The role of interleukin-5 in host defense during influenza infection

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A Dissertation Presented to the Graduate Faculty
of the University of Virginia in Candidacy for the Degree of
Doctor of Philosophy

Department of Microbiology, Immunology and Cancer Biology

University of Virginia
July 2013

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ABSTRACT

Respiratory virus infection, such as influenza, typically induces a robust type 1 (pro-inflammatory cytokine) immune response, however, the production of type 2 cytokines has been observed. Type 2 cytokine production during respiratory virus infection is linked to asthma exacerbation, however, type 2 cytokines may also be tissue protective. Interleukin (IL)-5 is a prototypical type 2 cytokine that is essential for eosinophil maturation and egress out of the bone marrow. However, little is known about the cellular source and underlying cellular and molecular basis for the regulation of IL-5 production during respiratory virus infection. We sought to identify the cellular source of IL-5 during influenza infection and determine the role of IL-5 during respiratory virus infection. Using a mouse model of influenza virus infection, we found a robust transient release of IL-5 into infected airways along with a significant and progressive accumulation of eosinophils in the lungs, particularly during the recovery phase of infection, i.e. following virus clearance. The cellular source of the IL-5 was group 2 innate lymphoid cells (ILC2) that infiltrated the infected lungs. Interestingly, the progressive accumulation of eosinophils following virus clearance was reflected in the rapid expansion of c-kit^{+} IL-5 producing ILC2. We further demonstrated that NKT cells, as well as alveolar macrophages (AM), were endogenous sources of IL-33 that enhance IL-5 production from ILC2. Importantly, the production of IL-5 was essential for optimal recovery

from influenza as neutralization of IL-5 during the recovery phase resulted in delayed weight gain and impaired epithelial regeneration following virus clearance. This effect was independent of eosinophils and was instead, partially dependent on IL-5 acting through neutrophils expressing the IL-5 receptor. In the absence of IL-5 signaling, neutrophils exhibited heightened inflammatory activity and their depletion partially rescued the defect in recovery in IL-5 neutralized mice. Collectively, these results reveal that c-kit^{+} ILC2 interaction with IL-33 producing NKT and AM leads to abundant production of IL-5 by ILC2 and that IL-5 is essential for an optimal tissue repair response following influenza infection.

ACKNOWLEDGEMENTS

I would first like to express my gratitude for my mentor, Dr. Thomas Braciale, for his patience, expertise and guidance. Under his supervision and leadership, his lab has become a wonderful place to learn how to conduct exceptional science. The members of the Braciale laboratory have made coming into the lab a fun and fantastic experience each and every day. In particular, I am indebted to the teaching and eternal enthusiasm of Dr. Taeg Kim, who has always encouraged me in my work. I would also like to thank my thesis committee (Drs. Ian Glomski, Young Hahn, Borna Mehrad, Norbert Leitinger, and Kodi Ravichandran) for their insightful discussions and advice. Finally, I thank my family – my mother Patricia Nixon who has always been there for me in every way, and my aunt PJ, my Poppy and my Justin – they are a never-ending source of encouragement, patience, love, faith and humor.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AAM	Alternatively activated macrophage
AM	Alveolar macrophage
AEC	Alveolar epithelial cell
αIL-5	Anti-IL-5 neutralizing antibody
αGC	Alpha-galactosylceramide
BAL	Bronchoalveolar lavage
BrdU	Bromodeoxyuridine
CD	Cluster of differentiation
DASC	Distal airway stem cell
DCFA-DA	2′,7′-dichlorofluorescein diacetate
DMSO	dimethyl sulfoxide
d.p.i.	days post infection
FcεR	Fc epsilon receptor
FCS	Fetal calf serum
FLT3	fms-related tyrosine kinase 3 (CD135)
FMO	Fluorescence minus one
HPRT	hypoxanthine-guanine phosphoribosyltransferase
IAV	Influenza A virus infection
ICS	Intracellular cytokine staining
IL-	Interleukin-
ILC	Innate lymphoid cells

ILC2	Group 2 innate lymphoid cells
IFN	Interferon
iNOS	Inducible nitric oxide synthase
i.n.	Intranasal
i.p.	Intraperitoneal
i.v.	Intravenously
JAK2	Janus kinase 2
LIN	Lineage
LN	Lymph node
LPS	Lipopolysaccharide
LTi	Lymphoid tissue inducer cell (an ILC family member)
MFI	Mean fluorescent intensity
MHC II	Major histocompatibility complex, class II
MIG	Monokine induced by gamma interferon (CXCL9)
MMP	Matrix metalloproteinase
NHC	Natural helper cell
NK	Natural killer
NKT	Natural killer T cell
PCR	polymerase chain reaction
PR8	A/PR8/34 (H1N1 mouse-adapted influenza strain)
pSTAT5	phosphorylated, signal transducer and activator of transcription 5

RDC	Respiratory dendritic cell
REC	Respiratory epithelial cell
rIL-5	recombinant murine IL-5
ROS	Reactive oxygen species
RT-PCR	real time PCR
SCF	Stem cell factor
TGF-β	Transforming growth factor, beta
TNFα	Tumor necrosis factor, alpha

CHAPTER 1: INTRODUCTION

The human body is protected from microorganisms, toxins and other foreign entities by the immune system. The mammalian immune system is comprised of a variety of different cell types and a myriad of effector molecules that are designed to defend the body against any number of invading pathogens. Mucosal sites, such as the lungs, are vulnerable areas where pathogens may attempt to circumvent the body's defenses. The innate branch of the immune system is designed to rapidly respond to any such "invaders" and limit their replication locally and/or dissemination to other sites in the body. While the innate response is essential for limiting an infection initially, it is the adaptive arm of the immune system that is critical for developing a coordinated response that typically results in elimination of a pathogen and prevents re-infection. Thus in contrast to the innate system, which is rapid but non-specific in its effector activities, the cells of the adaptive immune system have a delayed response but are directed against specific foreign entities and are ultimately responsible for immunological memory.

