ROLE OF GROUP 2 INNATE LYMPHOID CELLS AND SHIP-1 IN MUCOSAL IMMUNITY

by

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Abstract

Mucosal surfaces present an important barrier between the host and environment. Maintenance of barrier function requires intricate cross-talk between a diverse array of immune cells and the epithelia, acting synergistically to respond to harmful antigens and maintain tolerance to innocuous antigens. In this thesis I utilized an array of transgenic animals to explore the cellular and molecular mechanisms that initiate adaptive immune responses in the lung and gut mucosa.

Recently, innate lymphoid cells have been characterized for their role in maintaining barrier immunity. Group 2 innate lymphoid cells (ILC2s) colonize the lung and provide a rapid source of IL-5 and IL-13 in a T and B cell independent manner in response to protease antigens. Using ILC2-deficient mice, I examined the role of these cells in mucosal inflammation using mouse models of allergic asthma and hypersensitivity pneumonitis (HP). ILC2s were critical in initiation of a Th2 response to locally, but not systemically delivered allergens and were completely dispensable for Th1 and Th17 dependent responses.

The PI3K pathway plays an important role in regulating leukocyte activation, survival, migration and cytokine release. It is negatively regulated by the lipid phosphatase Ship1, and Ship1\textsuperscript{-/-} mice develop a wide array of hematological disorders leading to a reduced lifespan. The severe phenotype associated with loss of Ship1 throughout the immune systems masks subtler roles it plays in specific leukocyte subsets. Using a conditional deletion approach, I examined the role of
Ship1 in T cells, B cells and dendritic cells (DCs) in mouse models of allergic asthma and helminth infection. While loss of Ship1 in B cells did not influence susceptibility to a HDM model of allergic asthma, loss of Ship1 in either the T cells or DCs protected from disease development due to an immune skewing to a Th1 response. Additionally, loss of Ship1 in DCs rendered mice susceptible to infection with the intestinal helminth Trichuris muris, further highlighting this Th1 immune skewing.
Preface

A version of Chapter 2 has been published: Gold MJ, Antignano F, Halim TY, Hirota JA, Blanchet MR, Zaph C, Takei F, McNagny KM. Group 2 innate lymphoid cells facilitate sensitization to local, but not systemic, TH2-inducing allergen exposures. Journal of Allergy and Clinical Immunology. 2014 Apr;133(4):1142-8, with permission from Elsevier. I designed and conducted 80% of experiments and wrote the manuscript. Dr. Frann Antignano conducted 10% of the experiments. Dr. Timotheus Halim conducted 10% of the experiments and provided figures 1A-C and 2G. Dr. Jeremy Hirota provided intellectual content. Dr. Marie-Renee Blanchet provided the SR antigen and intellectual content. Dr. Colby Zaph provided intellectual content. Dr. Fumio Takei provided intellectual content. Dr. Kelly McNagny designed experiments and edited the manuscript.

A version of Chapter 3 has been submitted for publication: Gold MJ, Hughes MR, Antignano F, Zaph C and McNagny KM. Lineage specific role of Ship1 in regulating allergic airway inflammation. I designed and conducted 90% of experiments and wrote the manuscript. Dr. Michael Hughes provided intellectual content. Dr. Frann Antignano designed and conducted 10% of the experiments, provided intellectual content and wrote the manuscript. Dr. Colby Zaph provided intellectual content. Dr. Kelly McNagny designed the experiments and wrote the manuscript.

The work presented in this thesis was approved by the UBC Animal Care Committee, certificate numbers; A06-1483, A11-0096 and A13-0010.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAI</td>
<td>allergic airway inflammation</td>
</tr>
<tr>
<td>AHR</td>
<td>airway hyperresponsiveness</td>
</tr>
<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BMT</td>
<td>bone marrow transplant</td>
</tr>
<tr>
<td>CHILP</td>
<td>common helper-like innate lymphoid progenitor</td>
</tr>
<tr>
<td>CILP</td>
<td>common innate lymphoid progenitor</td>
</tr>
<tr>
<td>CLP</td>
<td>common lymphoid progenitor</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>MLP</td>
<td>formyl-methionyl-leucyl-phenylalanine</td>
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<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HDM</td>
<td>house dust mite</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cell</td>
</tr>
<tr>
<td>ICOS</td>
<td>inducible co-stimulatory molecule</td>
</tr>
<tr>
<td>Id2</td>
<td>inhibitor of DNA binding 2</td>
</tr>
<tr>
<td>i.n.</td>
<td>intranasal injection</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal injection</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous injection</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
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<td>immunoglobulin</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>ILC</td>
<td>innate lymphoid cell</td>
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<tr>
<td>Lin</td>
<td>lineage</td>
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<tr>
<td>LTI</td>
<td>lymphoid tissue-inducer</td>
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<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>MTG</td>
<td>monothioglycerol</td>
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<td>NCR</td>
<td>natural cytotoxicity triggering receptor</td>
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<tr>
<td>PAS</td>
<td>periodic acid-Schiff</td>
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<td>PAMP</td>
<td>pathogen associated molecular pattern</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PH</td>
<td>plekstrin homology</td>
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<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
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<td>PI3K</td>
<td>phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>PTB</td>
<td>phosphotyrosine-binding domain</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PX</td>
<td>phox homology</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative PCR</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<td>--------------</td>
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<tr>
<td>ROR</td>
<td>retinoic acid receptor orphan receptor</td>
</tr>
<tr>
<td>Sca-1</td>
<td>stem cell antigen 1</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2 domain</td>
</tr>
<tr>
<td>SHIP/Inpp5d</td>
<td>Src homology 2-containing inositol 5'-phosphatase</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TSLP</td>
<td>thymic stromal lymphopoietin</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
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</table>
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Dedication

This thesis is dedicated to my parents.
Chapter 1. Introduction
1.1 The immune system

The immune system functions through a tightly orchestrated interactions between the epithelial (barrier), endothelial (vascular) and hematopoietic systems to both protect and elicit inflammatory responses to harmful pathogens, while promoting and maintaining tolerance to innocuous antigens, whether they arise from self antigens or environmental antigens. Impaired regulation of these responses can result in autoimmune or allergic inflammation. Vertebrates contain two arms of the immune system, innate and adaptive immunity.

Innate responses are more ancient and act as a first line of defense to an inflammatory insult. Their recognition of antigens is largely through expression of pattern recognition receptors (PRR) that recognize pathogen associated molecular patterns (PAMPs), and do not require the re-arrangement of genes to form the more antigen specific receptor characteristic of the adaptive immune response[1]. Cellular constituents of the innate immune system include a physical barrier (such as the skin and mucosal surfaces), which acts to compartmentalize and separate the host from the environment, and should that be breached, leukocytes from the hematopoietic system that respond to the breach; macrophages, dendritic cells, neutrophils, basophils, mast cells, eosinophils and innate lymphoid cells (ILCs). The main function of the innate response is to limit the extent of the inflammatory insult and train and facilitate the development of an adaptive immune response to eventually clear the infection.
The adaptive immune system consists predominantly of T and B lymphocytes and these are responsible for antigen-specific responses through unique T cell receptors (TCR) or B cell receptors (BCR), respectively. The initial receptor repertoire is generated clonally through a variety of somatic combinatorial DNA rearrangements executed by recombination activating genes (RAG). B cells represent the humoral arm of the adaptive immune response, with their main role being the production of antibodies against various pathogens. T cells, in contrast, represent the cellular arm of adaptive immunity. These can be subdivided into two main groups; CD8+ cytotoxic T cells (CTL) that directly kill infected cells and CD4+ helper T cells (Th) that facilitate cytotoxic T cell, B cell and innate immune cell activities through the release of various cytokines. Cytokine production by these Th cells is not random and it was initially found that specific Th clones, termed T helper type 1 (Th1) and T helper type 2 (Th2), produced distinct cytokines[2]. Th1 clones were found to produce large amounts of the cytokine IFNγ, important for macrophage activation and the clearance of intracellular bacteria, while Th2 clones produce the cytokines IL-4, IL-5 and IL-13, facilitating allergic responses or helminth immunity through increased mucus production, smooth muscle contraction, IgE production and eosinophil expansion[3, 4]. Several more subtypes have since been characterized, and it is unclear whether these cells are terminally differentiated or if there is some plasticity in their phenotype[5, 6].

Unlike the innate immune system, which responds broadly to conserved PAMPs expressed by various pathogens, the adaptive immune response is remarkably specific and permits the detection of antigen (Ag) specific epitopes through the diversity of their TCRs and BCRs. This poses the challenge, however, of facilitating the exposure of these rare Ag-
specific T cells (approximately $5 \times 10^6\%$ of circulating lymphocytes, or 5 Ag-specific cells for every million circulating cells[7]) to the appropriate pathogen or antigen in a way that will facilitate their clonal expansion in time to respond to the invader. To overcome this challenge, the T cells are concentrated in lymphoid structures (such as lymph nodes, Peyer’s patches, spleen), and specialized innate immune cells such as dendritic cells (DCs) are tasked with sampling Ag’s from the periphery, processing these antigens for presentation of Ag-derived epitopes by major histocompatibility complex (MHC) cell surface proteins and transporting these cell bound epitopes to the lymphoid organs for presentation to naïve T cells, to facilitate their clonal expansion[7, 8]. MHC proteins come in two forms, MHC class I molecules present peptides originating from intracellular (cytosolic) proteins to CD8\(^+\) T cells and MHC class II molecules present exogenous or extracellular proteins to CD4\(^+\) T cells[9].

While MHC class I is expressed on a broad array of cells, MHC class II (MHC-II) expression is more restricted, with dendritic cells acting as the predominant MHC-II expressing antigen presenting cell (APC). A simplified model of DC-T cell interactions has DCs patrolling the periphery, sampling antigens and migrating to regional lymphoid structures, such as lymph nodes, where they can present these antigens to naïve T cells (summarized in figure 1.1). In addition to the initial signal of MHC-TCR interactions, DCs must also provide a second, co-stimulatory, signal to naïve T cells through the interaction of molecules such as B7.1/B7.2 (CD80/CD86) on DCs with CD28 on T cells[10, 11]. Instructional cytokines are also released by DCs or accessory cells that promote the differentiation of naïve CD4\(^+\) T cells to various “helper” lineages geared toward an
appropriate immune response, such as IL-12 for Th1 differentiation and IL-4 for Th2 differentiation[12]. A paradox is that, while DCs are known to produce IL-12 for Th1 differentiation [13], they are not known to produce IL-4, an important cytokine for Th2 polarization, although IL-4 and STAT6-independent mechanisms for Th2 polarization likely act in a complementary fashion[14-18]. This suggests that either naïve CD4\(^+\) T cells can produce IL-4 to act in an autocrine or paracrine role in the absence of other instructional cytokines[19], or that there exists a bystander cell or another APC (other than DCs), can supply the IL-4 required for efficient Th2 polarization. Candidate cells for such an accessory function include basophils[20-22], natural killer (NK) cells[23], eosinophils[24] or innate lymphoid cells (ILCs)[25, 26]. Despite this speculation, until recently, none of these candidate cell types have consistently proved sufficient to explain Th2 antigen polarization. A greater understanding of the cell types and signaling pathways involved in the generation of a robust Th2 response is clearly required to facilitate the development of novel therapeutics for the treatment of allergic, or Th2-driven, diseases.
Figure 1.1. T helper cell differentiation
Schematic of CD4⁺ T cell differentiation modified from O’Shea JJ et al.[5]. Dendritic cells acquire antigens and migrate through the lymphatics to the regional lymph node, where they present antigen epitopes in the context of MHC-II to naïve CD4⁺ T cells. MHC-II/TCR interaction, along with co-stimulation between CD80/CD86 and CD28 and instructional cytokines drive the differentiation and polarization of naïve CD4⁺ T cells to various “helper” lineages. These are associated with increased expression of specific transcription factors (T-bet, GATA-3, RORγt) and the secretion of specialized cytokines.

1.2 Innate lymphoid cells (ILCs)

Innate lymphoid cells are a newly identified constellation of differentially regulated cell types arising from a common precursor that are now thought to be involved in the initial innate immune response and in tissue homeostasis. The cardinal traits of ILC family members are; 1) a lack of lineage markers commonly used to denote committed myeloid and dendritic cell leukocytes (i.e. CD11b, CD11c, Gr-1), 2) an independence of
recombination activating gene (RAG) function for their development, 3) a lymphoid morphology and 4) a unique ability to produce the same immune-response-polarizing cytokines normally produced by adaptive immune cells. A common nomenclature has been developed to classify distinct ILC subsets into 3 main groups based on similarity of the cytokine profiles produced by polarized T cells (ILC1s corresponding to Th1 cells, ILC2s corresponding to Th2 cells, etc.). Specificity for production of these cytokines usually results from specific transcription factors associated with their survival (Figure 1.2)[27]. Group 1 ILCs comprise NK cells and ILC1s and are associated with expression of T-bet. These cells are potent producers of IFNγ, an important cytokine affecting macrophage activation and phagocytosis, and are associated with protection against intracellular bacteria[28-31]. Group 2 ILCs, or ILC2s, are dependent on the transcription factors GATA-3[32, 33], Gfi1[34], TCF-1[35] and RORα[36, 37] and produce high levels of Th2-associated cytokines IL-5 and IL-13 in response to epithelial derived innate cytokines IL-25, IL-33 and TSLP. These cells are enriched in the lung and intestinal mucosa and facilitate allergic responses and immunity to helminth infections. Group 3 ILCs contain both lymphoid tissue-inducer cells (LTi) as well as IL-17A, IL-22 and IFNγ producing ILC3s and depend on the transcription factor RORγt and are important for immunity to extracellular bacteria and parasites such as *Citrobacter rodentium* [38-40].
Innate lymphoid cell development

Schematic of ILC development modified from Klose et al[31]. A common ILC progenitor (CHILP, Lineage’Id2⁻CD127⁺α₄β₇⁺CD25⁻) exists in the bone marrow that can give rise to all ILC lineages but not T or B cells or NK cells. These CHILP can then differentiate into various helper-like ILCs that facilitate immunity to various pathogens. Multipotent progenitor (MPP), common lymphoid progenitor (CLP), common innate lymphoid progenitor (CLP), natural killer cell (NK), common “helper-like” innate lymphoid progenitor (CHILP), inhibitor of DNA binding 2 (Id2).

1.2.1 Group 2 innate lymphoid cells

Innate lymphoid cells that secrete large amounts of cytokines associated with an adaptive Th2 response were initially discovered in the early 2000’s as a lineage negative subset that responded to IL-25 to produce eosinophilia and IL-13[41, 42]. Later, multiple labs independently characterized similar cells in several peripheral tissues; in the mesenteric lymph nodes, spleen and liver they were termed innate helper type 2 cells (Ih2)[43], in fat associated lymphoid clusters (FALC) and lungs they were termed natural
helper cells (NH)[44, 45] and in the lymph nodes, intestines and lungs they were termed nuocytes[46]. Collectively, these cells are now named group 2 innate lymphoid cells (ILC2s) based on their similar surface receptor expression profiles. ILC2s are identified as lineage negative cells dependent on IL-7 for the development through expression of the IL-7Rα (CD127). They express the inducible co-stimulatory molecule (ICOS), Thy1 (CD90), IL-2Rα (CD25) as well as receptors to IL-25 (IL-17RB), IL-33 (T1/ST2) and thymic stromal lymphopoietin (TSLP, TSLPR), stimulation of which leads to robust production of IL-5 and IL-13.

1.2.2 ILC2 development

ILC2 progenitors are present in the bone marrow and arise from the common lymphoid progenitor (CLP, lineage−CD127+Flt3+), and require IL-7Rα signaling for their development[37]. ILC2s can be derived in vitro from CLPs through stimulation with Notch, IL-7 and IL-33[37]. There has been a concerted effort to identify committed ILC and ILC2 progenitors distinct from CLPs. Development of all ILC lineages, but not T and B cells, was found to be dependent on the transcriptional regulator inhibitor of DNA binding 2 (Id2)[44], and recently a common helper-like innate lymphoid progenitor population (CHILP, lineage−Id2+CD25−αβ+Flt3−CD127+) has been identified that gives rise to all ILCs, but not T cells, B cells or NK cells[31]. The transcription factor GATA-3 has also been found to be important for development of IL-7Rα+ ILCs[47], and is required for ILC2 development and survival of mature ILC2s[32, 33]. GATA-3 is also expressed in mature Th2 cells, leading to the search for a unique transcription factor specific to ILC2s to distinguish them from Th2 cells. Two
independent studies found the transcription factor RAR-related orphan receptor alpha (RORα) to be specifically expressed on ILC2s but not Th2 cells, and disruption of RORα function leads to a selective loss of ILC2s[36, 37].

