 7 days compared to controls. Expression of CD200R on alveolar macrophages peaked at 7 days and remained elevated at 13 days. These data indicate that distinct molecular pathways maybe responsible for induction and maintenance of allergic inflammation and AHR.

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The role of alveolar macrophages during the resolution of house dust mite induced allergic airways disease

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Allergic asthma is a chronic inflammatory disease of the lung. Deficiencies in pro-resolving mechanisms may contribute to the persistence of inflammation in the lung. Alveolar macrophages are considered to have a critical regulatory role in the lung but their interactions in the allergic lung are not well understood. Experimental models of asthma have shown that depletion of alveolar macrophages prior to allergen sensitisation results in exacerbated disease. However, their role during resolution has not been fully examined. Using a mouse model of house dust mite (HDM) induced allergic airway disease we investigate the role of alveolar macrophages during the resolution of allergic inflammation. Disease parameters were measured at 4 hours, 7days and 13days following allergen exposure. Airway hyper-reactivity was sustained 7days post challenge compared to PBS treated controls, returning to baseline by 13 days, accompanied with concomitant levels of Th2 lymphocytes and eosinophils. Levels of neutrophils increase following HDM exposure and resolve by 7days. CD11c+ alveolar macrophage numbers increased at 4 hours and are significantly elevated at 7 and 13 days, suggesting a role during resolution. Depletion of alveolar macrophages during the resolution phase was carried out using i.t administration of clodronate encapsulated liposome. This resulted in delayed resolution of Th2 lymphocytes and airway neutrophils. However, no changes were observed in Th2 cytokines or airway hyper-reactivity. Conversely, following the adoptive transfer of alveolar macrophages during resolution, total lung tissue cell numbers and neutrophils were decreased. Levels of Th2 lymphocytes and airway hyper-reactivity remain unchanged. Levels of CD11b+ CD11c- lung tissue macrophages were reduced upon alveolar macrophage transfer suggesting a cross talk between these pulmonary subsets. These data indicate that distinct pathways are responsible for the resolution of allergic airways disease and alveolar macrophages have a specific role to play in regulating the allergic inflammatory response.

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Activation of Annexin A1-FPR2 axis promotes resolution of house dust mite induced airway hyper-reactivity

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Allergic asthma is a chronic inflammatory disease of the conducting airways. Exacerbations in allergic asthmatics are induced by inhalation of an innocuous protein that promotes an aberrant immune response. Current therapy has focussed on the initiation of inflammation but less is understood about the mechanism that control the regulation of allergen induced mucosal injury. Indeed, deficiencies in endogenous pro-resolving mechanisms may contribute to the persistence of inflammation in the lung. One such endogenous molecule is Annexin A1, a 37KDa peptide with potent anti-inflammatory and pro-resolving properties. It exerts its effects primarily through FPR2. However, expression of Annexin A1 and FPR2 in the pulmonary allergic inflammatory environment is not well described. Using a mouse model of house dust mite (HDM) induced allergic airways disease (AAD) we investigated the role of Annexin A1. BALB/c mice receive intranasal administration of HDM 3 times a week for 3 weeks. Firstly, we established in a naive mouse that Annexin A1 is highly expressed in alveolar macrophages and at a lower levels, in airway epithelium and polymorphonuclear cells. The receptor FPR2 had similar localisation. Following HDM treatment the expression and numbers of Annexin A1 + cells in the cellular infiltrate significantly increased. Interestingly, levels of Annexin A1, quantified by mRNA and IHC analysis, demonstrated that Annexin A1 were found to remain significantly elevated during the resolution of phase inflammation. To investigate the importance of Annexin A1 in AAD, we challenged Annexin A1 knock-out mice with HDM. The results revealed that mice deficient in Annexin A1 presented an exacerbated AAD phenotype, with increased airway hyper reactivity (AHR), airway eosinophilia and elevated Th2 immunity. To corroborate the importance of Annexin A1 in our model we treated allergic mice with an Annexin A1 mimetic for the final week of HDM exposure. These mice demonstrated ameliorated AHR and reduced lung tissue inflammation. Conversely, the use of FPR2 antagonist WRW4 promoted worse AHR and increased lung inflammation. These data indicate that Annexin A1 and FPR2 are critical regulators of the pulmonary mucosal response following HDM challenge. Targeting Annexin A1 and its receptor FPR2 may provide a novel target to treat airway hyper-reactivity in allergic individuals. There are many Annexin A1 mimetics and FPR2 antagonists in development that may hold great therapeutic potential and this study further highlights the importance of understanding this pathway.

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Appendix III.

Publications

Lisa G. Gregory, Carla P Jones, **Sara A. Mathie**, Sophie Pegorier, & Clare M. Lloyd **Endothelin-1 directs airway remodeling and hyperreactivity in a murine asthma model.** *Allergy, the European Journal of Allergy and Clinical Immunology 2013*

Saglani S, Lui S, Ullmann N, Campbell GA, Sherburn RT, **Mathie SA**, Denney L, Bossley CJ, Oates T, Walker SA, Bush A, Lloyd CM. **IL-33 promotes airway remodelling in paediatric patients with severe steroid-resistant asthma.** *J Allergy Clin Immunol. 2013*

Lisa G. Gregory, **Sara A. Mathie**, Simone A Walker, Sophie Pegorier, Carla P Jones & Clare M. Lloyd (Jul 2010). **Overexpression of Smad2 drives HDM mediated airway remodelling and AHR via Activin and IL-25.** *Am J Respir Crit Care Med.*

GregoryLG; Causton B; Murdoch, JR; **Mathie SA**; O'Donnell V; Thomas CP; Priest FM; Quint DJ; Lloyd CM. (Oct 2009) **Inhaled house dust mite induces pulmonary Th2 cytokine production.** *CLIN EXP ALLERGY.*

Saglani S; **Mathie SA**; Gregory LG; Bell MJ; Bush A; Lloyd CM. (Sept 2009). **Pathophysiological Features of Asthma Develop in Parallel in House Dust Mite Exposed Neonatal Mice.** *Am J Respir Cell Mol Biol.*

Morgan AH; Vincent Dioszeghy V; Maskrey BH; Thomas CP; Clark SR;**Mathie SA**; Lloyd CM; Kühn H; Topley N; Coles BC; Taylor PR; Jones SA; O'Donnell VB. (Aug 2009) **Phosphatidylethanol-esterified eicosanoids in the mouse: tissue localisation and inflammation-dependent formation in the Th2 disease.** *J. BIO.CHEM*

Kearley J; Buckland KF; **Mathie SA**; Lloyd CM. (May 2009). **Resolution of Allergic Inflammation and AHR is Dependent upon Disruption of the T1/ST2-IL-33 Pathway.** *Am J Respir Crit Care Med*

Tourdot S; **Mathie S**; Hussell T; Edwards L; Wang H; Openshaw PJM; Schwarze J; Lloyd CM. (Jun 2008). **Respiratory syncytial virus infection provokes airway remodelling in allergen-exposed mice in absence of prior allergen sensitization.** *CLIN EXP ALLERGY.*