The respiratory tract

The respiratory tract is a dynamic organ whose role in gas exchange is vital for life. Because a large volume of air is exchanged by the lungs (i.e. up to 10 L/min), the respiratory tract is continuously exposed to microbial and chemical insults (Kohlmeier and Woodland, 2009). The respiratory tract is made up of the upper

airways, including the nasal cavity and pharynx, as well as the lower airways i.e. lungs, which includes all pulmonary tissue inferior to the Waldeyer's ring. The lower respiratory tract begins from the trachea, which then divides into large airways known as bronchi. The bronchi then repeatedly bifurcate into smaller bronchi and after approximately 3 divisions, end in bronchioles (Gardner et al., 1986). Unlike the bronchi, the smaller bronchiole airways contain no cartilage, but instead have relatively thick walls of smooth muscle. Eventually the bronchioles will terminate into the alveolar sacs (Figure 1.1), the functional unit of the lung. The alveoli are made up of thin, flat alveolar epithelial type I cells (AEC I) that have a large surface area to volume ratio. These cells are positioned in close proximity to blood vessels and this, along with their thin cytoplasm, facilitates efficient gas exchange of $C0_2$ out of the blood and 0_2 from the lung airspace into the blood. Type II alveolar epithelial cells (AEC II) are also present within the alveolar sac and these cells are largely responsible for surfactant production – the viscous substance that keeps the alveoli from collapsing during exhalation. While the alveoli cells are simple squamous epithelia, the respiratory cells of the bronchioles and bronchi are made up cuboidal and columnar epithelium, as well as an abundance of ciliated epithelia in the bronchi.

The surface area of the human lung is 70 meters^2 (Holt et al., 2008), which means it is continually exposed to a variety of stimuli – most of which are innocuous. Thus the homeostatic environment of the lung has evolved to be mostly immunotolerant to prevent overt inflammation from compromising air exchange. Immune cells

present in the basal state lung therefore have the difficult task of discriminating the relatively rare pathogen-associated stimuli from the myriad of harmless signals encountered. When potentially harmful antigens are detected, the immune system must be tightly controlled to minimize inflammation and damage done to the delicate epithelial cell surfaces. Most potentially invasive microorganisms like respiratory viruses are generally restricted to the upper respiratory tract where their replication is controlled by physical and chemical barriers as well as the cells of the innate immune system. Less frequently these organisms are able to gain access to the lower respiratory tract, and in the case of viruses like influenza, have the potential to cause substantial inflammation and hence tissue injury. Influenza A virus (IAV), in particular, can induce extensive inflammation when it infects the bronchial and alveolar epithelial cells, which subsequently results in wide-spread tissue injury and compromises respiratory function.

Influenza A Virus (IAV)

Influenza is responsible for greater than 100 million deaths in the past century (Ahmed et al., 2007). The viral genome is comprised of eight segments of negative-sense RNA. This genome organization classifies it as a member of the orthomyxovirus family. The virus can exist as one of three subtypes, A, B, or C, although it is only the A and B types that can infect humans and the A subtype is the most virulent. Because the virus exists as 8 separate genome segments, it can readily swap these segments with other influenza viruses during a co-infection (i.e.

infection of the same cell with two or more different virus strains)– thus explaining the phenomenon of antigenic shift, whereby an entirely new antigenically distinct virus emerges. IAV also have very error prone RNA-dependent RNA polymerases that allow them to rapidly mutate in a process known as antigenic drift. The ability to rapidly change viral proteins, particularly those surface proteins that are exposed to and targeted by the immune system (i.e. hemagglutinin and neuraminindase) allows IAV to quickly subvert immune responses.

It is through antigenic shift, and to a lesser extent drift, that new pandemic strains can arise. The 1918 influenza pandemic was one of the most devastating pandemics in modern history and resulted from an interspecies transmission from a circulating avian virus that mutated to be able to directly infect humans (Neumann et al., 2009). The virus was particularly virulent – killing between 50-100 million people within two years. Although the reasons for the exceptional pathology seen in 1918 have not been definitively demonstrated, a reconstructed 1918 virus was shown to induce an overexuberant immune response, occasionally referred to as cytokine storm. Infected mice and macaques displayed high levels of immune-cell infiltration into the lungs, particularly neutrophils and alveolar macrophages – cells of the innate immune system. These animals also had extremely elevated levels of pro-inflammatory cytokines, most notably IL-6. It has also been noted that >50% of specimens analyzed from 1918 had evidence of a concomitant secondary bacterial infection (Jakab, 1981), and although the incidence is not as high as seen in 1918, secondary bacterial pneumonias are also commonly seen in current seasonal IAV

infections (Alicino et al., 2011). The proposed mechanisms for the lethal synergism between IAV and certain bacterial strains are many (Ballinger and Standiford, 2010); however, one prominent theory suggests that the massive tissue injury sustained during IAV infection and a subsequent delay in epithelial repair leaves the lung vulnerable to otherwise innocuous bacteria (Ramphal et al., 1979). Thus the pathology associated with pandemic strains such as 1918 as well as current circulating seasonal strains suggests that the extent of tissue injury is a combination of virus and host-mediated responses.

Virus induced respiratory tract injury

A variety of respiratory cell types can potentially serve as targets of infection by IAV. These include lung resident cells: firstly, airway and alveolar respiratory epithelial cells (REC) whose destruction (or dysregulation) can, if severe, compromise respiratory function. Secondly, hematopoietic origin (bone marrow-derived $CD45^+$) inflammatory and immune cells can also be infected and which can, like the virus, induce tissue damage and compromise lung function (Table 1.1).

In most instances, productive infection of REC by influenza is necessary for virus propagation and as a consequence, contributes to respiratory tract inflammation and injury. IAV is a lytic virus and thus will result in a large number of necrotic and/or apoptotic REC. Infection of bone marrow-derived $CD45^+$ respiratory tract resident cells (e.g. respiratory dendritic cells (RDCs)) and recruited inflammatory

myeloid lineage cells (e.g. inflammatory mononuclear cells and neutrophils) may also profoundly influence the course and ultimate outcome of infection (Hao et al., 2008; Manicassamy et al., 2010). Highly pathogenic avian H5N1 IAV can productively infect cells of hematopoietic origin, which may account for the propensity of this particular virus strain to leave the respiratory tract and disseminate systemically (Spiegel et al., 2006; Thitithanyanont et al., 2007; Wang et al., 2008).