1.2.3 RORα and ILC2 development

The ROR family of transcription factors are orphan nuclear receptors containing both a ligand binding domain (LBD) and DNA binding domain (DBD) and consist of three family members: RORα, RORβ and RORγ[48]. RORγ (encoded by Rorc) plays an important role in development of Th17 cells[49] as well as ILC3s and is important for lymph node organogenesis due to the dependence of LTi cells on expression of RORγ[50]. RORα is expressed in several tissues but its highest expression is in the brain, particularly the cerebellar Purkinje cells[51], where it plays an important role in their maturation. A natural mutation in the RORα gene found in staggerer mice (Rora<sup>sg/sg</sup>) results in deletion of the LBD and a truncated non-functional protein. These staggerer mice exhibit severe developmental abnormalities and have a shortened life-span, predominantly resulting from a loss of RORα expression in the brain. RORα expression in the hematopoietic compartment, however, is tightly regulated, with selective expression in Th17 cells, where it synergizes with its family member RORγ[52], and in ILC2s[36, 37]. Loss of RORα in the hematopoietic system has no effect on normal development and life span but leads to a selective loss of ILC2s and no obvious influence on the development of Th17-mediated inflammatory responses[53]. Thus, this provides a useful strategy for examining the effect of ILC2 deficiency in development of adaptive immune responses.
1.2.4 ILC2s in lung immunity

The lung mucosa is regularly exposed to inhaled antigens that stimulate the lung epithelium to produce IL-25, IL-33 and TSLP. Lung resident ILC2s potently respond to this allergen-induced activation, and serve as an important linkage between the innate and adaptive immune response as they represent an early source of the type 2 cytokines IL-5 and IL-13 prior to the development of adaptive immunity[54]. IL-5 plays an important function in eosinophil biology, enhancing their survival, increasing eosinophilsopoiiesis in the bone marrow and facilitating their migration[55-57]. In allergic asthma, IL-5 and the resulting eosinophil dominated inflammation facilitate the development of airway hyper-responsiveness and lymphocyte infiltration[58]. Allergic airway inflammation also results in increased production of IL-13. Although it shares a common receptor with IL-4, IL-13 stimulates mucus production and airway hyper-responsiveness in an IL-4 and IgE independent manner[59, 60]. It is unclear what role ILC2 produced IL-5 or IL-13 has in facilitating the development of Th2 adaptive immune responses. Recent studies have suggested that IL-13 production by ILC2s is necessary and sufficient for developing a robust Th2 response[25]. This does not rule out a potentially important role for ILC2 produced IL-5, and the resulting influx in eosinophils, in modulating the adaptive immune response as eosinophils have previously been shown to promote dendritic cell maturation and the development of Th2 immunity[61]. There are also reports that ILC3s express MHC-II and can directly present antigen to T cells to influence T cell-mediated immune responses[62, 63]. ILC2s also express MHC-II, but whether these cells contribute to the
antigen presentation and adaptive immune response following allergic sensitization has yet to be evaluated[26].

In human subjects, ILC2s have been found in the skin[64], peripheral blood, gut and lungs [65, 66], and are enriched at sites of atopic skin inflammation[64], sputum of asthmatic patients[67] and nasal polyps of patients with rhinosinusitis[65], suggesting an important link between ILC2s and allergic disease, however their specific role in modulating the immune system is still unclear. Further exploration into how ILC2s promote allergic responses, using mouse models deficient in ILC2s, will hopefully provide insight into the early stages of the development of type-2 adaptive immune response. It should be noted, however, that while ILC2s may prove to be pathogenic in instances of allergy, they also possess important protective functions as well. They’re important for immunity from helminth infection, through the production of IL-13[46, 68], as well as from lung tissue injury in influenza viral infections through the production of several factors facilitating wound repair, including amphiregulin[66]. Novel targeting strategies can be imagined that promote or inhibit ILC2 activity for the treatment of a variety of diseases.

1.3 Dendritic cells in lung immunity

Dendritic cells (DCs) are a specialized leukocyte population that serves as the primary antigen presenting cell (APC) of the immune system. They were originally identified in Nobel prize winning work by the late Dr. Ralph Steinman in both the spleen and lymph nodes in the early 1970’s[69], but have since been found throughout the body, primarily at
barrier sites such as the skin and mucosal surfaces, where they constantly sample antigens. Their ability to phagocytose and transport antigen from peripheral sites to regional lymph nodes is a specialized property not shared, to the same extent, by other innate immune cells, signifying the importance of DCs as vital APCs[70]. Indeed, in the lung, depletion of DCs severely impairs the development of Th2 responses, suggesting that at this mucosal site, DCs are the primary APC for initiating Th2 responses[71]. Additionally, following sensitization, DCs play an important pro-inflammatory role in allergic mice, and again, their depletion results in a reduction of airway hyperresponsiveness, mucus production and eosinophil infiltration, hallmarks of allergic asthma[72].

Dendritic cells exist as a heterogeneous population and are composed of multiple subsets with specialized functions[73]. The lung contains predominantly two distinct subsets at steady state, characterized by their expression of CD11b (integrin αm) or CD103 (integrin αE, a component of the E-Cadherin ligand αEβ7), in addition to the pan-DC and hematopoietic markers MHC-II and CD11c (integrin αX)[74]. These DC subsets are concentrated at distinct locations in the lung and have specialized functions. CD103+ DCs are able to tightly associate with the lung epithelium through their interactions with E-cadherin and are primarily associated with cross-presentation of viral antigens to CD8+ T cells in the lymph node through MHC-I, as well as promoting T regulatory cell development[75-77]. CD11b+ DCs produce a wide array of pro-inflammatory mediators and specialize in MHC-II presentation to CD4+ T cells and are found in the lamina propria or perivascular regions of the lung[74, 78, 79]. Recent studies, using the physiologically relevant allergen house dust mite (HDM) have found that the CD11b+ DCs and an
inflammatory DC population that infiltrates the lung following HDM challenge, marked by the expression of the monocyte marker Ly6C and the high-affinity IgE receptor (FcεRI), are the predominant DC subsets for the trafficking of HDM antigen to the draining lymph node and the priming of an adaptive Th2 response associated with allergic asthma[80]. Increasing our understanding of the signaling pathways and proteins involved in the trafficking and migration of DCs will lead to new therapeutic targets to modulate DC function for the treatment of allergic diseases.

1.4 PI3K pathway

Phosphoinositides are ubiquitously present on the inner cell membrane of many cell types and play important roles in numerous cellular functions, such as survival, migration and activation. Due to this, the generation and hydrolyzation of these phosphoinositides is tightly regulated. Phosphoinositide 3-kinases (PI3Ks) add a phosphate group to the 3’-hydroxyl position of phosphatidylinositol (PtdIns), phosphatidylinositol(4)monophosphate (PtdIns[4]P₁) and phosphatidylinositol(4,5)bisphosphate (PtdIns[4,5]P₂), leading to the recruitment of proteins to the cell membrane through interactions with pleckstrin homology (PH)[81] and phox homology (PX)[82] domains, and a subsequent downstream signaling cascade (reviewed in [83-85]).

PI3Ks have been grouped into 3 classes, class I (consisting of IA and IB), class II and class III. The class I PI3Ks are the most well studied and understood and are the only PI3K class able to produce the important second messenger PtdIns[3,4,5]P₃ from the
substrate PtdIns[4,5]P₂, suggesting a prominent role for this class in regulating cellular survival and activation. Class II PI3Ks are act downstream of integrin, chemokine and some growth factor receptors and utilize PtdIns as their primary substrate producing PtdIns[3]P₁[86] and class III PI3K are involved in protein and vesicle trafficking through the exclusive production of PtdIns[3]P.

Class I PI3Ks form as heterodimers containing a regulatory and catalytic subunit. Class IA enzymes are activated by receptor tyrosine kinases (RTKs) and contain three different catalytic subunits (p110α, p110β and p110δ) and five different regulatory subunits (p85α, p55α, p50α, p85β and p55γ). While p110α and p110β are more broadly expressed, p110δ is enriched in leukocytes[87]. Class IB enzymes associate with G-protein coupled receptors (GPCRs) and contain a single enzyme type consisting of the p110γ catalytic subunit and either the p101 or recently characterized p87 regulatory subunit[84, 88]. While more broadly distributed than p110δ, p110γ is enriched in leukocytes[89]. Activation of receptor tyrosine kinases (IA) or through the Gβγ subunits of GPCRs leads to the membrane accumulation of the cytosolic PI3Ks, resulting in localized accumulation of their main product, PtdIns[3,4,5]P₃[90].

1.4.1 PI3K signaling

PI3K-induced production of PtdIns[3,4,5]P₃ leads to the recruitment and activation of the Tec kinase family as well as the AGC family of serine/threonine kinases, consisting of the phosphoinositide dependent kinase-1 (PDK-1), Akt (also referred to as PKB), protein
kinase C (PKC) and S6 kinase. Akt is in part activated by PDK-1 through phosphorylation of its Thr308 site[91], although phosphorylation of Akt’s Ser473 residue by the mammalian target of Rapamycin complex 2 (mTORC2) is required for maximal activity[92]. Activation of Akt results in the phosphorylation of its substrates, typically leading to activation, survival and proliferation (summarized in figure 1.3)[93-96]. Additionally, the accumulation of PI(3,4,5)P$_3$ and PI(3,4)P$_2$ at the leading edge of cells, predominantly through class IB activation of p110$\gamma$ through GPCRs, is important for facilitating cytoskeletal reorganization, directional migration and motility[97, 98].
Figure 1.3. PI3K pathway
Simplified schematic of activation of class IA and IB and generation of PI(3,4,5)P$_3$. Receptor tyrosine kinase (RTK, class IA) or G protein coupled receptor (GPCR, class IB) activation lead to activation of PI3k, which produce PI(3,4,5)P$_3$ from the substrate PI(4,5)P$_2$. Newly formed PI(3,4,5)P$_3$ acts as a second messenger, recruiting PH-domain containing proteins, such as Akt, leading to their activation and downstream effects on cellular survival and activation. Levels of PI(3,4,5)P$_3$, and in turn Akt activation, are tightly regulated by the inositol phosphatases PTEN (which hydrolyzes the 3'-phosphate to generate PI(4,5)P$_2$, and SHIP-1 and SHIP-2 (which hydrolyze the 5'-phosphate to generate PI(3,4)P$_2$.
1.4.2 PI3K in inflammation

Understanding the contribution of the PI3K pathway in leukocyte function is hampered by the overlapping expression of the various PI3K isoforms and the embryonic lethality associated with genetic ablation of p110α and p110β[99-101]. While leukocytes express all class I PI3Ks, there is a preferential expression and enrichment of the p110δ and p110γ isoforms, leading to the use of genetic models and isoform-specific inhibitors to examine their roles in leukocyte function.

Genetic deletion of p110δ or the generation of a kinase dead mutant results in an impairment in B cell development and maturation in vivo, reduced serum antibody levels and a severely abrogated T cell-dependent and independent humoral response [102-104]. It is unclear, however, whether or not this observed defect in B cell function and germinal center (GC) development is due to a B cell intrinsic role for p110δ or an effect of p110δ loss on supporting leukocyte populations. Recent reports using a conditional deletion approach have shown that it is p110δ expression in T cells, and not B cells, that is responsible for the loss of GCs due to a severe impairment of follicular helper T cells (T\textsubscript{FH})[105]. T\textsubscript{FH} are a unique T cell population able to migrate to germinal centers through expression of CXCR5 where they can support the development and provide instructional cues to aid in B cell differentiation[106]. Deletion of p110γ has revealed important functions in neutrophil and macrophage function, particularly migration[98, 107]. Indeed, p110γ\textsuperscript{−/−} (Pik3cg\textsuperscript{−/−}) mouse neutrophils displayed impaired polarization and migration to stimuli such as fMLP (N-formyl-methionyl-leucyl-phenylalanine)[98, 108].
The vital role PI3K isoforms play in inflammatory processes has lead to the development of isoform or dual-isoform specific inhibitors targeting p110δ and p110γ for the treatment of various diseases. While these inhibitors have offered promising results in pre-clinical and early clinical trials, there is concern that targeting such a ubiquitous pathway could result in toxic side-effects[109].

1.4.3 SHIP-1 and negative regulation of PI3K

Levels of PI(3,4,5)P₃ are tightly regulated and are maintained at low levels in unstimulated cells. While PI(3,4,5)P₃ levels rise abruptly after stimulation and activation of PI3Ks, they are negatively regulated predominantly by two lipid phosphatases: PTEN (phosphatase and tensin homolog), which removes the 3'-inositol phosphate and converts PI(3,4,5)P₃ to PI(4,5)P₂; and SHIP (SH2-containing inositol 5'-phosphatase), which removes the 5'-inositol phosphate converting PI(3,4,5)P₃ to PI(3,4)P₂. SHIP occurs as two distinct gene products, SHIP-2 is more widely expressed while SHIP-1 expression is enriched in the hematopoietic compartment[110, 111]. Due to its restricted expression in leukocytes, SHIP-1 represents an interesting therapeutic target to regulate the PI3K pathway for the treatment of inflammatory diseases and cancer[112, 113]. Indeed, small molecule activators and inhibitors of SHIP-1 activity have been developed for the treatment of inflammatory diseases and cancer[113-117].
SHIP-1 was initially cloned in 1996 as a 145 kDa protein containing an N-terminal SH2 domain, a 5' phosphatase domain and a C-terminal proline rich region containing two NPXY motifs (figure 1.4)[118, 119]. Targeted disruption of SHIP-1 (Ship1\(^{−/−}\)) leads to mice that are viable, but suffer from severe hematological abnormalities including enhanced myelopoiesis and a myeloid infiltration in the lung that eventually leads to a reduced survival [120, 121].

![Figure 1.4. SHIP-1 structure](image)

**Figure 1.4. SHIP-1 structure**
Simplified protein structure of SHIP-1, modified from Rohrschneider LR et al[118]. SHIP-1 contains an N-terminus SH2 domain, for interactions with phosphorylated tyrosine residues, a central phosphatase domain for removal of the 5' inositol phosphate, two NPXY motifs which may serve as binding sites for proteins containing PTB or SH2 domains, and a proline-rich C-terminus (PxxP motifs) that serve as potential interacting sites with SH3 domain containing proteins.

The reduced life-span of Ship1\(^{−/−}\) mice, and the severe pathologies associated with ubiquitous, constitutive deletion of Ship1 throughout the hematopoietic system likely masks subtle, but important functions of Ship1 in discreet leukocyte subsets and at distinct times in vivo. To overcome this barrier, researchers have begun using targeted deletion of Ship1 in specific leukocytes via the Cre/loxP genetic system[122, 123]. Consensus loxP sites were added within the Ship1 allele, and these mice have been crossed to mice expressing the
Cre recombinase under the control of various leukocyte specific promoters, allowing for the examination of the role of Ship1 in various specific leukocyte subsets (figure 1.5).

**F₀ Generation**

Promoter specific Cre transgene (i.e. Itgax, Cd4, Cd19.....)  

“Floxed” target gene

**F₁ Generation**

Target gene is disrupted in cells expressing lineage specific promoter  

Normal expression of target gene in cells not expressing Cre

**Figure 1.5. Cre-loxP system**

Transgenic mice are generated expressing Cre under the control of various inducible (Mx1, interferon inducible) or cell/tissue specific promoters (i.e. Itgax for CD11c expressing dendritic cells, Cd4 for T cells, Cd19 for B cells). Consensus loxP sites are added to flank segments of the target gene ("flox"), and are designed not disrupt or alter normal transcription. Crossing the Cre-expressing ("deleter") mouse to mice with a floxed target gene result in the site-specific recombination of the loxP sites in cells or tissues expressing Cre, but untouched and normal target gene of expression in the remaining cell types.
1.4.4 SHIP-1 in B, T and dendritic cell function

SHIP-1 plays an important role in regulating B cell function, predominantly through its association with the inhibitory receptor FcγRIIB and CD22 on B cells[124-126]. Germline deletion of Ship1 results in reduced frequencies of circulating B cells due to an overproduction of IL-6 by SHIP-1 deficient myeloid cells[127, 128]. Specific deletion of Ship1 in B cells has confirmed some of these phenotypes are due to an intrinsic role for SHIP-1 since these mice also develop increased isotype switching following immunizations but a failure to produce high affinity antibodies, although an influence of these low affinity antibodies in the progression of inflammatory diseases has not been explored[129].

SHIP-1 plays an important role in the negative regulation of T cell receptor (TCR) signaling through its association with downstream of kinase (Dok-1/2)[130]. While Ship1−/− mice have reduced circulating T cells, there is an increase in the frequency of T regulatory cells (Tregs) [131-133]. Surprisingly, however, mice with a lineage-specific deletion of Ship1 in T cells found no role for Ship1 in regulating TCR signal strength or the frequency of Tregs[134]. There was, however, an immune skewing towards Th1 responses at the expense of Th2 responses due to increased expression of T-bet in Ship1-deficient T cells[134]. Interestingly, deletion of Ship1 at earlier stages of T cell development, as well as in myeloid cells, does result in an increase in Treg numbers, suggesting both a lineage intrinsic and extrinsic role in controlling Treg numbers[135, 136].
Despite its well-known role regulating macrophage growth[137], phagocytosis[138, 139] and cytokine release[140], the role of SHIP-1 in regulating dendritic cell function, and its significance in vivo, has largely been ignored. SHIP-1 restricts the proliferation or survival of bone marrow derived dendritic cells in response to granulocyte macrophage colony stimulating factor (GM-CSF) or fms-like tyrosine kinase 3 ligand (Flt3L)[141, 142]. SHIP-1 also facilitates the maturation of DCs following stimulation with various TLR ligands, and SHIP-1 deficient DCs have an impaired ability to promote antigen specific T cell proliferation and Th1 responses, due to reduced IL-12 secretion. The role of SHIP-1 activity in DC function in vivo, however, has yet to be explored.

1.5 Mucosal immunity

Mucosal surfaces, predominantly in the lung and gut, represent a large interface site between the host and environment that, while allowing gas and nutrient exchange, are continuously exposed to various bacterial, viral and fungal antigens[143]. To overcome this challenge, the mucosal surfaces play host to a highly specialized and tightly regulated immune system, composed of the epithelial barrier itself, innate lymphoid cells (ILCs), dendritic cells, T and B lymphocytes, mast cells, eosinophils and others. These cells must coordinate their functions and maintain a delicate balance between inflammation (responsiveness) and tolerance (non-responsiveness). Key mediators of these responses are CD4+ T cells, which can differentiate into a wide array of helper subtypes and a plethora of immune-modulatory cytokines. T-helper 2 cells produce large amounts of IL-4, IL-5 and IL-13, promoting class switching to IgE, eosinophilia, mucus production, epithelial cell turnover and smooth muscle expansion and contraction. While these responses are
beneficial when exposed to harmful pathogens, such as intestinal helminth infections, inappropriate mucosal Th2 responses are associated with allergy and asthma. A greater understanding of the cell types and mechanisms involved in is needed to facilitate therapeutic manipulation of adaptive immune responses at mucosal sites.

1.5.1 Allergic airway inflammation

Allergic asthma is a chronic inflammatory disease of the lung typically affecting the main conducting airways. It is characterized immunologically by a Th2 biased immune response, increased mucus production and eosinophil infiltrate into the lung and airways. Physiologically it is characterized by reversible airflow obstruction, or airway hyperresponsiveness (AHR), caused by an expansion of the airway smooth muscle (ASM) surrounding the bronchi[144]. Allergic asthma represents a large socioeconomic burden, and currently affects approximately 8.5% of the population and up to 13% of children[145]. In the United States, this results in an annual economic burden of up to $56 billion, comprising direct health care costs and costs associated with loss of work and economic activity[146]. Therapeutic options have remained mostly unchanged since the 1970’s, with inhaled corticosteroids, to dampen inflammation, and long-acting β-agonists, to induce smooth muscle relaxation, being the mainstay drugs used today[147]. There are subsets of patients with severe asthma that do not respond to the current therapeutic options, requiring new research to uncover new therapeutic targets.
Mouse models have been key in evaluating the cell types and molecular pathways involved in allergic inflammation. Early studies used chicken ovalbumin (OVA) as a model antigen, sensitizing animals by adsorbing OVA to the Th2 inducing adjuvant alum and delivering the OVA/alum complex intraperitoneally. Mice are then challenged through the intranasal exposure of OVA, eliciting airway eosinophilia, a Th2-biased immune response and airway hyperresponsiveness, all hallmarks of allergic asthma. This model has uncovered important roles for numerous cell types, such as B and T cells[148], mast cells[149] and eosinophils[150], as well as numerous cytokines, such as IL-4[151] and IL-13[152] in the pathogenesis of allergic asthma.

While the OVA model has been useful for uncovering important therapeutic targets, it does not represent a physiologically relevant model of allergic disease due to the route of administration (intraperitoneal systemic delivery versus inhaled sensitization in the lung) and the requirement for an artificial adjuvant like alum to elicit an inflammatory response. Up to 85% of asthmatic patients respond to house dust mite (HDM), a common household allergen, and animal models utilizing HDM as the inducing allergen are gaining in popularity as a more physiologically relevant model of allergic asthma. Intranasal exposure and sensitization results in an epithelial barrier breach and epithelial release of innate cytokines such as IL-25, IL-33 and TSLP in a TLR-4 dependent manner[153-155]. These released cytokines stimulate resident innate immune cells, leading to an inflammatory cascade that primes the lung dendritic cells to transport antigen to the draining lymph node for antigen presentation to naïve CD4+ T cells and development of a Th2 adaptive immune response (figure 1.6). This HDM model of allergic asthma has replaced the OVA model as the gold
standard for uncovering the roles of various cell types and mediators in the pathogenesis of allergic asthma.

Figure 1.6. HDM allergic sensitization
Localized delivery of common allergens (derived from house dust mites, cockroaches, cats) results in an epithelial barrier breach and release of innate inflammatory mediators such as IL-25, IL-33 and TSLP. These act on lung resident innate immune cells, such as ILC2s, to promote DC activation and migration to the draining lymph node, where antigen presentation can efficiently occur leading to the development of an adaptive immune response.

Hypersensitivity pneumonitis (HP) is another chronic inflammatory lung disease, and while it is less prevalent than allergic asthma its incidence is likely underestimated[156]. HP is caused by the repeated inhalation of environmental allergens, typically bacterial derived, and results in a Th1/Th17-biased inflammatory response characterized by a lymphocyte dominated infiltration, the formation of parenchymal granulomas and antigen-specific IgG2a antibodies, making it a quite distinct disease from allergic asthma[157, 158]. A common mouse model of this disease results from the repeated intranasal exposure of mice to the
gram-positive thermophile *Saccharopolyspora rectivirgula* (SR), a bacteria commonly found in moldy hay that causes “farmers lung” in humans, a type of HP[159]. This model has become very useful modeling the initiation of Th1/Th17 adaptive immune responses in the lung, and identifying cytokines and cell types that facilitate this unique response.

### 1.5.2 Intestinal helminth infection

While inappropriate Th2 immune responses are typically associated with allergy, mucosal Th2 responses are indeed needed for protection from helminth infection. A common helminth infection in humans is the whipworm *Trichuris trichuria*, with up to a billion people infected worldwide[160]. Mouse models of human whipworm infection have utilized the murine helminth *Trichuris muris*, with similar life cycle and route and mode of infection to the human parasite[161]. *Trichuris* eggs are delivered via oral gavage, and they travel down the intestinal tract where they hatch in the cecum, eventually releasing eggs through the feces to continue the infectious cycle. Clearance of *Trichuris* infection is critically dependent on the formation of a Th2 response, leading to “resistance” to infection. This is due to production of the Th2 cytokine IL-4, leading to IgE production, and IL-13, leading to mucus production, epithelial cell turnover and smooth muscle contraction to expel the helminth[162, 163]. Alternatively, if *Trichuris* infection leads to the formation of a Th1 response, the end result is the inability to clear infection, leading to “susceptibility” to infection[164]. This model has become immensely useful in dissecting the mechanisms involved in initiating Th2 responses that can have broad implications not just for intestinal
immunity, but also for the initiation of Th2 responses at other sites, such as the lung environment in understanding the pathogenesis of asthma.

1.6 Summary

The development of adaptive immune responses at mucosal surfaces must be tightly controlled. While appropriately formed Th2 responses are protective in instances of helminth infection, the inability to initiate tolerance and the aberrant responses to harmless antigens results in allergic diseases, such as asthma. A greater understanding of the cells involved in this process and the proteins and signaling pathways that regulate their function is needed to identify new therapeutic targets. ILC2s are enriched at mucosal sites and are an immediate source of inflammatory mediators, but their contribution to adaptive immune responses are unknown. I hypothesized that, in addition to their role in innate immunity, they would have a significant impact on the development of subsequent adaptive immune responses. Through the use of ILC2-deficient mice, I found that ILC2s play a critical role in priming the adaptive Th2 response following inhaled allergen challenge. Surprisingly, these cells were dispensable for the development of Th2 responses to systemically delivered antigens, suggesting an important role for ILC2s specifically in the mucosal environment. Additionally, ILC2s were dispensable for sensitization to Th1/Th17 responses following inhalation of bacterial antigens, suggesting a specific role in promoting Th2 immunity. The lipid phosphatase SHIP-1 is an important regulator of the PI3K pathway, and deletion of SHIP-1 throughout the hematopoietic compartment results in increased airway inflammation. I hypothesized that despite the increased inflammation observed in Ship1−/−
mice, loss of Ship1 in specific leukocyte subsets could in fact have a more subtle and potentially protective effect in allergic responses. I found that loss of Ship1 in either T cells or DCs results in protection from the development of HDM-induced allergic airway inflammation through a skewing of the immune response to a Th1-dominated response. This was further confirmed in a Trichuris infection model where loss of Ship1 in DCs resulted in susceptibility to infection through an inappropriate Th1 response.
Chapter 2. Group 2 innate lymphoid cells facilitate sensitization to local but not systemic Th2-inducing allergen exposures
2.1 Introduction

Allergic airway inflammation/asthma, is a chronic inflammatory disease characterized by a Th2-biased immune response, antibody class-switching to IgE and reversible airflow obstruction. Its prevalence has increased by 12% between 2001-2009, currently affecting 8.2% of the population in the US alone and resulting in an annual economic burden of 56 billion dollars [146]. The most prevalent therapeutic strategies were developed in the 1970’s and include the use of inhaled corticosteroids (ICS) to dampen inflammation and long-acting β-agonist (LABA) to induce smooth muscle cell relaxation [147]. Though effective, these drugs fail to target the underlying immune deregulation that leads to disease. Similarly, in developing new therapeutics, the current emphasis has been on biologics targeting downstream effectors of allergic responses; primarily antibodies directed at IgE and Th2 cytokines. These new treatment options have only shown marginal clinical success and the data suggest that targeting the effector response of allergic inflammation may not represent an optimal approach [165]. In summary, there is a current unmet need in the treatment of allergic airway disease. It is likely that a greater understanding of the mechanisms involved in the initial priming and sensitization phase could unveil novel therapeutic targets.

House dust mite (HDM) is one of the most common indoor allergens and up to 85% of asthmatics respond to this allergen. Inhaled HDM acts on airway epithelial cells in a TLR4-dependent manner, inducing the release of cytokine danger signals including IL-25, IL-33, thymic stromal lymphopoietin (TSLP) and granulocyte macrophage colony-stimulating factor (GM-CSF) [153-155]. Understanding how allergens are able to promote naïve CD4⁺
T-cell polarization to a Th2 phenotype has been hampered by the lack of a clearly defined innate cell type that produces Th2-associated cytokines (such as IL-4, IL-5 and IL-13) during the sensitization phase. Studies utilizing $\text{Rag}2^{-/-}$ mice, which lack T-cells and B-cells, identified a novel innate cell type that produced IL-5 and IL-13 in response to IL-25 [41]. Several groups have since characterized populations of innate lymphoid cells that act as robust sources of IL-5 and IL-13, but not IL-4, in various tissues, including spleen, adipose tissue, lymph nodes, lungs and the nasal mucosa [43-46, 54, 65, 166]. While initially referred to as nuocytes, natural helper cells or innate helper type 2 cells, they are now more simply referred to as ILC2s based on their unique expression profile of cell surface antigens (CD45$^{+}\text{Lineage}^{-}\text{CD127}^{+}\text{CD25}^{+}\text{CD90.2}^{+}\text{ST2}^{+}\text{Sca1}^{+}$) and their production of the type 2 cytokines IL-5 and IL-13 in response to epithelial derived IL-25, IL-33 and TSLP. ILC2s also require the transcription factors, retinoic acid receptor-related orphan receptor-α (RORα) [36, 37], GATA-binding protein 3 (GATA-3) [32, 33] and T-cell factor 1 (TCF-1)[35] for their specification and maturation. Previously, we showed that RORα-deficient mice have an impaired innate response to protease allergens in the lung [36]. However the selective role of ILC2s in the priming of different CD4$^{+}$ T helper (Th) subsets and development of adaptive immune responses in the lung has yet to be examined. We sought to investigate the influence of ILC2s and the route of antigen priming on the development of an adaptive type-2 immune response to lung allergens. Using distinct models of inducing adaptive immunity, we demonstrate a specific and highly selective role for ILC2s in promoting Th2, but not Th1 or Th17 lung inflammation in response to inhaled antigens.
2.2 Methods

2.2.1 Mice and bone marrow transplantation

C57BL/6J, B6.SJL-Ptprc<sup>a</sup> Pepc<sup>b</sup>/BoyJ (Ly5.1) and B6.C3(Cg)-Rora<sup>sg</sup>/J were purchased from The Jackson Laboratories (Bar Harbor, ME) and were bred and maintained in a specific pathogen-free environment at The Biomedical Research Centre. Pups from Rora<sup>sg/+</sup> breeders were genotyped using DNA obtained from ear-clips using primer sequences and protocols from the JAX online database. Bone marrow cells were collected from wild-type and Rora<sup>sg/sg</sup> littermates and 1x10<sup>7</sup> bone marrow cells were injected intravenously into lethally irradiated Ly5.1 recipient mice (900 rads in split doses). Peripheral blood chimerism was verified 8 weeks after transplantation by saphenous vein bleeds and all mice used had greater than 90% hematopoietic cells originating from the Ly5.2 donor mice. All protocols were approved by the animal care committee of the University of British Columbia.

2.2.2 Antibodies and flow cytometry

Staining and antibody dilutions were prepared in PBS containing 2% FCS, 2mM EDTA and 0.05% sodium azide. Samples were first blocked in buffer containing 5μg/mL anti-CD16/32 (2.4G2) to block non-specific antibody binding. Alexa-450 conjugated CD3ε (145-2C11), CD11c (N418) CD11b (M1/70), CD19 (1D3), CD45R/B220 (RA3-6B2), NK1.1 (PK136), Gr1 (RB6-8C5) and Ter119 (TER-119), PE Conjugated CD25 (PC61.5), PE-Cy7 conjugated CD3ε, B220, Sca-1 (D7), APC conjugated IL-5, APC-efluor780 conjugated B220 and efluor650-conjugated CD90.2 (53-2.1) were purchased from eBioscience (San Diego, CA). PE conjugated Siglec-F (E50-2440), PerCP-Cy5.5 conjugated CD45.2 (104) and V500
conjugated CD45 (30-F11) was purchased from BD Biosciences (San Jose, CA). FITC conjugated anti-Neutrophil (7/4) was purchased from Abcam (Cambridge, MA). FITC conjugated ST2 (DJ8) was purchased from MD Bioproducts (St Paul, MN). Pacific Blue conjugated CD45 (I3/2) and Alexa-647 conjugated CD11c (N418) and CD45.1 (Ly5.1) was produced in-house. Intracellular cytokine staining was performed with the Cytofix/Cytoperm kit (BD Bioscience) and viable cells were identified using the efluor-450, 506 or 780 fixable viability dye (eBioscience). Samples were acquired on a BD LSRII and data analysis was performed using Flowjo (Treestar, San Carlos, CA).

2.2.3 **In vitro T cell polarization**

CD4+ T-cells were isolated from the spleens and lymph nodes of naïve WT or Rora<sup>sg/sg</sup> BMT mice by negative selection using Robosep (StemCell Technologies Inc.). 2.5x10^5 CD4+ cells were cultured for 6 days in culture media (IMDM supplemented with 10% FBS, 100U/mL penicillin, 100μg/mL streptomycin and150μM MTG) with 1μg/mL each of plate bound anti-CD3 (145-2C11) and anti-CD28 (37.51) in the presence of neutral (10ng/mL IL-2), Th2 (10ng/mL IL-2, 10ng/mL IL-4 and 10ug/mL anti-IFNγ [XMG1.2]) or Th17 (10ng/mL IL-1β, 20ng/mL IL-6, 10ng/mL IL-23, 10ng/mL TNF-α, 1ng/mL TGF-β, 10ug/mL anti-IL4 [11B11] and 10ug/mL anti-IFNγ [XMG1.2]) conditions. Cell free supernatants were collected at day 6 for cytokine measurements and total RNA was extracted using Trizol for quantitative RT-PCR. Immature ILC2s were sort purified from C57Bl/6 bone marrow (CD45<sup>+</sup>Lineage<sup>-</sup>CD127<sup>+</sup>CD25<sup>-</sup>ST2<sup>+</sup>) and cultured for 7 days in media supplemented with 10ng/mL each of IL-7 and IL-33.
2.2.4 Lung explant culture

Mice were euthanized by \text{CO}_2\text{ asphyxiation} and lungs were inflated with 1.5mL \text{DMEM} containing 10\% FCS, 2-ME, Pen/Strep and 1\% low melting point agarose kept at 37\°C and cooled on ice. Lungs were sliced with a razor into \sim0.5\text{mm} thick sections placed in 2mL of culture media stimulated with either phosphate buffered saline or 50\mu\text{g/mL} house dust mite antigen. Golgi-Plug (BD Biosciences) was added to the explant cultures 6 hr before collection and a single cell suspension was made by passing the lung slices through a 70\mu\text{m} cell-strainer. Lung ILC2s were identified as Lineage\textsuperscript{−}\text{CD45\textsuperscript{+}}\text{CD90.2\textsuperscript{+}}\text{CD25\textsuperscript{+}}\text{Sca1\textsuperscript{+}}\text{ST2\textsuperscript{+}} viable cells and gated for IL-5 expression based on FMO controls. Secreted IL-5 collected in the cell free supernatant was quantified by ELISA using purified and biotin antibody pairs to IL-5 (TRFK5, TRFK4) from eBioscience.

2.2.5 Induction and assessment of allergic airway inflammation

House dust mite (HDM, \textit{Dermatophagoides pteronyssinus}) extract was obtained from Greer Labs (Lenoir, NC, containing 0.034\mu\text{g} \textit{Der p 1} and 0.095EU per \mu\text{g} of total protein) and disease was induced as described\cite{167}. Mice were treated intranasally with endotoxin-free phosphate buffered saline (PBS) or HDM on days 0, 1 and 2 with 25\mu\text{g} of total protein and on days 14, 15, 16 and 17 with 5\mu\text{g} and mice were euthanized 24 hours after the final challenge.

For Ovalbumin/Alum induced AAI, mice were injected intraperitoneally with 50\mu\text{g} chicken ovalbumin (Grade III, Sigma, St Louis, MO) adsorbed to 650\mu\text{g} aluminum hydroxide (Sigma) on days 0 and 7. Ovalbumin contained less than 0.125 EU per 100ng of protein as
measured by PYROGENT™ Gel Clot assay (Lonza, Walkersville, MD). Mice were treated intranasally on days 21, 22, 23, 25 and 27 with 50μg OVA and mice were euthanized on day 28 via intraperitoneal injection of avertin.

Hypersensitivity pneumonitis (HP) was induced as described [168]. *S. rectivirgula* was obtained from the ATCC (29034). Bacteria cultures were grown in Trypticase Soy Broth at 55°C, before being spun down and washed with endotoxin-free water. Bacterial cells were lysed and lyophilized before being re-suspended to 4mg/mL in PBS for use. Mice were exposed intranasally to 40μl (160μg) of SR antigen three times a week for 3 weeks. Mice were euthanized four days after the last intranasal exposure.

Mice were euthanized via intraperitoneal injection of avertin. Bronchoalveolar lavage (BAL) was collected by three aspirations with 1mL of sterile saline, followed by blood collection via cardiac puncture. BAL cells were enumerated and differentiated by FACS using antibodies to CD3e, CD11c, CD45, CD45R/B220, SiglecF and 7/4.

2.2.6 Detection of antigen-specific IgG$_1$, IgG$_2a$ and IgE

ELISA for total serum IgE was performed according to the manufacturer’s instructions (BD Bioscience). For detection of Ag-specific IgG$_1$ and IgG$_2a$, plates were coated with 25μg/mL SR or HDM-antigen in carbonate buffer (0.1M sodium carbonate, pH 9.5). Serum was diluted in assay diluent (PBS with 3% BSA) and added to the plates for 2 hours at 37°C. Ag-specific IgG’s were detected with HRP-conjugated antibodies to mouse
IgG₁ or IgG₂a, biotin-conjugated anti-mouse IgE (R35-72), Streptavidin-HRP and TMB substrate (BD Pharmingen).