As more evidence has emerged, it is becoming increasingly clear that the pathogenicity associated with influenza virus infection reflects not only the efficiency of virus replication and the tropism of a given strain for particular cell types within the lung but also the magnitude and characteristics of the host anti-viral immune response. Recovery from influenza infection requires the elimination of virus/virus-infected cells, the resolution of injury-associated inflammation, and perhaps most importantly, cellular and molecular repair mechanisms necessary for restoration of normal lung structure and function.

Re-establishing the epithelial barrier and maintaining barrier integrity

A hallmark of influenza infection is replication of virus in and the subsequent destruction of the airway epithelium. Therefore, by necessity, the repair of the epithelium is essential for recovery. The stages of airway repair have been studied in great detail for a variety of chemically induced injury models (Beers and

Morrisey, 2011; Crosby and Waters, 2010); however, the unique set of conditions imposed by influenza infection (e.g. viral load and the tropism of a given virus strain for a particular respiratory cell type) can potentially modify the repair process in ways that are not well understood. New research has highlighted the importance of initiating and maintaining a proper repair response during and following respiratory virus infection and has demonstrated a renewed interest in an active repair process, rather than simply a passive dampening of inflammation (Kumar et al., 2011).

IAV infection results in large numbers of apoptotic and necrotic epithelial cells, leaving denuded basement membranes of the upper and/or lower airways. In addition to virus-induced cell death, infiltrating leukocytes secrete large quantities of matrix metalloproteinases (MMP) that damage and degrade the basement membranes of the endothelium and epithelium, which results in the loss of the microarchitecture of the conducting airways and alveoli. Therefore, the lung must initiate a robust repair response to reconstitute the extracellular matrix, return to homeostasis, and rebuild barrier function. Furthermore, impaired repair processes in the IAV-infected lung may also enhance susceptibility to secondary microbial infection.

The restoration of the respiratory epithelium following injury can be divided into three sequential stages: provisional matrix deposition, epithelial proliferation, and epithelial differentiation. In order for new epithelial cells to regenerate, fibroblasts

and epithelial cells surrounding the infected foci secrete a provisional matrix made predominantly of the structural protein fibronectin (Herard et al., 1996). Transforming growth factor (TGF) -β, another potent stimulator of the fibro-proliferative response, is released by influenza infected epithelial cells (Roberson et al., 2011), which can subsequently stimulate secretion of provisional matrix proteins from fibroblasts and other non- hematopoietic cell types. Upon completion, the newly formed extracellular matrix can provide a platform for epithelial progenitor cells to proliferate and give rise to new epithelial cells that can regenerate those lost to infection. Finally, once the cells have proliferated to cover the denuded areas, they then receive signals to differentiate into the specific cell types found within the airways (Buckley et al., 2008; Rock et al., 2011; Rogel et al., 2011). Thus, there are many pathways that converge to mount a proper repair response in the infected respiratory tract; however, emerging studies have also highlighted the role of the innate immune system in this process.

The role of the innate immune system: the second act

Although the innate immune system plays a clear role in the induction of inflammation and injury associated with IAV infection during the acute phase, a number of studies have demonstrated the importance of innate immune cells, particularly of the newly described family of innate lymphoid cells (ILC), to the maintenance and regeneration of mucosal, especially pulmonary, epithelia (Allen and Wynn, 2011; Chen et al., 2012a; Kumar et al., 2013; Monticelli et al., 2012).

ILC are now recognized as a family of various innate cell subsets of lymphoid origin. These subsets include NK cells, lymphoid tissue-inducer (LTi) cells, LTi-like cells, which are phenotypically similar to LTi cells but also express the NK cell receptor NKp46 in adults (often referred to as NKR+LTi, ILC22, or NK22), as well as group 2 innate lymphoid cells (ILC2; also referred to as natural helper cells (NHC)) (Figure 1.2).

ILC2 were variously described by several groups (Moro et al., 2010; Neill et al., 2010; Price et al., 2010; Saenz et al., 2010), and in all settings were shown to be potent producers of type 2 cytokines, namely IL-5 and IL-13. Although originally described in fat associated lymphoid clusters (Moro et al., 2010), ILC2 have been identified in many organs including the spleen, bone marrow, and various regional lymph nodes. Of interest, a relatively large number are also found within the lung (Ikutani et al., 2012; Price et al., 2010). The ability of these cells to secrete large quantities of IL-5 and IL-13 (on the order of 30 ng per 5000 cells) has made them a target for limiting virus induced asthma exacerbations (Chang et al., 2011). However, ILC2 are also essential for epithelial integrity, lung function, and proper airway remodeling during IAV infection via their secretion of the epidermal growth factor ligand, amphiregulin (Monticelli et al., 2011). Amphiregulin can limit lung inflammation during bleomycin-induced injury (Fukumoto et al., 2010); however, this new demonstration of its role in actively participating in and/or regulating airway repair following IAV infection merits further research. ILC2 are also early

producers of the type 2 cytokine IL-9 (Wilhelm et al., 2011), which although a culprit in asthma and allergy, can protect epithelial cells from apoptosis by upregulating Bcl2 (Singhera et al., 2008). In addition to amphiregulin and IL-9, it is also formally possible that ILC2 are secreting other factors that directly or indirectly modulate the repair response. ILC2 are located near the bronchi and bronchioles (Ikutani et al., 2012) and thus are well situated to mediate the repair response following a respiratory virus infection. ILC2 produce large amounts of IL-5 and IL-13 when stimulated by IL-33 or IL-25. IL-33 is present in the IAV-infected lung, with the predominant sources being necrotic epithelial cells (Le Goffic et al., 2011), alveolar macrophages (Chang et al., 2011), and NKT cells (Gorski, 2013). Thus IL-33 may be the signal to initiate ILC2 into the repair phase. Whether IL-25 has a direct role in any respiratory virus infection, outside of virus-induced asthma exacerbation (Kaiko et al., 2010), is not yet clear.