2.2.7 Immune analysis

Lungs were minced and digested with 200U/mL collagenase IV (Sigma) for 1 hour at 37°C and a single cell suspension was obtained by passing the tissue through a 70μm cell strainer and red blood cells were lysed and leukocytes were enriched by percoll separation. Leukocytes were counted and stimulated at 4-8x10⁶ cells/mL in culture media (DMEM with 10% FCS, Pen/Strep, 50μM 2-ME and 25mM HEPES) with the indicated concentration of HDM or SR antigens for 72 hours. Cell-free supernatants were collected and cytokine levels were measured by ELISA using purified and biotin-conjugated antibody pairs to IL-13 (eBio13A, eBio1316H) or IL-17A (eBio17CK15A5, eBio17B7) from eBioscience.

2.2.8 RNA isolation and quantitative RT-PCR

Lungs were homogenized in Trizol (Life Technologies, Carlsbad, CA) using a Qiagen TissueLyser II (Valencia, CA). Total RNA was extracted and reverse transcribed using a high capacity cDNA kit (Life Technologies) and quantitative RT-PCR was performed using Sybr green (KAPA Biosystems, Woburn, MA). The primers used for qPCR are listed in table 2.1. Reactions were carried out in an ABI 7900 real-time PCR machine (Life Technologies) and values are expressed relative to Actb.

2.2.9 Histology

Lung tissue was fixed in 10% buffered formalin overnight and embedded in paraffin. Sections (5μm thick) were stained with hematoxylin and eosin. A histologic disease score
from 0-4 was attributed based on severity for peribronchial, perivascular and parenchymal
immune cell infiltration, for a total score of 12.

2.2.10 Statistics

Results represent mean±s.e.m. Student’s t test was used to determine statistical
significance.
### Table 2.1. Primer list
Forward and reverse primer pairs for gene specific detection by quantitative RT-PCR. Primers were used at a final concentration of 200mM.
2.3 Results

2.3.1 HDM induces lung ILC2 activation and IL-5 secretion

To test whether HDM could elicit ILC2-dependent production of Th2 cytokines, we utilized lung explant cultures, which maintain the lung microenvironment and epithelial-leukocyte interactions. Lung explants were stimulated with HDM antigen in vitro and ILC2 production of intracellular IL-5 was measured by flow cytometry. Lung ILC2s were identified based on their unique expression of cell surface markers: positively reacting with the pan-hematopoietic marker CD45, the stem cell antigen-1 (Sca-1) and the IL-33 receptor (ST2, Fig 2.1, A) and lack of expression of common leukocyte proteins (CD3, CD11b, B220, Gr1, NK1.1, Ter119). Gated ILC2s were also found to express IL-2Rα (CD25, Fig 2.1, B). HDM treatment of wild type explants led to a rapid induction of IL-5 synthesis compared to saline controls (Fig. 2.1, C-D), suggesting that these cells could serve as downstream effectors in response to a common human allergen.
Figure 2.1. HDM induces lung ILC2 production of IL-5
Lung explants from wild-type mice were stimulated with HDM or PBS for 18 hours, with Golgi-Plug added for the last 6 hours. Following stimulation, lung explants were dissociated and cells were prepared for flow cytometry (A) with ILC2s identified as Lineage^CD45^ST2^Sca1^ and intracellular staining for IL-5 production by ILC2s, compared to fluorescence minus one (FMO) controls. (B) Expression of CD25 by lung ILC2s. (C) Percent IL-5 positive ILC2s from lung explants stimulated with HDM or PBS. (D) IL-5 released in the supernatant of PBS or HDM stimulated lung explants was measured by ELISA at 6, 12 and 24hrs. Results are pooled from 3 independent experiments (n=3). * Error bars are means ± SEMs, * p<0.05.

2.3.2 ILC2s facilitate HDM-induced allergic airway inflammation

To examine the role of ILC2s in the development of allergic asthma, we utilized a HDM mouse model of allergic airway inflammation (AAI, Fig 2.4, A). In this model, repeated intranasal exposure of HDM antigen induces several hallmark features of clinical asthma, including airway and lung parenchymal eosinophilia, a Th2-biased immune response with high levels of IL-4, IL-5 and IL-13 secretion and antibody class-switching to IgE [153].

While ILC2s are known to produce IL-5 in response to protease antigens in the lung [45], their role in regulating the development of an adaptive immune response to physiologically relevant antigens is still unknown. To address this issue, we generated mice selectively lacking ILC2s by transplanting wild type mice (CD45.1) with bone marrow from Rora^{sg/sg} mice (CD45.2) to produce hematopoietic chimeras. These chimeric mice have a very selective defect in ILC2 production (although rare, residual, radio-resistant CD45.1^ ILC2s can be detected in transplanted chimeras (Fig 2.2, A-B)).
Figure 2.2. Reduced lung ILC2s in Rora^{sg/sg} BMT Mice
(A) Flow cytometric analysis of lung ILC2s from WT and Rora^{sg/sg} BMT mice. (B) Quantification of lung CD45.2^+ ILC2s from donor mice (WT or Rora^{sg/sg}) or residual radio-resistant ILC2s (CD45.1^+). Data are representative of 2 independent experiments (n=2-3). Error bars are means ± SEMs.

Indeed, RORα expression is tightly regulated in the hematopoietic compartment, with high expression found only on ILC2s as well as Th17, but not Th2 cells (Fig 2.3, A). Additionally, we found that isolated CD4^+ T cells from wild type or Rora^{sg/sg} BMT mice displayed no difference in their ability to polarize to Th2 cells, as marked by equal production of the Th2 cytokines IL-5 and IL-13 as well as equal expression of the transcription factor Gata3 (Fig 2.3, B-C).
Figure 2.3. RORA expression profile and function in Th2 polarization

CD4+ T cells isolated from WT or Rora<sup>sg/sg</sup> BMT mice were stimulated under neutral, Th2 or Th17-polarizing conditions for 6 days. (A) Expression of Rora transcript from T cell polarization cultures or purified ILC2s was measured by using quantitative PCR relative to Actb. (B) Secreted IL-5 and IL-13 from Th2 polarization conditions was quantified by ELISA. (C) Expression of Gata3 transcript from Th2 polarization conditions was measured by using quantitative PCR relative to Actb. Data are representative of 2 independent experiments (n=2). Error bars are means ± SEMs.

When exposed to a HDM model of AAI, Rora<sup>sg/sg</sup> chimeras exhibited a significant impairment in Th2 response induction, with a marked reduction in leukocyte infiltration, particularly eosinophils, into the airways 18 days after initial HDM exposure while challenges with saline (PBS) resulted in very low levels of airway infiltrate comprised almost exclusively of macrophages (Fig 2.4, B-C). Additionally, HDM exposure resulted in a significant expansion of ILC2s in the lungs of WT BMT mice and a low level expansion in Rora<sup>sg/sg</sup> BMT mice (Fig 2.4, D). ILC2-deficient mice also exhibited significantly reduced levels of both total serum IgE, as well as HDM antigen-specific IgE and IgG<sub>1</sub> (Fig 2.4, E), and significantly reduced expression of lung transcripts for the Th2-associated cytokines Il4

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and \(\text{Il}13\) (Fig 2.4, \(F\)). To evaluate the activity of Th2 cells in the inflamed lung tissue, isolated lung leukocytes were re-stimulated ex-vivo with HDM antigen. ILC2-deficient mice displayed a strikingly reduced recall response as indicated by decreased antigen-induced production of IL-13 (Fig 2.4, \(G\)).

**Figure 2.4. HDM-induced AAI in WT and ILC2-deficient mice**
(A) Schematic of HDM disease model. (B) Total cells and (C) eosinophils collected in BAL samples of WT or ILC2-deficient mice. (D) Total CD45.2\(^+\) lung ILC2s from PBS and HDM-exposed WT or ILC2-deficient mice. (E) Serum total IgE and HDM-specific IgE and IgG\(_1\) levels were quantified by ELISA. (F) Transcript levels of \(\text{Il}4\) and \(\text{Il}5\) from lung RNA relative to \(\text{Actb}\). (G) Lung leukocytes were isolated and restimulated \textit{ex vivo} with 50\(\mu\)g/mL HDM for 72hrs. IL-13 levels were quantified by ELISA. Data are representative of 3 independent experiments (B, C, E-G; \(n=4\)-8) or combined from 2 independent experiments (D; \(n=4\)-5). Error bars are means ± SEMs. *\(p<0.05\).

Histologically, we found that loss of ILC2s also resulted in a significant reduction in lung histopathology, with reduced leukocyte infiltration into the lung tissue, particularly in the peribronchiolar and perivascular space (Fig 2.5, A-B). Consistent with the significant reduction in BAL eosinophils in ILC2-deficient mice, the frequency of tissue infiltrating parenchymal eosinophils was also reduced as quantified by the evaluation of eosinophil specific mRNA \(\text{Prg}2\) (encoding major basic protein-1) in the lung (Fig 2.5, \(C\)). Thus, our
results suggest that RORα-dependent ILC2s facilitate the development of allergic lung inflammation.

**Figure 2.5. Airway inflammation in HDM-exposed WT and ILC2-deficient mice**

(A) Hematoxylin and eosin staining of PBS or HDM-treated WT and ILC2-deficient mice. *Scale bar = 100 μm.* (B) Lung sections were scored for peribronchial, perivascular and parenchymal infiltration (a score of 0-4 was given for each parameter, for a maximum score of 12). (C) Infiltrating lung eosinophils were quantified by *Prg2* transcript levels in total lung RNA. Data are representative of 3 independent experiments (n=4-8) *Error bars are means ± SEMs. *p<0.05.

### 2.3.3 ILC2s are dispensable for the development of Th2 responses to systemically delivered antigens

Although these data suggest that ILC2s are important mediators for the induction of Th2 cell responses to physiologically relevant antigens when delivered locally through the natural intranasal route, they do not address whether there is a similar role for these cells in priming the adaptive immune system to systemically administered antigens in the presence of adjuvants. To test this we utilized the well-described ovalbumin (OVA)/alum model of AAI
In this model, mice are primed intraperitoneally (systemically) with OVA and alum followed by intranasal challenges (local) with OVA. In contrast to our results with intranasal HDM antigen, we found that ILC2s were completely dispensable for the induction of AAI in this model. ILC2-deficient mice had similar numbers of airway infiltrating leukocytes and displayed normal recruitment of eosinophils into the airways and lung tissue (Fig 2.6, B-C, E). Likewise, we detected no significant differences in serum IgE levels or Il5 expression (Fig 2.6, D-E), suggesting a normal development of an adaptive Th2-response.

**Figure 2.6. OVA/alum-induced AAI in WT and ILC2-deficient mice**
(A) Schematic of disease model for OVA/alum-induced AAI. (B) Total cells and (C) eosinophils collected in BAL samples of WT or ILC2-deficient mice. (D) Serum total IgE levels were quantified by ELISA. (E) Transcript levels of Il5 and Prg2 from lung RNA relative to Actb. Data are representative of 2 independent experiments (n=1-7). Error bars are means ± SEMs.

**2.3.4 Systemic delivery of antigens without adjuvant elicits ILC2-independent Th2-response**

Alum acts as a strong Th2 inducing adjuvant that causes release of various danger signals, such as uric acid, that could bypass ILC2s and act on resident macrophages and
DCs. To examine whether ILC2s are required for systemic antigen priming of allergic responses in the absence of external adjuvant, we primed $Rora^{sg/sg}$ chimeras intraperitoneally with OVA, in the absence of alum, followed by intranasal challenges with OVA (Fig 2.7, A). Surprisingly, here too, ILC2-deficient mice had equivalent levels of leukocyte and eosinophilic infiltrates into the airways and tissues and exhibited no difference in IgE levels or $Il5$ expression (Fig 2.7, B-E). This suggests that systemic antigen delivery, irrespective of the addition of an exogenous adjuvant, is able to induce an ILC2-independent allergic airway inflammatory response.

![Figure 2.7](image)

**Figure 2.7. OVA-induced AAI in WT and ILC2-deficient mice**

(A) Schematic of disease model for OVA-induced AAI. (B) Total cells and (C) eosinophils collected in BAL samples of WT or ILC2-deficient mice. (D) Serum total IgE levels were quantified by ELISA. (E) Transcript levels of $Il5$ and $Prg2$ from lung RNA relative to $Actb$. Data are representative of 1 independent experiments (n=4). Error bars are means ± SEMs.

### 2.3.5 ILC2s are dispensable for local priming of Th1/Th17 responses

Although we find ILC2s are essential for Th2 sensitization to locally delivered antigens in the lung, it remains unclear whether they play any positive or negative role in the development of Th1/Th17 responses. Hypersensitivity pneumonitis (HP) is a chronic lung
inflammatory disease caused by repeated airborne exposure to predominantly organic antigens and is characterized by a Th1/Th17-biased immune response [157, 168, 169]. In humans, exposure to *Saccharopolyspora rectivirgula* (SR), a bacteria present in moldy hay, leads to Farmer’s lung (a subtype of HP), and this is recapitulated in mice after repeated intranasal exposure to this antigen (Fig 2.8, A).

Wild type and ILC2-deficient mice were treated as outlined in Fig 6A and clinical features of HP were assessed on day 20 post-induction. We found that loss of ILC2s had no effect on the ability to mount an adaptive Th1/Th17 response to bacterial SR antigens. ILC2-deficient mice developed similar levels of alveolar infiltrate with no difference in alveolar lymphocyte accumulation (Fig 2.8, B-C). Unlike allergic asthma, which is characterized by high levels of serum IgE and IgG1 typical of a Th2 immune response, HP induces production of antigen-specific IgG2a. ILC2-deficient mice had normal levels of SR-specific IgG2a and IgG1 (Fig 2.8, D). There was also no difference in Th17 responses in the lung, as measured by *Il17a* transcript levels and IL-17A production from re-stimulated lung cultures (Fig 2.8, E-F). We conclude that ILC2s are largely dispensable for Th1/Th17 inflammatory responses.

**Figure 2.8.** *S rectivirgula* (SR)-induced HP in WT and ILC2-deficient mice
2.4 Discussion

The initiation of Th2 responses, and the innate cell types responsible for the production of cytokines prior to the polarization of naïve CD4+ T-cells to a Th2 phenotype, is not well understood, and an important area of investigation. Lung ILC2s represent a recently characterized leukocyte population that reside in the naïve lung and rapidly produce Th2 associated cytokines IL-5 and IL-13 in response to inhaled proteases[36]. Here we have formally examined their role in initiation of an adaptive immune response in the lung to Th1/Th17 and Th2 antigens (summarized in Fig 2.9). For these studies we utilized Rora<sup><s>sg/sg</s></sup> bone marrow chimeras as mice that selectively lack ILC2s. Rora<sup><s>sg/sg</s></sup> mice carry a mutation in the ligand-binding domain of RORα that leads to severe neurologic defects but, within the hematopoietic compartment, also results in an extremely selective defect in ILC2 production [36, 37]. Utilizing these ILC2-deficient mice, we have shown that ILC2s represent a critical relay between HDM-induced epithelial barrier damage and the subsequent generation of an adaptive Th2 response. Interestingly, we found that while allergen exposure elicits a robust expansion of ILC2s in the lungs of wild-type mice, it also induces a modest but significant increase of ILC2s in Rora<sup><s>sg/sg</s></sup> BMT mice. This may result from functional compensation by other transcription factors (GATA-3, TCF-1, Gfi1[32-35]) known to be important for ILC2 development in situations where large amounts of factors associated with ILC2 proliferation and survival, such as IL-25, IL-33 and TSLP are
produced. While we have found ILC2s to be important for the formation of an adaptive Th2 response, they do not produce the prototypical Th2-inducing cytokine IL-4, and instead produce large amounts of IL-5 and IL-13, suggesting a role for one or both of these cytokines in the ILC2-mediated Th2 response. ILC2s have been shown to be potent inducers of eosinophil migration, either through their production of IL-5 or potentially through production of eosinophil chemotactic agents, such as eotaxin-1 or eotaxin-2. Eosinophils have been found to induce DC maturation through release of their granule protein EDN (eosinophil-derived neurotoxin) that can act as an agonist of DC TLR2 receptors[61]. In addition, ILC2 production of IL-13 could also stimulate lung resident DCs and promote either their migration to the draining lymph node or their ability to promote Th2 differentiation[170].

Human ILC2 equivalent cells have also been identified and while they are found in healthy human lungs, they are enriched in the nasal polyps of patients with rhinosinusitis, suggesting their possible relevance in disease pathology[65]. Indeed, circulating and peripheral CD34⁺ progenitor cells have also been characterized that share several key features of ILC2s (such as TSLP-mediated release of type 2 cytokines IL-5 and IL-13), and are more abundant in sputum samples from allergic patients, highlighting their clinical importance[67]. Finally, genome-wide association studies have also found polymorphisms in the RORA locus to be associated with greater risk of asthma (along with IL33 and IL13), suggesting a potential link between ILC2s and disease[171]. In summary, our data using Rora⁶⁶/⁶⁶ bone marrow chimeras now provide a formal link between these previously detected innate cytokine producing cells and the generation of adaptive allergic immune responses to local antigen stimulation.
To test whether these cells are also required in models of systemic exposure to allergens we primed mice intraperitoneally with Ova as a model antigen. Surprisingly, we found that systemic administration of antigen, along with an alum adjuvant, obviated the requirement for ILC2s in the development of a robust Th2 response. It is possible that the strong immune-stimulatory effects of alum, such as the release of uric acid and stimulation of inflammatory monocytes [172] overcame any requirement for ILC2s in the sensitization phase. To test this, we repeated the same model of systemic antigen delivery in the absence of adjuvant and again found that this too led to an ILC2-independent Th2 response. Thus, our data would argue that although ILC2s play a critical role in the initiation of local responses to antigen exposures, they are dispensable in responses to systemically delivered antigens which likely short-circuits the need for DC dependent antigen delivery to the draining lymph nodes. This finding also highlights the important interplay between lung resident ILC2s and the epithelium in shaping the innate and adaptive immune response to inhaled allergens. The lung epithelium is a potent producer of ILC2 activating cytokines, such as IL-25, IL-33 and TSLP, and HDM-induced experimental asthma is dependent upon epithelial derived signals[154].