Numerous studies have linked ILC2 to asthma and allergy (Kim et al., 2012; Klein Wolterink and Hendriks, 2013), however, the propensity of these cells to produce large quantities of predominantly type 2 cytokines in the absences of any allergic phenotype suggests they may also be playing additional roles. The recent finding that ILC2 produce amphiregulin to promote epithelial integrity during IAV infection supports this. Whether their production of type 2 cytokines might also play a beneficial role is unknown. However, it is being increasingly appreciated that type 2 immune responses can also be thought of as a reparative response to tissue injury (Allen and Wynn, 2011).

Type 2 immunity: an innate response to tissue injury

The ability of ILC2 to produce extremely high levels of type 2 cytokines, not just in response to parasites or as a result of allergy, but also in response to respiratory virus infection begs the questions of why? Type 2 immune responses typically involve the cytokines IL-4, -5, and -13. These cytokines are effective at supporting mast cell growth, stimulating eosinophil maturation and egress from the bone marrow, as well as inducing smooth muscle contraction, respectively. Mast cells, eosinophils and basophils are all important type 2 effector cells for combating parasitic infection. All three of these cell types express the high affinity Fcε receptor I, which upon engagement with IgE, induces degranulation of effector molecules that assist in expelling larger organisms such as worms or helminths. Conversely, these effector activities also contribute to the pathogenesis of asthma and allergy. However, recent evidence has emerged, which suggests that type 2 responses evolved not only to combat parasites but also to limit tissue injury and stimulate repair (Chen et al., 2012a).

Type 2 immune responses largely depend on signaling through the IL-4R alpha chain (IL-4Rα). Both IL-4 and IL-13 signal through IL-4Rα, and mice deficient in IL-4Rα have delayed wound repair responses (Chen et al., 2012a; Loke et al., 2007). In addition, IL-13 is highly pro-fibrotic, which when present in small amounts and under tight regulation, may be able to promote a repair response, particularly in

generating a provisional matrix. Signaling through the IL-4Rα via IL-4 and/or IL-13 is also important for the generation of alternatively activated macrophages (so-called 'M2' macrophages) (Gordon and Martinez, 2010). M2 are known to be anti-inflammatory and involved in tissue repair in a variety of injury models (Varin and Gordon, 2009). Much of the emerging data has focused on the necessity of IL-4 and IL-13 in the tissue repair response; however, what role, if any, IL-5 may be playing in this process is not yet known.

IL-5 and Eosinophils

Interleukin (IL)-5 was initially described as a B cell and eosinophil growth factor *in vitro* (Howard et al., 1982; Yamaguchi et al., 1988), but was quickly shown to also be essential for eosinophil survival and maturation *in vivo*. In the bone marrow, IL-5 is essential for terminal differentiation of eosinophil progenitors and promotes eosinophil survival by delaying apoptotic death (Sanderson, 1992). Systemic IL-5 is also required for eosinophil egress out of the bone marrow and into circulation (Collins et al., 1995). Transgenic mice engineered to overexpress IL-5 demonstrate elevated levels of blood eosinophils as well as increased numbers of B1-B cells (Dent et al., 1990; Vaux et al., 1990). These mice have enhanced airway hyperreactivity following exposure to allergens and typically have greater resistance to parasitic infection, but are otherwise free from pathology (Borchers et al., 2001; Lee et al., 1997). Conversely, IL-5 or IL-5 receptor deficient animals show marked reductions in B1-B cells in the peritoneum and circulating eosinophils and have reduced

allergic sympoms, but enhanced susceptibility to parasitic infection (Foster et al., 1996; Sher et al., 1990).

Following the discovery of IL-5 and its physiologic effects, it was identified as being a product of predominantly CD4+ Th2 cells with minor contributions from mast cells and eosinophils themselves. However, the recent discovery of ILC2 is leading to a paradigm shift. IL-5 can now be viewed as an innate cytokine that does not require an adaptive immune response for its production. The production of IL-5 by ILC2 has been shown to be essential to clearing *Nippostrongylus brasiliensis* (Moro et al., 2010), and is a hallmark of protease allergen induced airway hyperreactivity (Halim et al., 2012).

IL-5 exerts its biological effects through binding to its high affinity receptor, which is made up of the unique IL-5 receptor alpha subunit (IL-5Rα) and the common beta chain (βc) (Mita et al., 1989). The βc is also a shared subunit with the receptors for IL-3 and GM-CSF (Takaki et al., 1991). IL-3, GM-CSF, and IL-5 are all required for eosinophil differentiation in the bone marrow, but IL-5 is by far the most lineage-restricted to eosinophils. By itself, the IL-5Rα binds IL-5 with low affinity, but upon binding, recruits the βc to form the high affinity receptor (Mita et al., 1989) and initiates a signaling cascade via JAK2 and STAT5 (Figure 1.3). Eosinophil progenitors and mature eosinophils constitutively express the high affinity receptor as do B1-B cells (Yamaguchi et al., 1990). A recent report also indicated that IL-5Rα expression can be induced on macrophages during sepsis (Linch et al., 2012).

Because of their high frequency in the sputum of asthmatics, eosinophils (and by association, IL-5) were shown to be causative agents in asthma pathogenesis (Spahn, 2012; Wegmann, 2011). Numerous mouse models confirmed the role of IL-5 in asthma, however, the generation of two distinct eosinophil deficient mouse models provided conflicting data on the exact contribution of eosinophils (Humbles et al., 2004; Lee et al., 2004). Large-scale clinical trials in asthmatics of a human monoclonal antibody against IL-5 also proved to be disappointing, with only limited success in a small subset of patients (Pelaia et al., 2012). While the contribution of IL-5 and eosinophils during asthma remains controversial, their role during respiratory virus infections remains almost completely unknown. The presence of eosinophils and IL-5 has been noted during IAV infection, although no report has investigated the purpose. During respiratory syncytial virus, eosinophils were shown to have anti-viral activity through their release of eosinophil-derived neutrotoxin (EDN)(Domachowske et al., 1998; Phipps et al., 2007). Such a role for the cell type in experimental IAV infection seems unlikely since maximum accumulation of eosinophils occurs after infectious virus clearance. On the other hand, the tempo of eosinophil accumulation and increased IL-5 production during the resolution phase of infection raises the possibility that this type 2 response could have a positive role in restoration of pulmonary epithelium and function by promoting tissue repair.