While we found ILC2s to facilitate sensitization to the Th2-inducing antigen HDM, they were completely dispensable for sensitization to Th1/Th17-inducing antigens in a murine model of hypersensitivity pneumonitis (HP). This was surprising as RORα, as well as its related transcription factor RORγ are important for proper Th17 differentiation[52], and mice deficient in IL-17 or its receptor are protected from development of HP[157, 169]. While we found IL-17A production is reduced in Rora^{sg/sg} BMT mice in both the HDM and OVA models of allergic airway inflammation (data not shown), we found no difference in IL-
17A production in the Th17 model of HP. Thus, it is likely that SR exposure is able to induce a strong Th17-polarizing stimulus and that the RORγ expression in RORα-deficient T cells is sufficient for Th17 development, whereas HDM and OVA produce a weak Th17 inducing signal that requires synergistic activity of both RORα and RORγ for proper Th17 differentiation. The ability of ILC2-deficient mice to mount a normal response in a Th1/Th17 model of lung inflammation does not, however, preclude the potential role for other lung resident innate lymphoid cell populations in modifying responses to bacterial antigens. Our data do, however, rule out a significant role for ILC2s in this process.

In summary, our data highlight a pivotal role for ILC2s in the initiation of Th2 responses to natural routes of antigen exposure. They also highlight these cells as potential targets for therapeutics aimed at blocking the initiation of allergic responses prior to development of adaptive immunity.
Exposure of the lung epithelium to HDM antigens results in loss of barrier integrity and release of innate danger signals (TSLP, IL-25 and IL-33), which in turn activate lung resident ILC2 release of IL-5 and IL-13. This results in eosinophil recruitment and activation of immature lung resident dendritic cells (iDC). Activated lung DCs pick up antigen for transport to the draining lymph node and activation of a Th2 adaptive response. Systemic immunization with allergen obviates the need to ILC2s to mount an adaptive Th2 response. Local exposure to bacterial Th1/Th17 inducing antigens (SR) activate a pathway independent of group 2 ILCs, but may rely on other lung resident innate lymphocyte populations, such as the IL-17A and IL-22 producing ILC3s.

Figure 2.9. Model for ILC2 function in lung inflammation
Exposure of the lung epithelium to HDM antigens results in loss of barrier integrity and release of innate danger signals (TSLP, IL-25 and IL-33), which in turn activate lung resident ILC2 release of IL-5 and IL-13. This results in eosinophil recruitment and activation of immature lung resident dendritic cells (iDC). Activated lung DCs pick up antigen for transport to the draining lymph node and activation of a Th2 adaptive response. Systemic immunization with allergen obviates the need to ILC2s to mount an adaptive Th2 response. Local exposure to bacterial Th1/Th17 inducing antigens (SR) activate a pathway independent of group 2 ILCs, but may rely on other lung resident innate lymphocyte populations, such as the IL-17A and IL-22 producing ILC3s.
Chapter 3. Lineage-specific regulation of allergic airway inflammation by the lipid phosphatase SHIP-1
3.1 Introduction

Allergic asthma is a chronic inflammatory disease of the airways characterized, immunologically, by a T-helper type 2 (Th2) biased inflammatory response, high levels of IL-4, IL-5 and IL-13, eosinophilic infiltration into the airways and lung tissue, antibody class switching to IgE and increased mucus production. Despite the ever-increasing incidence of this disease, treatment options have remained stagnant over the years with inhaled corticosteroids remaining the frontline treatment option. A greater understanding of the mechanisms surrounding allergic sensitization would facilitate the discovery of novel therapeutic targets.

The phosphoinositide 3-kinase (PI3K) pathway[173, 174] is pivotal to the activation, survival and migration of allergy promoting cells, and several studies have shown the therapeutic benefit of targeting this pathway in animal models[175-179]. Unfortunately, the ubiquitous expression of PI3Ks makes them challenging to target clinically and potentially obscures cell type-specific effects of targeting this pathway at different stages of allergic airway inflammation (AAI) pathogenesis. Additionally, PI3K signaling is negatively regulated by the SH2-containing inositol 5’-phosphatase 1 (Ship1, Inpp5d), which hydrolyzes the 5’ phosphate of PI(3,4,5)P₃ generated by PI3K to produce PI(3,4)P₂, thereby inhibiting signaling downstream of PI(3,4,5)P₃. Thus, therapies aimed at targeting the PI3K pathway would benefit from a deeper understanding of how perturbing this hematopoietic-restricted negative regulator influences disease.
Ship1-deficient mice develop spontaneous airway inflammation[120, 121] as well as a host of other hematological abnormalities including enhanced myelopoiesis and reduced lymphopoiesis and erythropoiesis[120], expansion of myeloid-derived suppressor cells (MDSCs)[180] and regulatory T cells (Tregs)[132], and altered natural killer (NK) cell development[181]. While the diverse immune disorders caused by germline deletion of Ship1 reveal a key role in hematopoietic homeostasis, they likely mask subtle, yet important, functions in specific leukocyte subsets. We hypothesized that deleting Ship1 expression specifically in lineages known to be crucial for adaptive Th2 responses would uncover distinct roles that could either positively or negatively regulate susceptibility to AAI. To explore this possibility further, we selectively deleted SHIP-1 in distinct hematopoietic lineages and examined the effects on the development of AAI. Strikingly, we found that loss of SHIP-1 in either T cells or DCs attenuated the development of the Th2 responses to HDM-induced allergic inflammation while loss in B cells showed no influence on the development of disease. Our data reveal an unanticipated therapeutic effect of targeting SHIP-1 in T cells and DCs and suggest that targeting this enzyme either temporally or lineage-specifically may offer a novel approach to therapy.

3.2 Methods

3.2.1 Mice

Inpp5d^{F/F} mice (Ship1^{F/F}) were provided by Dr. W. Kerr[181]. Cd19-Cre (B6.129P2(C)-Cd19^{tm1(cre)Cgn/J}), Cd4-Cre (B6.Cg-Tg(Cd4-Cre)1Cwi/BfluJ), Itgax-Cre (Cd11c-Cre, B6.Cg-Tg(Itgax-cre)1-1Reiz/J), C57BL/6J and OT-II (B6.Cg-Tg(TcraTcrb)425Cbn/J) mice were obtained from the Jackson Laboratories (Bar Harbor,
Animals were bred and maintained in a SPF environment at The Biomedical Research Centre. All protocols were approved by the Animal Care Committee of the University of British Columbia.

### 3.2.2 Histology

Lungs were fixed in 10% buffered formalin and paraffin embedded. 5μm thick sections were stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS).

### 3.2.3 HDM-induced allergic airway disease

House dust mite (HDM, *Dermatophagoides pteronyssinus*) was obtained from Greer Laboratories (Lenoir, NC). Extracts contained 34 ng of Derp 1 and 0.095 EU/μg of total protein and were re-suspended in sterile PBS. Mice were anesthetized with isoflurane and sensitized with 25μg of HDM protein intranasally on days 0-2 and challenged with 5μg of HDM on days 13-17. Mice were euthanized 24 hours after the last HDM challenge on day 18. Bronchoalveolar lavage (BAL) fluid was collected by 3 repeated instillations and aspirations of 1mL sterile PBS and blood was collected by cardiac puncture for serum antibody analyses. BAL cells were enumerated and classified by flow cytometry.

### 3.2.4 Flow cytometry

Samples were first incubated in staining buffer containing 10% goat serum and 5μg/mL anti-CD16/32 to block non-specific binding. IL-13, IFNγ, CD11c, MHC-II, CD4, CD11b and CD45R/B220 antibodies were obtained from eBioscience (San Diego, Calif). CD103, CD40, Siglec-F, FoxP3, CD86 and CD45 antibodies were purchased from BD
Biosciences (San Jose, Calif). anti-neutrophil antibody (7/4) was purchased from Abcam (Cambridge, Mass). Intracellular staining was performed using the intracellular fixation and permeabilization buffer set or the FoxP3/Transcription factor buffer set from eBioscience. Dead cells were excluded using the eFluor fixable viability dyes (eBioscience). Samples were acquired on a BD LSRII and data analysis was performed with FlowJo software (TreeStar, San Carlos, Calif).

### 3.2.5 RNA isolation and quantitative RT-PCR

Lungs were homogenized in Trizol (Life Technologies, Carlsbad, Calif) using a TissueLyser II (Qiagen, Valencia, Calif). Total RNA was extracted and reverse transcribed using a high-capacity cDNA synthesis kit (Life Technologies) and quantitative RT-PCR was performed with Sybr green (KAPA Biosystems, Woburn, Mass). Reactions were carried out in an ABI 7900 real-time PCR machine (Life Technologies) and values are expressed relative to Actb. Primer sequences are shown in table 3.1.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actb</td>
<td>GGCTGTATTCCTCCCATCG</td>
<td>CCAGTTGTAACATGATGATG</td>
</tr>
<tr>
<td>Il4</td>
<td>TGGCTGATTTGAACAGGTC</td>
<td>CAAGCATGGATTTCCCATG</td>
</tr>
<tr>
<td>Il5</td>
<td>GATGAGGCTTCCTGCTCTACTC</td>
<td>TGGCCACACTTCTCTTTTGG</td>
</tr>
<tr>
<td>Il13</td>
<td>CCTGGCTCTTGCTTGCTT</td>
<td>GGTCTTGTGATGGTTCTC</td>
</tr>
<tr>
<td>Ifng</td>
<td>GGATGCACTTCATGATTGCC</td>
<td>CTTTTTCCTCTCGAGG</td>
</tr>
<tr>
<td>Prg2</td>
<td>ATGGGTGACTTCGATGCAAAG</td>
<td>GGGACTGGATCCGAAGT</td>
</tr>
<tr>
<td>Muc5ac</td>
<td>CAGGACTTCTCTAAATCGATCAA</td>
<td>GAAGGCTCGTACCACAGGG</td>
</tr>
<tr>
<td>Muc5b</td>
<td>GTGGCCTTCTGATGGTGT</td>
<td>GCCTCATGCTAGGGAAGACAG</td>
</tr>
</tbody>
</table>

**Table 3.1. Primer list**
Forward and reverse primer pairs for gene specific detection by quantitative RT-PCR. Primers were used at a final concentration of 200mM.
3.2.6 Detection of serum IgE, IgG₁ and IgG₂ₐ

ELISA for total serum IgE was performed according to the manufacturer's instructions (BD Biosciences). For detection of HDM-specific IgG₁ and IgG₂ₐ, plates were coated with 25 μg/mL of HDM antigen in carbonate buffer. Antigen-specific IgG’s were detected with HRP-conjugated antibodies to mouse IgG₁ or IgG₂ₐ and TMB substrate (BD Pharmingen) and reactions were stopped by the addition of 1N HCl.

3.2.7 Immune analysis

Lungs were minced and digested in 200U/mL collagenase IV (Sigma, St Louis, Mo) for 1 hour at 37°C and passed though a 70-μm cell strainer. RBCs were lysed and leukocytes were enriched using a percoll (Sigma) separation. mLNs were passed through a 70-μm cell strainer to form a single cell suspension. Isolated leukocytes were re-suspended in culture media (IMDM with 10% FBS, penicillin/streptomycin and 150 μM monothioglycerol) containing 750ng/mL ionomycin and 50ng/mL PMA (Sigma) in the presence of brefeldin A (eBioscience) for 4 hours before intracellular staining for flow cytometry. BAL fluid cytokine levels were quantified by cytometric bead array (BD Biosciences).

3.2.8 In vivo DC migration

Mice were challenged i.n. with 50μg of DQ-OVA (Molecular Probes, Eugene, OR) and 100μg of HDM antigen. Draining mediastinal LNs were collected 24 hours later and analyzed by flow cytometry.
3.2.9 OT-II adoptive transfer

Mice were sensitized i.n. on days 0-2 with 25μg HDM and 100μg OVA (Grade V, Sigma) and again with 5μg of HDM and 100μg of OVA on days 13-15. Naïve CD4+ T cells were isolated from the spleens and LNs of OT-II mice using magnetic separation (StemCell Technologies, Vancouver, Canada) and stained with CFSE (Molecular Probes). CFSE labeled CD4+ OT-II cells (2.5x10^6) were injected i.v. on day 13 and mice were sacrificed on day 16.

3.2.10 Measurement of airway resistance

Airway hyperresponsiveness was measured on day 18, 24 hours following the final HDM challenge. Mice were anaesthetized with tribromoethanol (Avertin, Sigma, 250mg/kg i.p.), tracheotomized with a blunted 18G needle and connected to a FlexiVent small animal ventilator (SCI REQ, Montreal, QC, Canada). Animals were paralyzed with pancuronium bromide (Sigma, 0.5mg/kg i.p.) and were administered increasing doses of methacholine (Sigma) through the jugular vein. Total resistance in response to methacholine was measured using the snapshot perturbation.

3.2.11 DC culture and adoptive transfer

Splenic DCs were expanded in vivo by injecting Flt3L expressing B16 melanoma cells subcutaneously (1x10^7 cells) into the lower back of Ship1^F/F and Ship1^ADC mice. Splenic CD11c+ DCs were purified using magnetic separation (StemCell Technologies). DCs were stimulated with 100μg/mL of HDM antigen or co-cultured with CD4+ OT-II cells in the presence of 100μg of OVA and 100μg of HDM for 72hrs and secreted IL-12p40 and
IFNγ was quantified by ELISA (eBioscience). Adoptive transfer experiments were performed as described previously[80], with some modifications. DCs were pulsed for 16-24hrs with 100μg of HDM and 2.5x10^5 Ag-pulsed DCs were delivered i.n. into naïve C57Bl/6 mice. One week later, mice were challenged for five consecutive days with 10μg of HDM and were sacrificed 24hrs after the final HDM challenge.

3.2.12 Statistics

Results are presented as means ± s.e.m. or means ± s.d. (Fig 7A-C). The Student t test was used to determine statistical significance (*: p≤0.05).

3.3 Results

3.3.1 Lineage-specific deletion of Ship1 fails to induce spontaneous lung inflammation

Ubiquitous deletion of Ship1 leads to a myeloproliferative disorder, spontaneous lung inflammatory disease and shortened lifespan that prohibit evaluation of its role in adaptive immune responses to inhaled allergens. To examine Ship1’s role in specific leukocyte subpopulations, mice carrying a Ship1 gene flanked by LoxP sites were crossed to mice expressing the Cre recombinase under the control of the Cd19, Cd4 and Itgax (Cd11c) promoters. This lead to selective deletion of Ship1 expression in B cells (Ship1^ΔB cell), T cells (Ship1^ΔT cell) and DCs (Ship1^ΔDC), respectively. Interestingly, none of these mice developed spontaneous lung inflammation (Fig 3.1), suggesting deletion of Ship1 in another cell type (or a combination of cell types[182]) is required to induce the naïve lung inflammation observed in ubiquitous knockouts.
Figure 3.1. Lineage specific deletion of Ship1 does not result in spontaneous lung inflammation

H&E and PAS stained lung sections from PBS exposed Ship1^{F/F}, Ship1^{ΔB cell}, Ship1^{ΔT cell} and Ship1^{ΔDC} mice. Original magnification is 100X, scale bars = 100μm, n=3.

3.3.2 B cell specific deletion of Ship1 has no effect on progression of AAI

SHIP-1 plays an important role in the negative regulation of BCR signaling through its association with the inhibitory motif of FcγRIIB and previously Ship1^{ΔB cell} mice were reported to have a mildly Th1-biased antibody response and a lower threshold for negative selection, leading to lower titres of antigen-specific antibodies following systemic immunization[129].
We therefore examined whether B cell specific deletion of Ship1 would alter the severity of house dust mite (HDM)-induced allergic airway inflammation (AAI). Surprisingly, wild-type (WT, Ship1^{F/F}) and Ship1^{ΔB cell} mice exhibited similar numbers of total leukocyte and eosinophilic infiltrates into the alveolar space (Fig 3.2A), and into the lung tissue as measured by expression of the eosinophil specific transcript Prg2 (MBP, Fig 3.2B). Ship1^{ΔB cell} mice also had no observable defect in their humoral response to HDM challenge and produced WT titres of total IgE and antigen specific IgG1 (Fig 3.2C-D). There was also no apparent defect in the formation of adaptive Th2 responses, since these mice exhibited WT levels of the transcripts for Th2-associated cytokines Il4, Il5 and Il13 and the Th1-associated cytokine Ifng in total lung RNA (Fig 3.2E). Correspondingly, they exhibited near-WT cytokine production from CD4^+ cells isolated from the draining lymph node as measured by intracellular flow cytometry (Fig 3.2F). In accordance with WT levels of Th2 cytokines, there was no difference in mucus production in the lungs as measured by expression of Muc5ac and Muc5b genes (Fig 3.2G). Histological examination of lung sections from HDM-exposed wild-type and Ship1^{ΔB cell} mice further confirmed equivalent levels of inflammation and mucus production (Fig 3.2H). In summary, deletion of SHIP-1 in B cells does not alter the development of HDM-induced AAI.
Figure 3.2. B cell expression of Ship1 does not influence HDM-induced AAI
(A) BAL leukocyte differentials and cell counts from HDM-exposed mice. (B) Lung
eosinophil Prg2 mRNA expression relative to Actb. Levels of serum (C) IgE and (D) HDM-
specific IgG1. (E) Lung mRNA expression of Il4, Il5, Il13 and Ifng relative to Actb. (F)
Intracellular cytokine expression of mLN CD4+ cells measured by flow cytometry. (G) Lung
mRNA expression of mucus associated mRNA Muc5ac and Muc5b relative to Actb. (H)
H&E and PAS stained lung sections from HDM exposed Ship1F/F and Ship1ΔB cell mice.
Original magnification is 100X, scale bars = 100μm. Data are means±s.e.m. and are
representative of 2 independent experiments, n=3-5 mice per experiment.