SPECIFIC AIMS

The previous sections have summarized our current understanding of influenza pathogenesis with regards to virus attributes and host-mediated immune responses. Emerging data appears to indicate that the host repair response is equally important in limiting morbidity and mortality associated with IAV. Type 2 immune responses, particularly those dependent on IL-4 and IL-13, have recently been shown to promote tissue repair following lung injury. However, the role of the type 2 cytokine IL-5 during IAV infection remains unknown and fundamental questions regarding the full range of its biological activity remain unanswered. **Therefore the specific aims of this thesis are to characterize IL-5 production and its role during recovery from experimental IAV infection**. These aims include:

1) Identify the source of IL-5 in the lung during IAV infection.

    Determine the cell types actively secreting IL-5 during IAV infection using qPCR, ICS, and cell depletion.

2) Elucidate the cellular and molecular mechanisms leading to IL-5 production

    Using our novel *ex vivo* ICS method to monitor cellular IL-5 production, we will compare knockout mouse strains and cultures treated with recombinant cytokines previously reported to enhance IL-5 levels.

3) Determine the role of IL-5 during the recovery from IAV-induced pulmonary injury.

Evaluate weight gain, lung function, and pulmonary inflammation in mice treated with IL-5 neutralizing antibody compared to IgG controls.

4) Identify IL-5 responsive cells in the lung and subsequent functional consequences of IL-5 signaling.

Identify cells within the IAV infected lung that express the IL-5 receptor and determine how those cell(s) respond to *in vitro* rIL-5 stimulation and *in vivo* IL-5 neutralization.

Figure 1.1: The alveolus is the functional unit of the lung. The lower airways of the lung terminate in the alveolar sacs. Gas exchange occurs through the alveolar epithelial type I cells (AEC I). The alveolar epithelial type II cells (AEC II) are the major producers of surfactant. Image adapted from (Warburton et al., 1998).

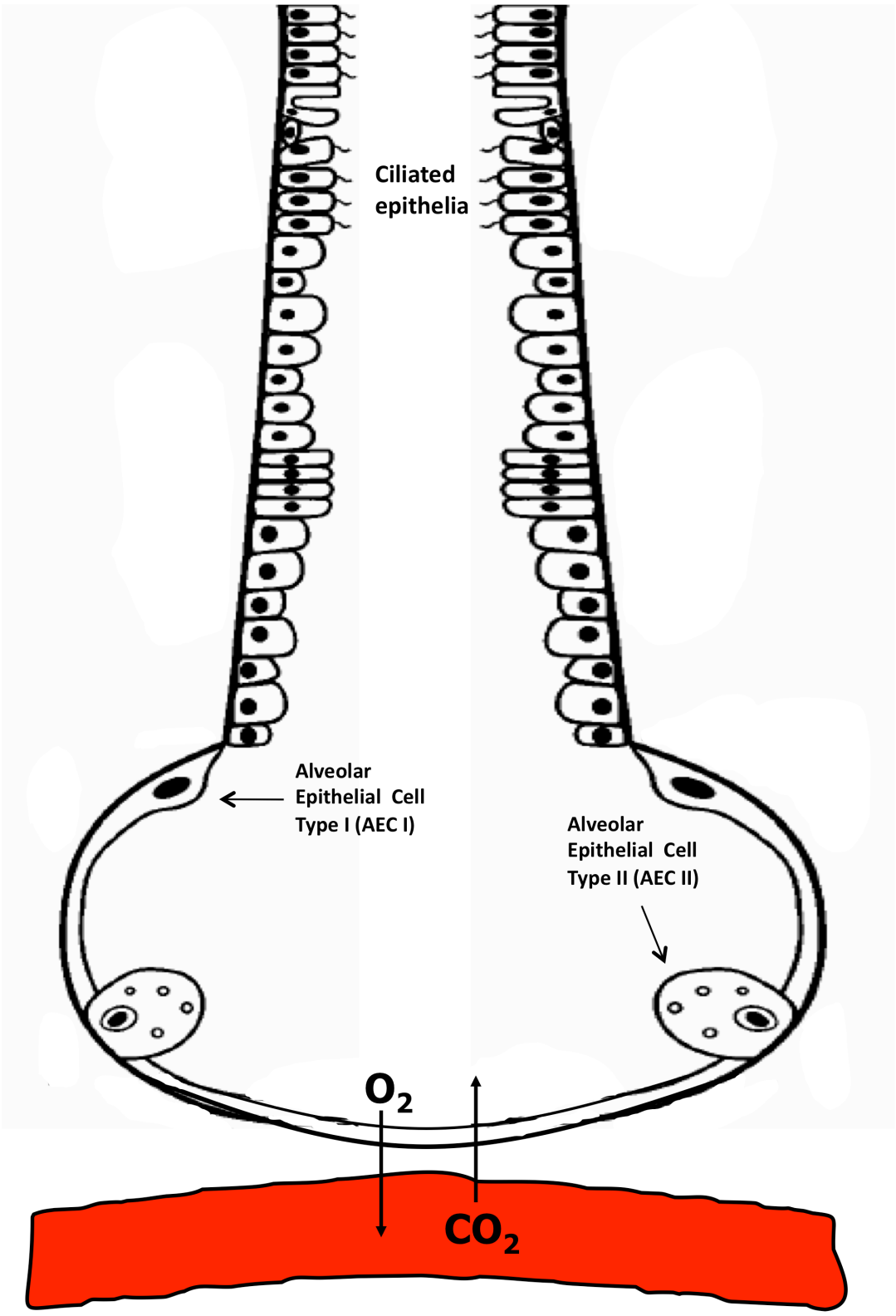

Ciliated
epithelia
Alveolar
Epithelial Cell
Type I (AEC I)
Alveolar
Epithelial Cell
Type II (AEC II)
O2
CO2

Table 1.1 Direct effects of IAV infection on respiratory target cells. Influenza A virus (IAV) primarily infects the respiratory epithelia ($CD45^-$ cells), however, can also infect cells of hematopoietic origin ($CD45^+$). The cellular consequences of infection are described. Table from (Gorski et al., 2012).