3.3.3 T cell specific deletion of Ship1 leads to a Th1-biased response and protection
from AAI

While conventional SHIP-1 deficient mice (Ship1−/−) exhibit expansion of the
regulatory T cell (Treg) pool and a myeloproliferative disorder, mice with a T cell-specific
deletion of SHIP-1 develop normally but have an increase in cytotoxic T cells and a biased
polarization towards a Th1 response due to increased levels of T-bet expression[134]. To
determine how this influences AAI, Ship1ΔT cell mice were primed and challenged with HDM
antigen. In contrast to WT mice, Ship1ΔT cell mice exhibited reduced eosinophilic
inflammation in both the airways and lung parenchyma (Fig 3.3A-B). This reduction was not
due to changes in lung Treg frequencies since we found no significant difference between
WT and Ship1ΔT cell mice (Fig 3.3C). Ship1ΔT cell mice also failed to develop robust Th2
responses, and we were unable to detect Th2 cytokines IL-4 and IL-13 in the
bronchoalveolar lavage fluid (BALF, Fig 3.3D) and observed significantly reduced levels of
Th2 cells in the lungs and draining mediastinal lymph nodes of HDM-exposed animals.
These were replaced by a significant increase in Th1 cells in HDM-exposed Ship1ΔT cell mice
as measured by CD4+ IFNγ+ cells in the lung and mLN (Fig 3.3E-F) and in lung tissue RNA
(Fig 3.3G). Consistent with the enhanced IFNγ expression, Ship1ΔT cell mice were found to
have increased titres of HDM-specific serum IgG2a and reduced titres of IgE (Fig 3.3H-I).
Transcription of goblet cell-associated mucus genes was also attenuated in HDM-challenged Ship1\textsuperscript{ΔT}\textsubscript{cell} mice due to the significantly attenuated Th2 response and reduced IL-13 expression (Fig 3.3J). HDM-exposure did lead to significant inflammation in Ship1\textsuperscript{ΔT}\textsubscript{cell} mice, as evidenced by inflammatory cell infiltration into the lung at levels comparable to HDM-exposed mice, but this was not accompanied by an increase in mucus production due to the Th1-skewed response (Fig 3.3K). In summary, the data suggest that Ship1\textsuperscript{ΔT}\textsubscript{cell} mice are protected from HDM-induced AAI through a generalized skewing of their adaptive immune response away from a Th2 response towards a Th1-biased response.
Figure 3.3. T cell expression of Ship1 restricts Th1 adaptive responses to HDM
(A) BAL and (B) lung leukocyte differentials and cells counts from HDM-exposed mice. (C) Flow cytometry of lung Tregs (FoxP3^+CD25^+) as a percentage of CD4^+CD45^+ leukocytes. (D) BALF levels of IL-4, IL-13 and IFNγ were measured by CBA. (E) Lung and (F) mLN intracellular cytokine expression by CD4^+ cells measured by flow cytometry. (G) Lung mRNA expression of Il13 and Ifng relative to Actb. Serum (H) total IgE and (I) HDM-specific IgG1 and IgG2a was measured by ELISA. (J) Lung mRNA expression of Muc5ac and Muc5b relative to Actb. (K) H&E and PAS stained lung sections from HDM exposed Ship1^{F/F} and Ship1^{ΔT} cell mice. Original magnification is 100X, scale bars = 100μm. Data are means±s.e.m. and are representative of 2 independent experiments, n=3-4 mice per experiment, * p<0.05.

3.3.4 Dendritic cell specific deletion of Ship1 protects from AAI

While in vitro studies have suggested an important role for SHIP-1 in regulating dendritic cell (DC) activation and maturation[141, 142, 183], the role of SHIP-1 in DC function in vivo has yet to be explored. Surprisingly, in response to HDM challenge, Ship1^{ΔDC} mice also displayed reduced eosinophilic infiltration in both the airways and lung parenchyma (Fig 3.4A-B). This was accompanied by a slight but significant increase in lung macrophage and DC numbers. Mucus production in the airways, a hallmark of severe allergic lung inflammation, was also reduced in Ship1^{ΔDC} mice (Fig 3.4C). Lung DCs serve as important sentinels for inhaled pathogens, and undergo an intricate maturation and migration process in order to efficiently prime an adaptive immune response. Resident lung DCs first phagocytose and process inhaled antigens, followed by up-regulation of co-stimulatory molecules prior to migration to the draining lymph node where they induce naïve T cell proliferation and activation. SHIP-1’s ability to regulate PIP3 levels could influence each of these steps since it has been reported to have functions in phagocytosis[138], maturation[141], directional migration[184] and T cell presentation[183].
To track the phagocytic and migratory activity of resident WT and $Ship1^{ΔDC}$ DCs in vivo, mice were exposed intranasally to a mixture of HDM antigen and fluorescently-labeled ovalbumin (DQ-OVA) prior to flow cytometric evaluation of labeled cells in the draining lymph node 24 hours later. Interestingly, $Ship1^{ΔDC}$ mice exhibited no defect in the ability to acquire antigen and migrate to the mLN (Fig 3.4D); there was no difference in the relative frequency of antigen positive CD11b+ or CD103+ DC subsets in the mLN (Fig 3.4E) or in the total influx of DCs into the mLN (Fig 3.4F). Additionally there was no difference in the expression of the surface markers CD40 and CD86 on mLN DCs following HDM exposure (Fig 3.4G). Thus, we conclude that the protective effect of $Ship1$ loss was not due to a defect in DC migration or activation. The ability of DCs to induce antigen specific T cell proliferation was also examined in vivo using adoptively transferred CFSE labeled CD4+ T cells from OT-II transgenic mice. Labeled OT-II cells were intravenously transferred into WT and $Ship1^{ΔDC}$ mice sensitized with HDM and OVA antigens, followed by subsequent intranasal challenge with HDM and OVA. After 72 hours, mLNs were removed and OT-II proliferation was examined by CFSE dye dilution. There was no detectable difference in T cell proliferation in WT and $Ship1^{ΔDC}$ mice (Fig 3.4H), which further confirmed similar DC migration and activation following HDM exposure.
Figure 3.4. Loss of Ship1 in DCs protects from development of HDM-induced AAI

(A) BAL and (B) lung leukocyte differentials and cells counts from HDM-exposed mice. (C) Lung mRNA expression of Muc5ac and Muc5b relative to Actb. (D) Flow cytometry gating strategy for CD11b+ and CD103+ mLN DCs from HDM/DQ-OVA exposed Ship1<sup>F/F</sup> and Ship1<sup>ΔDC</sup> mice. (E) Frequency of DQ-OVA+ CD11b+ and CD103+ DCs in the mLN 24hrs after HDM/DQ-OVA exposure. (F) Total number and expression (MFI) of (G) CD40 and CD86 on mLN DCs (CD11c<sup>+</sup>MHC-II<sup>+</sup>) 72hrs after HDM exposure. (H) CFSE dilution of adoptively transferred OT-II CD4<sup>+</sup> T cells in the mLN of HDM/OVA exposed mice. Data are means±s.e.m. and are representative of 2 independent experiments, n=3-6 mice per experiment. * p<0.05.
3.3.5 *Ship1*-deficient DCs preferentially prime Th1, versus Th2, responses *in vivo* in response to HDM-exposure

Having ruled out a role for SHIP-1 in DC migration and activation, we examined their ability to generate an appropriately polarized adaptive T cell response to HDM antigen. CD4+ T cells isolated from HDM-exposed *Ship1ΔDC* mice exhibited significantly less IL-13 production and instead produced high levels of IFNγ (Fig 3.5A-B). These data suggest that *Ship1ΔDC* mice have an impaired ability to polarize naïve CD4+ to the Th2 phenotype that is typically elicited in HDM-induced models of AAI and instead promote the formation of a Th1 response. In further support of this notion we found significantly reduced expression of *Il4*, *Il5*, and *Il13* transcripts in the lung tissues of HDM exposed *Ship1ΔDC* mice (Fig 3.5C). This immune skewing was also observed in the humoral response, with reduced levels of the Th2-associated IgE and IgG1 and a compensatory increase in Th1-associated IgG2a (Fig 3.5D-E) in HDM exposed *Ship1ΔDC* mice when compared to their WT counterparts.
Figure 3.5. DC expressed Ship1 facilitates Th2 sensitization to HDM

(A) Gating strategy for intracellular IL-13 and IFN-γ expression in CD4+ mLN lymphocytes. (B) Frequency of cytokine expressing CD4+ cells in the lung and mLN of HDM-exposed mice. (C) Lung mRNA expression of Il4, Il5 and Il13 relative to Actb. Serum (D) total IgE and (E) HDM-specific IgG1 and IgG2a was measured by ELISA. Data are means±s.e.m and are representative of 2 independent experiments, n=4-6 mice per experiment (n.d., not detected), * p<0.05.

While the Th1-biased response in Ship1ΔDC mice led to a reduction in HDM-induced BAL infiltrates (Fig 3.4A), eosinophilic infiltration (Fig 3.4A-B) and mucus production (3.4C), these mice still exhibited similar numbers of total parenchymal infiltrates (Fig 3.4B) and equivalent lung inflammation determined by histologic evaluation of sections from HDM-exposed Ship1°F/F and Ship1ΔDC mice (Fig 3.6). This suggests that while Ship1ΔDC mice are
protected from HDM-induced eosinophilic infiltration into the airways (Fig 3.4A), they still
develop lung inflammation dominated by macrophages and lymphocytes but not
eosinophils.

![Image of lung sections from HDM exposed Ship1<sup>F/F</sup> and Ship1<sup>ΔDC</sup> mice.](image)

**Figure 3.6. Lung inflammation and mucus production in Ship1<sup>ΔDC</sup> mice**

H&E and PAS stained lung sections from HDM exposed Ship1<sup>F/F</sup> and Ship1<sup>ΔDC</sup> mice. Original magnification is 100X, scale bars = 100μm. Data are representative of 2 independent experiments, n=4-6 mice per experiment.

To evaluate whether there is a physiological benefit in modifying the type of lung inflammation experienced by Ship1<sup>ΔDC</sup> mice, we looked at changes in airway resistance following HDM exposure in response to methacholine challenge. Indeed, Ship1<sup>ΔDC</sup> mice were found to have significantly reduced airway resistance compared to Ship1<sup>F/F</sup> mice (Fig 3.7). This suggests that modification of the type of inflammatory response following HDM exposure in Ship1<sup>ΔDC</sup> mice to a Th1 dominated response, while resulting in equivalent amounts of lung inflammation (Fig 3.6), significantly reduces the airway hyperresponsiveness typically associated with the Th2-dominated HDM-induced allergic airway inflammation experienced in wild-type mice.
Figure 3.7. Reduced airway hyperresponsiveness in HDM exposed Ship1ΔDC mice
Total lung resistance in response to increasing doses of methacholine delivered through the jugular vein was measured in HDM-exposed Ship1F/F and Ship1ΔDC mice. Data are means±s.e.m and are representative of pooled results from 2 independent experiments, n=10-11 mice per group, * p<0.05.

3.3.6 Ship1-deficient DCs induce Th2 polarization in vitro and in vivo

Alveolar macrophages (AMΦs) constitutively reside in the lung and are integral to maintaining lung homeostasis. Since these express high levels of CD11c, it is likely that Ship1ΔDC mice delete SHIP-1 in this important leukocyte population as well. To ensure that the Th1 polarization phenotype observed in Ship1ΔDC mice was due to loss of Ship1 in DCs and not AMΦs, we isolated splenic DCs from Ship1F/F and Ship1ΔDC mice and evaluated their response to HDM stimulation in vitro. Both WT and Ship1-deficient DCs induced equivalent proliferation of antigen-specific T cells (Fig 3.8A), supporting our findings in vivo (Fig 3.4H). However, Ship1-deficient DCs produced significantly more IL-12p40 than their WT counterparts (Fig 3.8B) and, when co-cultured with naïve CD4+ T cells, produced
significantly more IFNγ (Fig 3.8C), suggesting an increased ability to promote Th1 responses. Adoptive transfer of in vitro HDM-pulsed Ship1-deficient DCs into naïve C57Bl/6 recipients resulted in reduced eosinophil infiltrates into the airways and lung parenchyma (Fig 3.8D-E), an increased frequency of CD4+IFNγ+ T cells in the mLN (Fig 3.8F) and a significant reduction in serum IgE (Fig 3.8G). Thus, adoptive transfer of Ship1-deficient DCs recapitulates the effects we observe in Ship1ΔDC mice.

Figure 3.8. Ship1-deficient DCs induce Th1 polarization in vitro and in vivo to HDM (A) Proliferation of CFSE-labeled CD4+ OT-II cells cultured with OVA/HDM pulsed Ship1F/F and Ship1ΔDC DCs. (B) IL-12p40 production from HDM stimulation Ship1F/F and Ship1ΔDC DCs. (C) IFNγ production from CD4+ OT-II cells cultured with non-pulsed (media) or OVA/HDM pulsed Ship1F/F and Ship1ΔDC DCs. (D) BAL and (E) lung leukocyte differentials and cell counts from mice adoptively transferred HDM-pulsed WT or Ship1ΔDC DCs. (F)
Frequency of intracellular cytokine expressing CD4$^+$ cells in the mLN of HDM-exposed mice. (G) Serum IgE levels in Ship1$^{F/F}$ and Ship1$^{ADC}$ DC transferred animals was quantified by ELISA. Data are means±s.d. (A-C) or means±s.e.m. (D-G) and are representative of 3 independent experiments (A-C) or 1 experiment (D-G, n=5 mice per experiment), * p<0.05.

3.4 Discussion

Allergen exposure activates the PI3K pathway and thereby facilitates inflammatory responses[185]. Correspondingly, inhibition of PI3K activity has been found to be effective in treating AAI in animal models[174-179, 185]. In leukocytes, the lipid phosphatase SHIP-1 acts as a key negative regulator of PI3K signaling, and mice lacking this enzyme develop spontaneous airway inflammation and have a reduced lifespan. These severe hematological defects could serve to obscure its function in specific leukocyte subsets and here we have used lineage-specific deletion to examine its composite role in allergic disease. We show that while deletion of Ship1 in the B cell lineage has no effect, deletion of Ship1 in either the T cell or DC compartment protects mice from Th2 disease by inducing a Th1-biased adaptive response to the, typically, Th2-inducing HDM allergen. This correlates well with recent clinical data showing that SHIP-1 is a viable and attractive therapeutic target for treating allergen induced airway inflammation[117]. It is possible that specific targeting of SHIP-1 activity, either positively or negatively, in a lineage specific manner could lead to greater reductions in allergic airway inflammation.

SHIP-1 is known to be a key intermediary of signaling through the inhibitory motif of the FcγRIIB receptor[124] and plays an important role in B cell development, survival, isotype class switching and antibody production[128, 129, 186, 187]. Surprisingly, loss of Ship1 in B cells did not affect HDM-induced AAI, as Ship1$^{ΔB}$ cell mice had similar levels of leukocyte infiltrates, mucus production and Th2-associated cytokines compared to their WT
counterparts. There was also no observable defect in their humoral response, with equal levels of both total serum IgE as well as HDM-specific IgG1. This is in stark contrast to previous findings documenting impaired antibody responses in B cell specific Ship1-deficient mice following infection or immunization[129]. These discrepancies could reflect the types of antigen used and resulting immune response elicited, as well as differences in the route of immunization.

Previously, lineage-specific deletion of Ship1 in T cells has been shown to enhance CD8+ cytotoxic T cell activity, increase T-bet expression and impair the ability to mount an effective Th2 response to helminth infections[134]. In support of these reports, we found that Ship1ΔT cell mice fail to mount a robust Th2 response to HDM antigen exposure and instead produce a strong Th1 response. Protection from Th2 disease is likely due to Th1 skewing since we did not observe significant differences in Treg frequencies in Ship1ΔT cell mice. This supports previous findings and suggests that while deletion of Ship1 in either the myeloid or early lymphoid lineages leads to increased Treg frequencies, deletion at later stages of lymphocyte development does not affect Treg numbers[134, 135, 188].

While in vitro experiments have suggested a role for SHIP-1 in regulating DC development, maturation and T cell interactions, its role in vivo has not been explored[141, 142, 183]. In this study, we used targeted deletion in dendritic cells to evaluate its function in development of adaptive immune responses. Ship1ΔDC mice failed to mount an effective Th2 response to HDM antigen and instead produced an aberrant Th1 response that lead to reduced eosinophilia, mucus production and airway hyperresponsiveness that are hallmarks
of HDM-induced AAI. While Ship1-deficient DCs were able to phagocytose and process antigen, migrate to the lymph node and induce T cell proliferation, they induced Th1 rather than Th2 polarization of naïve CD4⁺ T cells. This could be due to alterations in the factors secreted by Ship1-deficient DCs, since previous reports have found that loss of Ship1 in myeloid cells can result in aberrant production of IL-12 and a skewed Th1 response to intestinal helminth infection[189]. It is important to note that using Cre recombinase expressed under the control of the CD11c promoter likely leads to deletion of Ship1 expression in alveolar macrophages (AMΦs) in addition to DCs. Using in vitro and in vivo assays with DCs obtained from Ship1<sup>F/F</sup> and Ship1<sup>ΔDC</sup> mice, we showed that loss of Ship1 in DCs alone was sufficient to induce Th1 polarization, as Ship1-deficient DCs produced high levels of IL-12p40 following HDM stimulation and preferentially induced Th1 polarization of wild-type T cells in vitro and in vivo. This, however, does not rule out a potential role for SHIP-1 in AMΦ development, survival or function as we did see a small but significant increase in AMΦs in the lungs of Ship1<sup>ΔDC</sup> mice.

In summary, the severe pathologies found in constitutive Ship1<sup>−/−</sup> mice renders them difficult to evaluate in disease models. Here we have successfully used lineage-specific deletion of Ship1 from either B cells, T cells or DCs to further reveal its functional significance in allergic responses and have uncovered novel roles for T cells and DCs. In aggregate, our data suggest that the temporal inactivation of Ship1 in these lineages could prove therapeutic in instances of severe Th2-driven lung inflammatory disease and justify further investigation of the efficacy of pharmacological inhibitors of SHIP-1.
Chapter 4. Dendritic cell expression of *Ship1* regulates immunity to helminth infection
4.1 Introduction

Immunity to the intestinal helminth parasite *Trichuris muris* is critically dependent on the formation of an appropriate adaptive immune response. If parasite infection leads to a type 2-immune response, characterized by T-helper type 2 (Th2) CD4\(^+\) T cells, the outcome is a clearance of worms and “resistance” to infection. This is accomplished by production of the Th2 cytokines IL-4 and IL-13 that serve to promote isotype class switching and subsequent production of IgE and IgG\(_1\) by plasma cells, increase mucus production from goblet cells and increase smooth muscle contractility to facilitate clearance of the infection[162, 163, 190, 191]. Conversely, if infection leads to the formation of a T-helper type 1 (Th1) polarized immune response the end result is “susceptibility”, an inability to clear the parasite and a chronic infection[164, 192]. This is due to production of IL-12, IL-18 and IFN\(_\gamma\), which are ineffective in eliminating the parasite and, in some cases, facilitate the chronic infection.