Table 1

Direct effects of virus infection on respiratory tract target cells

Target Cell:	Primary Effect:	Direct Consequences:
Respiratory Epithelium	**Cell Death** (i.e. Apoptosis/Necrosis)	- Accumulation of Cellular Debris - Compromised Lung Function and Gas Exchange - Loss of Barrier Function & Epithelial Integrity - Stimulation (or Suppression) of Epithelial Stem Cell Response
	Induction of Innate Viral Recognition Pathways (e.g. PAMP Receptors)	- Anti-Viral State - Cytokine/Chemokine/IFN Production - Mucus Production
Hematopoietic Cells (e.g. Macrophages, Neutrophils, RDCs)	**Cell Death** (i.e. Apoptosis/Necrosis)	- Accumulation of Cellular Debris - Inhibition of Viral Clearance
	Induction of Innate Viral Recognition Pathways (e.g. PAMP Receptors)	- Activation/Maturation (e.g. RDCs, Macrophages) - Anti-Viral State - Cytokine/Chemokine/IFN Production - Reduced Immune Suppression (e.g. Alveolar Macrophages)
	Migration (e.g. Macrophages, RDCs)	- Induction of Adaptive Immune Responses - Systemic Spread (i.e. H5N1, SARS-CoV)

PAMP = pathogen associated molecular pattern; RDCs = respiratory dendritic cells.

Figure 1.2: ILC2 are a part of the innate lymphoid family. Group 2 innate lymphoid cells (ILC2) were originally identified in fat associated lymphoid clusters as major producers of IL-5, which supports B1-B cells survival in the peritoneum, and IL-13, which promotes mucus production. ILC2 production of IL-5 and IL-13 is highly responsive to IL-33 and IL-25. All innate lymphoid family members (including ILC1, ILC2, ILC17, and ILC22) are dependent on the transcription factor *Id2*. Images adapted from (Rankin et al., 2013; Strober, 2010).

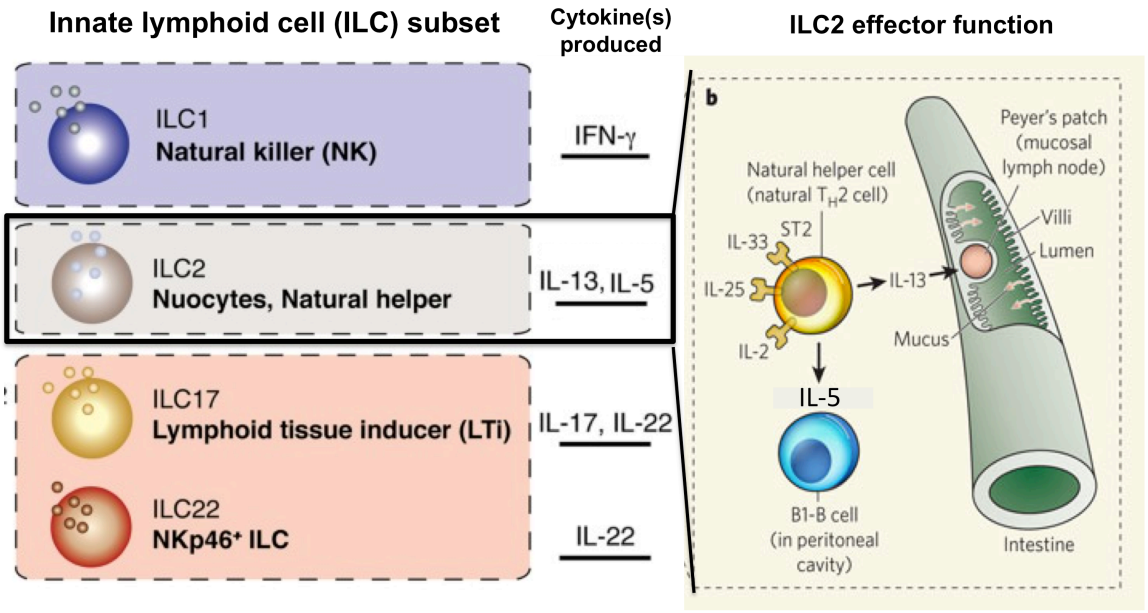
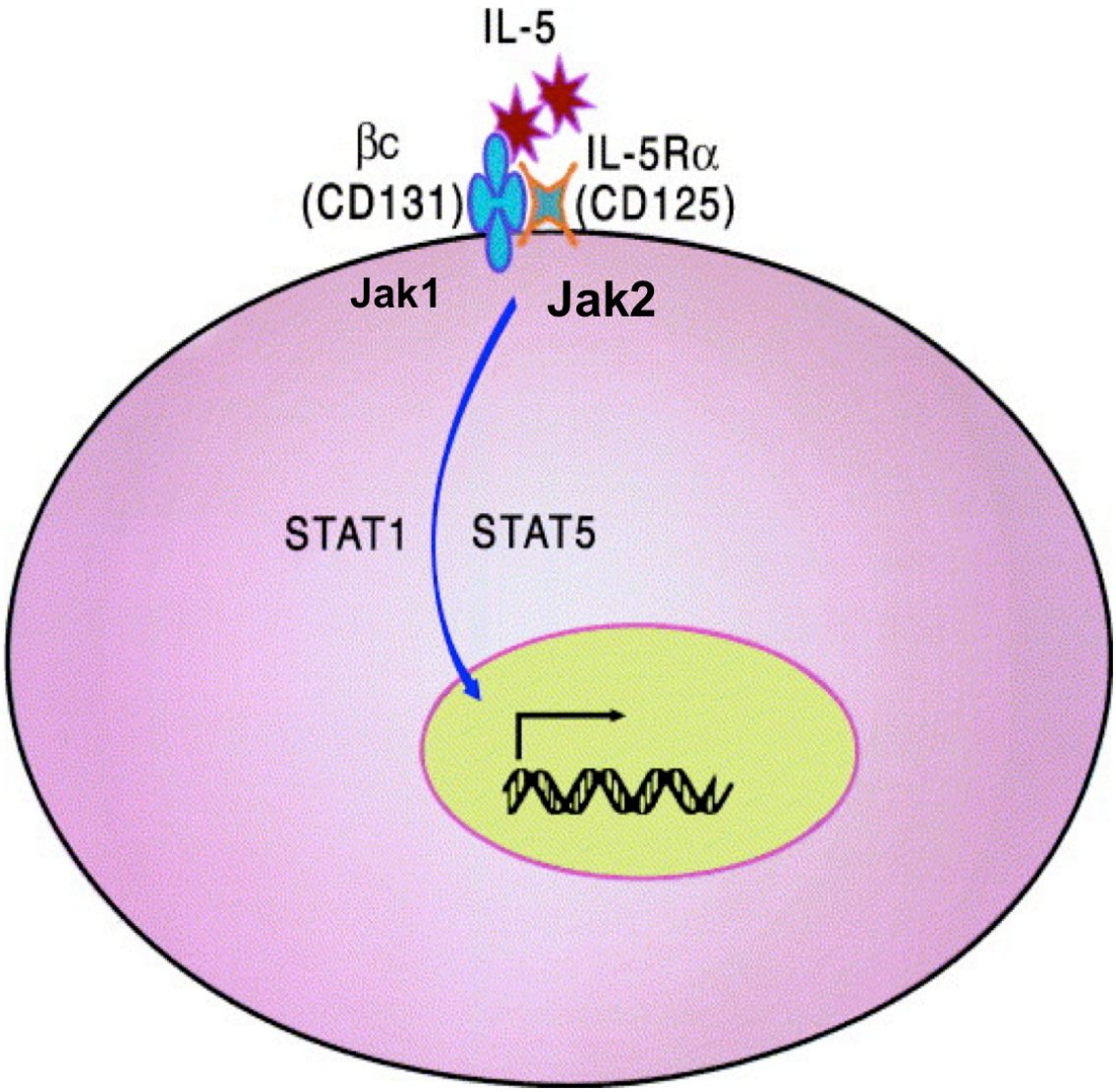