While it’s clear that a Th2 response is required for development of protective immunity, the events leading to the induction of a Th2 response are still under debate. Dendritic cells (DCs) are the predominant antigen-presenting cells (APC) of the immune system, responsible for detecting pathogen-associated molecular patterns (PAMPs) from invading species through expression of various pattern recognition receptors (PRRs). DCs rapidly infiltrate the intestine following *Trichuris* infection[193], and significantly more DCs are found in the intestines of resistant animals (Balb/c, C57Bl/6) compared to susceptible mice that fail to clear the infection (AKR), suggesting an important role for DCs in priming an appropriate adaptive response[194]. While this suggests an important role for DCs in
facilitating immunity to *Trichuris* infection, few studies have examined which DC subsets and which functional molecules are required and how they guide the immune response to be protective or to facilitate chronic infection. Previously, we examined the response of mice lacking the DC and T cell specific integrin, CD103, which facilitates the trafficking of these cells to mucosal epithelia and is known to be expressed by a subset of T cells that regulate tolerance. Surprisingly, loss of CD103 from DCs (as well as CD103*+* T cells in *Itgae<sup>-/-</sup>* mice) had no effect on the ability to clear the infection[195]. In summary, the role of DCs in resistance or susceptibility to *Trichuris* infection remains enigmatic.

The phosphoinositide 3-kinase (PI3K) pathway is involved in a number of cellular processes and its activity is typically associated with cellular survival, migration and cytokine production. Negative regulation of PI3Ks is achieved by the SH2-containing inositol 5'-phosphatase 1 (*Ship1, Inpp5d*), which serves to hydrolyze the 5' phosphate of PI(3,4,5)P<sub>3</sub> generated by PI3K activity to produce PI(3,4)P<sub>2</sub>. *Ship1* expression is restricted to the hematopoietic lineages, and *Ship1*-deficient mice (*Ship1<sup>-/-</sup>* ) suffer a host of hematological disorders, including enhanced myelopoiesis, reduced lymphopoiesis and erythropoiesis, Th1-biased lymphocyte responses, lung consolidation and a shortened lifespan[120, 121, 134]. While SHIP-1 is known to influence DC maturation and function *in vitro*, the role of SHIP-1 in DC function *in vivo* has yet to be explored[141, 142, 183]. Previous reports have found that *Ship1* expression increases dramatically in the intestine following *Trichuris* infection[189]. Additionally, loss of *Ship1* throughout the hematopoietic system, or specifically in myeloid cells (macrophages and neutrophils), leads to aberrant IL-12 production and a chronic infection[189]. Here, we have generated dendritic cell-specific
Ship1-deficient mice (Ship1ΔDC) and show that loss of Ship1 expression specifically in DCs is sufficient to induce increased IL-12 production and susceptibility to Trichuris infection. Our results indicate that perturbation of DC function through the loss of Ship1 impairs the ability to mount an appropriate Th2 adaptive immune response to Trichuris. These results argue for a critical role of SHIP-1 in the early phase of DC-dependent T cell polarization and subsequent protective immunity.

4.2 Methods

4.2.1 Mice

Inpp5dff (Ship1ff) mice were described previously and provided by W. Kerr[181]. Itgax-Cre mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Animals were bred and maintained in a SPF environment at the Biomedical Research Centre and all animal work was approved by the Animal Care Committee of UBC.

4.2.2 Spleen and peripheral blood leukocyte analysis

Peripheral blood was sampled by saphenous vein and collected into microvette EDTA coated tubes (Sarstedt, Montreal, QC). Mice were euthanized via CO2 exposure and spleens were passed through a 70μm cell-strainer to obtain a single cell suspension. Red blood cells were lysed in peripheral blood and splenocyte samples using ammonium chloride buffer (150mM NH4Cl, 1mM KHCO3, pH 7.3). Total splenocyte cells were enumerated using a hemocytometer. Leukocyte differentials were obtained using flow cytometry and fluorescently conjugated antibodies to CD3, CD11b and Gr1 (eBioscience,
San Diego, CA); CD11c, CD19 and CD45 (in-house). Samples were collected on BD LSR II (San Jose, CA) and analyzed using FlowJo (TreeStar, San Carlos, CA).

4.2.3 *T. muris* infection

Mice were infected on day 0 with 225-250 embryonated eggs and parasite burdens were quantified on day 21 post-infection. *Trichuris muris* excretory-secretory antigens and eggs were collected as described previously[196].

4.2.4 Histology

Cecal tissues were fixed overnight in 10% buffered formalin and paraffin-embedded. 5μm thick tissue sections were stained with periodic acid-Schiff (PAS) for analysis.

4.2.5 Immune response

Mesenteric lymph nodes (mLN) were excised and passed through a 70μm cell strainer to generate a single cell suspension. mLN cells (4x10⁶/mL) were cultured for 72hrs in media containing 1μg each of antibodies against CD3 (145-2C11) and CD28 (37.51, eBioscience). Cytokine production from cell free supernatant was quantified by ELISA using commercially available antibodies (eBioscience). Total serum IgE was quantified by ELISA (BD Biosciences, San Jose, CA). *Trichuris*-specific serum IgG’s were determined using *Trichuris* antigen coated ELISA plates (5μg/mL overnight in carbonate buffer) and HRP-conjugated mouse IgG₁ and IgG₂a antibodies followed by TMB (BD).
4.2.6 Flow cytometry

Isolated mLN cells were stimulated with 50ng/mL of PMA and 750ng/mL of ionomycin (Sigma, St Louis, MO) in the presence of Brefeldin A (eBioscience) for 4 hours. Intracellular flow cytometry was performed using antibodies to CD4, CD11c, CD45, IL-12p40, IL-13 and IFNγ. Dead cells were excluded using eFluor fixable viability dyes and fixation and permeabilization was performed using the IC staining kit (eBioscience). Samples were acquired on a BD LSRII and data analysis was performed with FlowJo software.

4.2.7 RNA isolation and qPCR

Proximal colon tissue samples were homogenized with Trizol and RNA was reverse transcribed using a cDNA synthesis kit (Life Technologies, Carlsbad CA). qPCR was performed with Sybr green (KAPA Biosystems, Woburn, MA) using gene specific primer pairs listed in table 4.1. Values are expressed relative to Actb.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
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<tbody>
<tr>
<td>Actb</td>
<td>GGCTGTATTTCCCTCCATCG</td>
<td>CCAGTTGGAACAAATGCCCATGT</td>
</tr>
<tr>
<td>Il4</td>
<td>TCGGCATTTCAGAGGTC</td>
<td>CAAGCATGGACATCCTTTG</td>
</tr>
<tr>
<td>Il5</td>
<td>GATGAGGCTTCCCTGACTCT</td>
<td>TCACACACTCTCTCTGG</td>
</tr>
<tr>
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<td>ACAGGGAGGTCCTGGTGTTCT</td>
</tr>
<tr>
<td>Il13</td>
<td>CCTGGCTCTTGTCCTGCTT</td>
<td>GGTCTTGTGTGATGTTTCT</td>
</tr>
<tr>
<td>Il4</td>
<td>GGATGCATTCCAGATATGGCC</td>
<td>CACCGTGCTCTTTCCCTT</td>
</tr>
<tr>
<td>Tnfa</td>
<td>CATCTTCTCAATTGAGTGACAA</td>
<td>TGGGAGTAGACAAAGGTACAACC</td>
</tr>
<tr>
<td>Relmb</td>
<td>ATGGGTGCTACTGGATGTGCTT</td>
<td>AGCAGTGGCAGTGCAAGTA</td>
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Table 4.1. Primer list

Forward and reverse primer pairs for gene specific detection by quantitative RT-PCR. Primers were used at a final concentration of 200mM.
4.2.8 *In vivo* antibody treatment

Isotype and IL-12p40 (clone C17.8) antibodies were purchased from Bio-X-Cell (West Lebanon, NH). Mice received 1mg of antibody i.p. every four days starting from day 4-20 post infection.

4.2.9 Statistics

Results are presented as mean±SEM. Statistical significance was determined by the Student’s *t*-test.

4.3 Results

4.3.1 DC specific deletion of *Ship1* results in splenomegaly and expansion of circulating DCs

Constitutive deletion of *Ship1* leads to a severe myeloproliferative disorder, resulting in lung consolidation and a shortened lifespan due to deregulated PI3K activity in multiple hematopoietic subpopulations[120, 121, 197]. This severe immune deregulation makes it difficult to examine the lineage-specific effects of SHIP-1 activity *in vivo*. While SHIP-1 has a well-established role in regulating dendritic cell (DC) function *in vitro* [141, 142, 183], the *in vivo* significance of SHIP-1 in DCs has yet to be explored. By crossing *Ship1*\(^{FF}\) (wild-type) mice with *Itgax-cre (Cd11c-cre)* transgenic mice we were able to generate mice with a specific deletion of *Ship1* in DCs (*Ship1*\(^{ADC}\)). *Ship1*\(^{ADC}\) mice do not develop the spontaneous lung consolidation found in *Ship1*\(^{−/−}\) mice (Fig 3.1) but do have enlarged spleens compared to wild-type (*Ship1*\(^{FF}\)) controls (Fig 4.1A). Differential leukocyte analyses reveal a moderate, but significant, increase in the frequency of B cells in the
spleens of $Ship1^{\Delta DC}$ mice at the expense of T cells and macrophages (Fig. 4.1B). Peripheral blood analyses of naïve $Ship1^{+/+}$ and $Ship1^{\Delta DC}$ mice reveals a significant increase in circulating CD11c$^+$ DCs in $Ship1^{\Delta DC}$ mice without any significant decrease in peripheral lymphocytes when compared to $Ship1^{+/+}$ mice (Fig 4.1C-D).

Figure 4.1. $Ship1^{\Delta DC}$ mice have enlarged spleens and increased circulating DCs
(A) Total and (B) differential leukocyte analysis from naïve $Ship1^{+/+}$ and $Ship1^{\Delta DC}$ mice. Peripheral blood (C) dendritic cell (CD11c$^+$) and (D) lymphocyte (CD3$^+$ and CD19$^+$) frequencies were quantified by flow cytometry. Data are representative of 2 independent experiments (n=3-6), * p<0.05.

4.3.2 $Ship1^{\Delta DC}$ mice are susceptible to $Trichuris muris$ infection

Previously we showed that $Ship1$ expression increases following helminth infection and that ubiquitous $Ship1^{-/-}$ (c$Ship1^{-/-}$) mice and mice lacking $Ship1$ expression in the myeloid and neutrophil populations (c$Ship1^{\Delta LysM}$) are susceptible to infection with the intestinal helminth, $Trichuris muris$[189]. Here we sought to examine the influence of DC
expression of Ship1 in response to infection with Trichuris muris. Ship1^{FF} and Ship1^{ΔDC} mice were infected with 250 embryonated Trichuris eggs and worm burdens were examined 21 days post infection. Surprisingly, Ship1^{ΔDC} mice failed to clear the infection (Fig 4.2A) while, as expected, wild-type mice were able to expel worms by day 21. Although the intestines of naïve Ship1^{FF} and Ship1^{ΔDC} mice were indistinguishable histologically prior to infection, Trichuris-infected Ship1^{ΔDC} mice displayed increased inflammatory cell infiltration, a reduced frequency of goblet cells, submucosal edema and parasites attached to the epithelium in cecal tissue samples (Fig 4.2B). From this we conclude that DC expression of Ship1 is required for clearance of Trichuris infection.
Figure 4.2. Dendritic cell expression of Ship1 is required for immunity to *Trichuris* infection

(A) *Ship1<sup>F/F</sup>* (wild-type) and *Ship1<sup>ADC</sup>* mice were infected with 250 *Trichuris muris* eggs. Worm burdens were determined microscopically from cecal contents 21 days post infection. (B) PAS stained cecal section from naïve and *Trichuris* infected *Ship1<sup>F/F</sup>* and *Ship1<sup>ADC</sup>* mice. Original magnification 100X. Data are representative of 1 independent experiment (n=5).
4.3.3 DC expression of *Ship1* facilitates Th2 dependent immunity following *Trichuris* infection

We next sought to evaluate the immune response elicited in *Trichuris* infected wild-type and *Ship1*ΔDC mice. We found that *Ship1*ΔDC mice mounted an aberrant Th1 response following infection, as re-stimulation of the draining mesenteric lymph node (mLN) displayed increased frequency of IFNγ+ CD4+ T cells (Fig 4.3A-B). In support of this Th1-bias in *Trichuris* infected *Ship1*ΔDC mice, proximal colon RNA samples showed a significant reduction in the expression of the Th2 associated genes *Il5* and *Il13* and a significant increase in expression of the Th1 associated genes *Il12b* and *Ifng* (Fig 4.3C), as well as a significant increase in secreted IFNγ and IL-12p40 from re-stimulated mLN (Fig 4.3D). We also found significantly increased expression of *Tnfa* and reduced expression of *Relmb* (resistin-like molecules beta) in RNA samples of *Trichuris infected Ship1*ΔDC mice (Fig 4.3C). In support of this Th1-biased response, infected *Ship1*ΔDC mice had significantly reduced serum levels of IgE and *Trichuris*-specific IgG1 than infected wild-type mice (Fig 4.3E-F). These data suggest the impaired expulsion of worms in *Ship1*ΔDC mice is due to an aberrant Th1-polarized adaptive immune response in lieu of the normal Th2 response elicited in wild-type mice that would normally result in worm expulsion and resistance to chronic infection.
Figure 4.3. Dendritic cell expression of *Ship1* facilitates Th2 polarization following *Trichuris* infection

(A) Gating strategy for IL-13 and IFNγ expressing cells from re-stimulated mLN s, gated on CD4⁺CD45⁺ cells. (B) Quantification of cytokine expressing CD4⁺ T cells in the mLN s of *Trichuris* infected *Ship1<sup>fl/fl</sup>* and *Ship1<sup>ΔDC</sup>* mice. (C) Quantitative RT-PCR from proximal colon RNA collected from naïve and *Trichuris* infected mice. (D) Secreted cytokines collected in the
supernatant of αCD3/CD28 re-stimulated mLN cells were quantified by ELISA. (E) Total serum IgE from naïve and *Trichuris* infected mice was quantified by ELISA. (F) *Trichuris*-specific IgG\(_1\) and IgG\(_{2a}\) levels were quantified by ELISA. *Trichuris* = Tm, mesenteric lymph node = mLN. Data are representative of 1 independent experiment (n=5), * p<0.05.

### 4.3.4 Neutralization of IL-12p40 in *Trichuris*-infected Ship1\(^{ΔDC}\) mice promotes resistance

Previous reports of *Trichuris* infection in Ship1\(^{Δ}\) mice have implicated deregulated IL-12 production as a factor leading to susceptibility to infection, although a cellular source of IL-12 was not determined. We have found here that Ship1\(^{ΔDC}\) mice have increased production of IL-12p40 in mLN cultures (Fig 4.3D) and sought to examine whether neutralizing IL-12p40 with an antibody would facilitate clearance of helminth infection in Ship1\(^{ΔDC}\) mice. Neutralization of IL-12p40 enhanced expulsion of *Trichuris* in Ship1\(^{ΔDC}\) mice as demonstrated by reduced worm burdens at day 21 when compared to mice that received an isotype-matched control antibody (Rat Ig) (Fig 4.4A). Treatment with antibodies against IL-12p40 also lead to a reduction in the Th1 response with reduced IFN\(γ^+\) CD4\(^+\) T cells, *Ifng* mRNA expression and IFN\(γ\) secretion from re-stimulated mLN cells (Fig 4.4B-D) and increased mRNA expression of the Th2 associated genes *Il4*, *Il5*, *Il13* (Fig 4.4C). In addition, neutralization of IL-12p40 also lead to a significant increase in serum *Trichuris*-specific IgG\(_1\) levels, although serum IgE levels were not significantly increased. From these data we conclude that Ship1 is involved in the negative regulation of IL-12p40 release by dendritic cells, and neutralization of IL-12p40 in Ship1\(^{ΔDC}\) mice facilitates resistance to *Trichuris* infection by establishing the appropriate Th2 response.
Figure 4.4. Neutralization of IL-12 in *Trichuris*-infected *Ship1ΔDC* mice facilitates immunity to infection

(A) Day 21 worm counts from *Ship1ΔDC* mice treated i.p. with antibodies to IL-12 or an isotype control (Rat Ig). (B) PAS stained cecal sections from *Trichuris* infected mice. Original magnification 200X. (C) Intracellular IL-13 and IFNγ expression in CD4⁺ T cells isolated from the mLN was measured by flow cytometry. (D) Quantitative RT-PCR from proximal colon RNA collected from *Trichuris* infected mice. (E) Secreted cytokines collected in the supernatant of
αCD3/CD28 re-stimulated mLN cells were quantified by ELISA. (F) Total serum was quantified by ELISA. (G) Trichuris-specific IgG1 levels were quantified by ELISA. Data are representative of 1 independent experiment (n=4-5), * p<0.05.

4.4 Discussion

Here, we demonstrate that the specific loss of SHIP-1 from DCs leads to splenomegaly and increased circulating DCs. DC-specific SHIP-1 loss also results in enhanced production of IL-12, promoting an adaptive Th1 response and chronic infection following Trichuris infection. Blockade of IL-12 in Ship1ΔDC mice with a neutralizing antibody restored the formation of a protective Th2 response and clearance of the infection. This highlights the important role DCs play in shaping the adaptive immune response to Trichuris, and reveals how a subtle perturbation in the normal dampening of PI3K activity can dramatically alter the development of anti-helminth immune responses.

While the initiation of a normal type 1 immune response is well established, with DCs acting as the dominant APC to naïve CD4+ T cells and producing IL-12 to promote Th1 polarization, the steps involved in the initiation of type 2 responses are still under debate. This is, in part, due to the fact that DCs do not produce large amounts of IL-4, a key factor required for promoting naïve CD4+ T cell polarization and maturation into a Th2 cell. DCs clearly have an important role in immunity to helminth infection; in elegant experiments where CD11c+ cells were inducibly-depleted using a diphtheria toxin-dependent ablation system, helminth-induced protective Th2 responses were severely impaired[198, 199]. Our results are consistent with this observation and show that deregulated PI3K signaling activity (through the deletion of Ship1 specifically in DCs, but not in other leukocyte
populations) results in increased production of IL-12 and a Th1-biased immune skewing that impaired immunity to *Trichuris*-infection.

Although these results suggest a pivotal role for DCs in helminth immunity, it is also likely that they require the assistance of other innate leukocyte populations in order to prime Th2 responses. Indeed, experiments aimed at examining the need for MHC class II expression by distinct subsets of APCs have shown that expression of MHC-II by DCs alone, and not by other leukocyte populations, is insufficient to confer resistance to chronic *Trichuris*-infection[21]. This suggests that other MHC class II positive innate populations (such as mast cells[200], basophils[20, 22], eosinophils[201, 202] and ILC2s[26] macrophages and neutrophils) are required to support DCs in the formation of a protective Th2 response.

The influence of PI3K activity in regulating IL-12 production from DCs is controversial, and could be due to differing effects and expression patterns of the various PI3K isoforms. PI3K exists as heterodimers containing a regulatory and a catalytic subunit and are grouped into three different classes: class I, class II and class III. The most widely studied are the class IA PI3Ks, which consist of three different catalytic subunits (p110α, p110β and p110δ) and five distinct regulatory subunits (p85α, p55α, p50α, p85β and p55γ). Consistent with the results presented here, siRNAs targeting p110α and p110β in human APCs in response to LPS stimulation, p110β expression was found to positively correlate with JNK activity and IL-12 production, suggesting enhanced PI3K activity could, indeed, support increased production of IL-12[203]. Surprisingly, however, others have shown that deletion of the regulatory subunit p85α in *Pik3r1−/−* mice (p85α−/−), or pharmacological
inhibition of PI3K activity using wortmannin leads to increased production of IL-12, suggesting a negative regulatory role for PI3Ks in IL-12 production[204]. Thus there are data to support both positive and negative roles for PI3K activity in IL-12 production and further studies are warranted to better delineate which PI3K isoforms and cell types regulate the net production of IL-12.

Previously, we reported that Ship1ΔLysM mice, which lack Ship1 expression in macrophages and neutrophils, also displayed heightened IL-12 production in resting mice and Trichuris-infected mice, suggesting an important role for Ship1 in regulating IL-12 production in multiple leukocyte populations[189]. The current data expand on this observation and suggest that, in addition, DCs too, can regulate expression of this critical effector and tip the balance from Th2 to Th1 dominated immune responses.

In summary, we show that selective deletion of Ship1 expression in DCs (Ship1ΔDC) renders mice susceptible to infection with Trichuris. This is the result of increased production of IL-12 in Ship1ΔDC mice, leading to a reduced Th2 response and instead a Th1-dominated response featuring high levels of IFNγ and a chronic infection. Neutralization of IL-12 in Ship1ΔDC mice enhanced the formation of a Th2 response and facilitated resistance to Trichuris infection.
Chapter 5. Discussion
5.1 Summary

The mucosal barriers of the lung and gut serve as important interfaces between the host and environment and are integral for gas and nutrient exchange. They are, however, constantly exposed to environmental antigens and must navigate a delicate balance between tolerance and inflammation to appropriately respond to harmless, or harmful antigens, respectively. This requires the coordinated cross talk between the epithelial barrier and resident leukocytes, including innate lymphoid cells (ILCs), dendritic cells (DCs), T cells and B cells, to integrate the antigenic stimulus and respond with an appropriate response. Using various transgenic mice, I explored the contributions of a recently characterized innate cell population, ILC2s, and the inositol phosphatase SHIP-1 in the initiation and maintenance of Th2 immunity at mucosal surfaces.

In Chapter 2, I addressed the role of the group 2 innate lymphoid cells (ILC2s) in the regulation of both Th2, and Th1/Th17 adaptive immune responses in the lung. Using bone marrow chimeras I was able to generate ILC2-deficient mice, and explore the contribution of ILC2s to the formation of adaptive immune responses in the lung. Interestingly, I found that ILC2s were critical for local sensitization and subsequent robust Th2 immunity in the lung in response to the physiologically relevant antigen HDM. Surprisingly, and despite their role in local sensitization, systemic deliver of antigen, with or without an additional adjuvant, obviated the requirement of ILC2s for initiating Th2 immune responses in the lung. This selective role for ILC2s in the lung during local sensitization was specific for the initiation of Th2 adaptive immune responses, as these cells were completely dispensable for local sensitization to the Th1/Th17-inducing antigen SR.
The implication of ILC2s as important facilitators of Th2 adaptive responses has significant clinical relevance due to the finding of increased ILC2s in the lungs of human allergic patients[32, 65]. While ILC2s are potent producers of IL-5 and IL-13 at early stages of the immune response, their contribution is taken over at later timepoints by Th2 cells[25]. This suggests that therapeutic targeting of ILC2s, after sensitization and a robust Th2 response has been initiated, may not yield improved clinical outcomes. The use of ILC2-deficient mice is hampered by the lack of a true ILC2-specific marker. Indeed, while Rora is highly expressed by ILC2s it is also expressed by Th17 cells, another cell type associated with allergic asthma, particularly steroid resistant and severe asthma[205]. While typically considered a pro-inflammatory cytokine, IL-17A has been associated with some anti-inflammatory properties, reducing TNF-induced production of some chemokines (CCL5, CCL27, CX3CL1) and vascular cell adhesion molecule-1 (VCAM-1) in mesenchymal cells[206-209]. In the context of allergic inflammation, IL-17-induced signaling is required for the initiation of inflammation, however administration of exogenous IL-17 after allergic sensitization reduces airway hyper-responsiveness and eosinophil infiltration, suggesting a time-dependent anti-inflammatory role of IL-17A[210, 211]. Some reports suggest the anti-inflammatory actions of IL-17A are dose dependent, with a greater inhibitory effect at lower concentrations[209]. Importantly, we did observe reduced IL-17A production in HDM-exposed Rora$^{sg/sg}$ BMT mice compared to their wild-type controls, which could conceivably have led to an enhanced anti-inflammatory effect leading to the reduced inflammation observed in these mice (Fig. 2.4). It should be noted, however, that similarly reduced levels of IL-17A were observed in OVA-exposed Rora$^{sg/sg}$ BMT mice, both with and without alum,
and this did not result in any measurable reduction in airway inflammation (Fig. 2.6-7). In some models of allergic airway inflammation, IL-17 production can promote airway hyper-responsiveness and smooth muscle contraction while having no measurable effect on airway inflammation[212]. It would be worthwhile to evaluate AHR responses in OVA-exposed $Rora^{sg/sg}$ BMT mice to examine if they have altered airway responsiveness despite comparable levels of airway inflammation.

To further discern whether the protection observed in the ILC2-deficient $Rora^{sg/sg}$ BMT mice is indeed due to a lack of ILC2s or a reduced Th17 response following HDM challenge, we could attempt an adoptive transfer of ILC2s into $Rora^{sg/sg}$ BMT mice to rescue the phenotype. Similar experiments have successfully been performed in papain challenged $Rora^{sg/sg}$ BMT mice[25], further suggesting that an altered Th17 response does not significantly contribute to allergic sensitization in these mice. Additionally, there was a small but detectable population of radio-resistant, radiation-insensitive, ILC2s found in $Rora^{sg/sg}$ BMT mice, identified by their staining for the host marker CD45.1. It’s possible that the low frequency ILC2s found in $Rora^{sg/sg}$ BMT mice facilitated sensitization to HDM antigen, and the contribution of ILC2s in our model may be undervalued. Transplantation of $Rora^{sg/sg}$ bone marrow into hosts already deficient in ILC2s, such as $Rag^{-/-}Il2rg^{-/-}$ mice that lack both the RAG gene as well as the IL-2Rγ chain, would likely yield a more robust deficiency in ILC2s. New ILC2-deficient mouse models have been recently generated that both allow for the temporal ablation of ILC2s via the administration of diphtheria toxin, or allow for the deletion of $Rora$ specifically in lymphocytes by using a “floxed” $Rora$ gene and an $Il7r-Cre$ expressing mouse[26]. While this eliminates the requirement for bone marrow
transplantation, it doesn’t address the issue of Rora expression in Th17 cells. An improved model would likely consist of Cre expression under control of the Rora gene to delete a gene specific for ILC2s and not Th17 cells, such as Gata3. The important role of RORα in ILC2 development could be explored as a therapeutic target through the screening and identification of agonists and antagonists. Natural and synthetic ligands have already been discovered[213, 214], and the development of antagonists, particularly ones that do not cross the blood brain barrier, could prove useful for inhibiting ILC2 development and/or Th17 responses.

In Chapter 3, I examined the lineage-specific role of the lipid phosphatase SHIP-1, a pan-hematopoietic negative regulator of PI3K signaling, in the initiation of Th2 immune responses in the lung. Although I was unable to evaluate the importance of this signaling mediator in ILC2s due to a lack of the appropriate Cre strains, I was able to evaluate its significance selectively in T cells, B cells and DCs. Loss of Ship1 specifically in B cells did not affect allergic sensitization or humoral immune responses to HDM antigen, arguing that it is dispensable in this scenario. Deletion of Ship1 in T cells, however, resulted in a striking protection from HDM-induced allergic airway inflammation (AAI) due to an immune skewing to a Th1-biased immune response, effectively reducing the influx of eosinophils in the airways and reducing the mucus and IgE production associated with AAI. Additionally, loss of Ship1 in DCs led to a protection from HDM-induced AAI. This was not due to a defect in the ability of Ship1-deficient DCs to pick up, process or transport antigen from the lung to the draining lymph nodes, or the ability to induce antigen specific T cell proliferation. Instead, Ship1-deficient DCs preferentially primed Th1, versus Th2 responses, as Ship1ΔDC
mice had reduced eosinophils, mucus production and IgE production but enhanced IFNγ production following HDM challenge.

In Chapter 4, I examined the role of Ship1 expression, specifically in DCs, in facilitating Th2 immunity to the intestinal helminth Trichuris muris. Ship1ΔDC mice were susceptible to Trichuris infection as they were unable to mount an effective Th2 response to infection. Instead, Trichuris infection led to a Th1 response likely produced by an overproduction of IL-12 resulting from the loss of Ship1 in DCs. Systemic neutralization of IL-12 in Trichuris infected Ship1ΔDC mice resulted in an enhanced Th2 response and a resulting increase in their ability to clear the infection.

With regard to the lineage specific role of SHIP-1 in balancing the immune response, the use of lineage specific, Ship1-deficient mice overcomes some of the previous limitations in analyzing the role for Ship1 in a host with such severe hematological abnormalities, and a host of lineage intrinsic, as well as extrinsic functions. The use of Cre-mediated deletion comes with its own complications however. There are several reports of toxicity associated with Cre expression. Indeed, high level Cre expression, on its own, is sufficient in certain cells such as mast cells and basophils to lead to their ablation[215, 216]. The use of a Ship1F/+ would allow for Cre expression in wild-type controls to compare with Ship1F/F knockout animals. A potential caveat to our findings, given that alveolar macrophages also express CD11c, is that Ship1 will also be deleted in these cells. Indeed, I did observe an expansion in alveolar macrophages in HDM-exposed Ship1ΔDC mice (Fig. 3.4A-B). To overcome this, we utilized in vitro and adoptive transfer experiments of isolated wild-type
and Ship1-deficient DCs to confirm our phenotype of enhanced Th1-skewing (Fig 3.8). Another option would be to utilize other, dendritic cell-specific, Cre expressing strains to selectively delete Ship1 expression[217]. It is important to note that we used over-expression of a naturally occurring DC growth factor expressed by a melanoma cell line, B-16-FIlt3L, to expand wild-type and Ship1-deficient DCs in vivo. Interestingly, we found that tumors grew much more rapidly in Ship1ΔDC mice, compared to their wild-type counterparts, which was surprising as one would imagine the Th1 skewed environment in these mice would likely act to inhibit tumor growth. This enhanced tumor growth likely leads to a much higher dose of Flt3L during DC expansion, which could influence DC development and function. The use of more primary DCs, such as those isolated from the lung draining lymph node, for in vitro and adoptive transfer experiments would likely yield more physiologically relevant responses, although their limited number would be restrictive for downstream assays. Signaling pathways could be examined using flow cytometry and various phosphorylation-specific antibodies, and could be screened with the use of a mass cytometry (CyTOF) approach. Previous in vitro studies have shown enhanced Akt phosphorylation in Ship1−/− DCs[142], and more PI3K pathway targets, and proteins associated with IL-12 production such as JNK (c-Jun N-terminal kinases) and NF-κB could be examined. However, the role of PI3K pathway activity in IL-12 production is controversial, with reported positive and negative regulatory roles in the production of IL-12[203, 204]. While our findings would suggest that the PI3K pathway is a positive regulator, it is also possible that protein-protein interactions with SHIP-1, or production of the SHIP-1 product PI(3,4)P₂ have important roles in regulating IL-12 production.
5.2 Future directions

The mechanisms involved in the initiation of Th2 adaptive immune responses are still unclear. Here, we have uncovered a role for ILC2s, a recently characterized leukocyte subtype, in the sensitization to HDM-induced AAI, however the mode in which ILC2s exert their function is still unclear. While recent reports have suggested ILC2-expression of MHC-II allows them to directly associate with T cells, their scarcity makes this an unlikely physiologically relevant mode of action. However, ILC2s have been shown to potently recruit eosinophils to sites of mucosal inflammation, and eosinophils have previously been described to promote DC activation towards Th2 immunity[61]. It is possible that ILC2-recruited eosinophils can facilitate DC maturation or potentially on ILC2s themselves, serving as a positive feedback loop. Using mice that selectively lack IL-5 in ILC2s, and eosinophil-deficient mice (such as the ΔdblGATA strain), would allow the examination of this possibility. If ILC2-recruited eosinophils do indeed facilitate a positive feedback effect on their activity or expansion, mice with an enlargement of the eosinophil pool, such as IL-5 transgenic mice, would likely have an expansion in tissue resident ILC2s[218]. Further studies could be imagined looking at eosinophil products, such as eosinophil-derived neurotoxin (EDN), facilitating ILC2 activity and expansion in vitro and in vivo. EDN has been shown previously to activate TLR2-signal transduction in DCs to enhance Th2 responses. While the ability of ILC2s to respond to TLR2-stimuli isn’t clear, other ILC populations have displayed responsiveness to TLR2 stimulation[219].
While we report here that loss of Ship1 in T cells and DCs leads to a protective Th1 response following HDM challenge, the use of SHIP-1 activators have been proposed and tested in clinical experiments with asthmatic patients, although the therapeutic effect was relatively minor and restricted to a small decrease in the late phase response[117]. Initial allergen stimulation results in a rapid degranulation of mast cells, whose mediators initiate the early phase response and lead to the recruitment of eosinophils, basophils and lymphocytes (Th2 cells) for a more sustained inflammation in the late phase response. This limited protection observed with SHIP-1 agonists in the late phase could be due to the role of SHIP-1 in leukocyte subsets such as mast cells, basophils or eosinophils, key mediators of the late phase response. Indeed, transfer of Ship1−/− mast cells into mast cell deficient mice leads to enhanced allergic inflammation, suggesting that SHIP-1 is an important regulator of mast cell degranulation and activation that results in enhanced asthmatic responses[220]. SHIP-1 also has been shown to be an important negative regulator of IL-4 production from basophils, and pharmacological activation of SHIP-1 could inhibit Th2 polarization and M2 macrophage skewing[221, 222]. Reduced PI3K pathway activity, through the use of pharmacological SHIP-1 agonists, could also prevent the survival and recruitment of eosinophils in the airways[223]. Further examination of the role of SHIP-1 in these specific subsets could be explored with recently generated Cre-deleter strains[224-227], although care must be taken as Cre-mediated toxicity has been observed in some of these strains[215, 216]. The minor clinical benefits associated with SHIP-1 agonists in the treatment of allergic asthma are potentially the result of opposing effects, with a beneficial response on mast cells, basophils and eosinophils and an adverse effect on DCs and T cells as I have shown here. While the specific targeting of SHIP-1, whether through
agonists or antagonists, in restricted leukocyte subsets would be ideal it may be difficult to accomplish. Instead, identifying unique molecular pathways controlled by SHIP-1 in these various populations could lead to the identification of new therapeutic targets with restricted activities. The enhanced IL-12 production observed with $Ship1^{ΔDC}$ mice was also seen with a myeloid-specific $Ship1$ deficient mouse, suggesting that SHIP-1 plays an important role in multiple lineages in regulating production of this important immunomodulatory cytokine. Further exploration into the signaling pathway influenced by SHIP-1 will hopefully lead to new therapeutic targets.
References


103. Okkenhaug, K., A. Bilancio, G. Farjot, H. Priddle, S. Sancho, E. Peskett, W. Pearce, S.E. Meek, A. Salpekar, M.D. Waterfield, A.J. Smith, and B.


Appendices
Appendix 1 Publications arising from my PhD work


Appendix 2 Bronchoalveolar lavage leukocyte enumeration and differentiation

FSC-A
SSC-A
beads

CD45-Pacific Blue
CD45-
AmCyan

FSC-W

CD4-

SSC-

FSC-A

CD11c-AF647

SiglecF-PE
7/4-FITC

SiglecF-PE

7/4-PE

CD11c

Alveolar Macrophages

Dendritic Cells

Singlets

T cells

Eosinophils

Neutrophils

B cells

B220-APCefluor780

CD4-PeCy7

CD4+ T cells

SiglecF 7/4

CD4+ B220-

CD8+ T cells

CD8a-PerCP-Cy5.5

Total cells = cells counted x (input beads / beads counted)