Innate lymphoid cell (ILC) subset
Cytokine(s) produced
ILC2 effector function
ILC1
Natural killer (NK)
IFN-γ
ILC2
Nuocytes, Natural helper
IL-13, IL-5
ILC17
Lymphoid tissue inducer (LTi)
IL-17, IL-22
ILC22
NKp46+ ILC
IL-22
b
Natural helper cell
(natural TH2 cell)
IL-33
ST2
IL-25
IL-2
IL-13
IL-5
B1-B cell
(in peritoneal
cavity)
Peyer's patch
(mucosal
lymph node)
Villi
Lumen
Mucus
Intestine

Figure 1.3: Interleukin (IL)-5 signaling cascade. IL-5 initially binds to the IL-5-specific IL-5Rα (CD125) subunit. Binding to IL-5Rα recruits the common beta chain (CD131), which is also shared with the receptors for IL-3 and GM-CSF. Binding of IL-5 to its receptor results in phosphorylation of STAT5 by Jak2, and minor signaling through Jak1/STAT1. Image adapted from (Foster et al., 2002).


IL-5
βc
(CD131)
IL-5Rα
(CD125)
Jak1
Jak2
STAT1
STAT5

CHAPTER 2: GROUP 2 INNATE LYMPHOID CELL PRODUCTION OF IL-5 IS REGULATED BY NKT CELLS DURING INFLUENZA VIRUS INFECTION.

Published in part in 'Gorski SA, Young SH, and Braciale TJ. 2013. *PLoS Pathogens* (in press).'

INTRODUCTION:

Type 2 immune responses are induced by parasitic and helminth infection and are characterized by the production of prototypical cytokines such as IL-4, -5, and -13 (Oliphant et al., 2011). IL-5 is one of the major type 2 cytokines that is essential for eosinophil survival (in humans) as well as B1-B cell development in mice (Takatsu, 2011). Although beneficial during parasitic or helminth infection, IL-5 may have a detrimental role in the development and severity of asthma and allergic diseases. Because of its essential role in eosinophil generation in the bone marrow and eosinophil egress out of the bone marrow, local production of IL-5 in the lungs during asthma exacerbation can result in pulmonary eosinophilia which can in turn enhance airway smooth muscle contraction and cause excess mucus production(Lee et al., 2004; Walsh et al., 2008).

Although viral infections are generally considered to elicit classic type 1 immune responses, features of the type 2 response are frequently present particularly in

individuals with pre-existing allergic diseases e.g. asthma (Bendelja et al., 2000; Hamada et al., 2013). The presence of type 2 cytokines during respiratory virus infection has been linked to asthma exacerbation; however, there is an emerging view that the type 2 responses might also play a tissue protective role (Allen and Wynn, 2011; Pulendran and Artis, 2012).

Group 2 innate lymphoid cells (ILC2) are innate immune lymphocyte-like cells that are capable of producing large amounts of IL-5 and IL-13 when stimulated by IL-25 and IL-33, two cytokines associated with the type 2 response (Spits et al., 2013). ILC2 were first identified in fat associated lymphoid clusters and subsequently other groups identified similar cells in the gut, spleen and lung (Moro et al., 2010; Neill et al., 2010; Price et al., 2010). ILC2 present in the lung of mice infected with influenza A virus (IAV) have been reported to produce abundant IL-13 during infection, which may contribute to airway hyperreactivity observed during experimental infection with respiratory viruses such as IAV (Chang et al., 2011). However, Monticelli et al. found that ILC2 are also capable of producing the epidermal growth factor family member amphiregulin, and this is essential to proper repair of the epithelial barrier following IAV infection (Monticelli et al., 2011).

In this chapter we demonstrate that during IAV infection there is abundant production of IL-5 in the infected respiratory tract, which stimulates the progressive recruitment and accumulation of eosinophils in the infected lungs, particularly late in infection (i.e. after infectious virus clearance) during the recovery phase. This IL-

5 is primarily the product of a small number of ILC2 recruited to the IAV infected lungs. Interestingly, while ILC2 produce IL-5 during the acute phase (i.e. 5-7 d.p.i., which corresponds to IAV-specific adaptive immune cell influx into the lungs), both ILC2 numbers and their ability to produce IL-5 increases dramatically following infectious virus clearance during the recovery phase (8–10 d.p.i. and beyond). This increase in IL-5 production by ILC2 is in part stimulated by IL-33 produced by both alveolar macrophages and, unexpectedly, NKT cells infiltrating the IAV infected lungs. The significance of these findings is discussed.

MATERIALS AND METHODS:

Ethics Statement

All animal experiments conducted in this study were carried out in accordance with the Animal Welfare Act (Public Law 91-579) and the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (OLAW/NIH, 2002). All experiments were approved by the University of Virginia Animal Care and Use Committee (Protocol Number 2230).

Mice and infection

BALB/c and C57BL/6 mice were purchased from the National Cancer Institute and maintained at the University of Virginia in a pathogen-free environment. Mice deficient in CD1d and Rag2 were a kind gift from Drs. Mark Okusa and Timothy

Bender (University of Virginia), respectively. Jα18$^{-/-}$ mice were provided by Dr. Victor Laubach (University of Virginia), and TCRγδ-/- mice were purchased from Jackson Laboratories. All mice used in experiments were between the ages of 8-12 weeks and matched for age and sex. Type A influenza virus A/PR/8/34 (H1N1) was grown in day 10 chicken embryo allantoic cavities as described previously (Lawrence and Braciale, 2004). Mice were infected with 300 egg infectious doses (EID_{50}) of A/PR/8/34 i.n. (corresponding to a 0.1 LD_{50} dose) unless otherwise stated.

Preparation of lung tissue

Lungs were perfused with 5 mL of PBS via the right ventricle of the heart to remove cells from the vasculature. To prepare a single cell suspension, lungs were minced and digested in media containing 183 U/ml type II collagenase (Worthington) for 45 minutes at 37°C. Lung tissues were then pushed through a steel screen and red blood cells were lysed with ammonium chloride buffer (e.g. ACK lysis buffer). Cells counts were performed with a hemocytometer and trypan blue was used to exclude dead cells. To prepare total lung RNA, lungs were homogenized in 1 ml TRIzol (Invitrogen) immediately following perfusion and stored at -80°C until analyzed.

Cytokine analysis of BAL fluid

Bronchoalveolar lavage (BAL) fluids were obtained by cannulating the trachea and flushing the lungs with 0.5 ml of sterile PBS three times. Cells were removed by centrifugation and supernatants were stored at -80°C until analyzed. IL-5 levels

were quantified by either ELISA (eBioscience) or a multiplex Luminex assay (Flow Cytometry Core Facility at the University of Virginia). Cytokines such as IL-33, IL-13, and IL-25 were analyzed at various d.p.i. using the Luminex assay.

RT-qPCR analysis

Total RNA was isolated from whole lung samples or FACS sorted cell populations frozen in TRIzol according to the manufacturer's instructions. RNA samples were treated with DNase I (Invitrogen) and complimentary DNAs (cDNA) were synthesized using random primers and Superscript II (all Invitrogen). Real time PCR of cDNA was performed in a StepOnePlus PCR system (Applied Biosystems) using SYBR Green master mix (Applied Biosystems). Primer sequences used were as follows: IL-5 F: 5′-GCTTCTGCACTTGAGTGTTCTG-3′, R: 5′-CCTCATCGTCTCATTGCTTGTC-3′ (Bamias et al., 2005); IL-33 primers F: 5′-TCCTTGCTTGGCAGTATCCA-3′, R: 5′-TGCTCAATGTGTCAACAGACG-3′; ST2 F: 5′-GCAATTCTGACACTTCCCATG-3′, R: 5′- ACGATTTACTGCCCTCCGTA-3′ (Pastorelli et al., 2010); Areg F: 5′-CTATCTTTGTCTCTGCCATCA-3′, R: 5′-AGCCTCCTTCTTTCTTCTGTT-3′ (Siegl and Uhlig, 2012).

***In vivo* antibody administration**

Mice were given 100 μg of anti-IL-5 (TRFK5, eBioscience) or control IgG (Jackson ImmunoResearch) i.p. in 100 μl of sterile PBS at indicated days post infection. Mice were given 300 μg of anti-CD8α (2.43, BioExpress), 250 μg of anti-CD4 (GK1.5, BioExpress), or control IgG i.p. at 3 d.p.i.. In some experiments, 250 μg of anti-NK1.1

(PK136, a kind gift from Dr. Young Hahn, University of Virginia) or control IgG was given at 5 d.p.i.. Levels of IL-5 in the BAL were assessed at 7 d.p.i..

Flow cytometry & antibodies

Single cells were suspended in FACS buffer containing PBS, 2% FBS, 10 mM EDTA, and 0.01% NaN_3. Cell suspensions were blocked with anti–mouse CD16/32, followed by incubation with either specific mAbs or isotype-matched control Igs for 20 min at 4°C. The following antibodies were used: CD11c, CD11b, CD45, Sca-1, C-kit, CD44 (Biolegend); Ly6G, SiglecF, CD49b, CD3, CD4, CD8, CD19, TCRβ, TCRγ, NK1.1 (BD Biosciences); and Thy1.2 (eBioscience). Lineage cocktail included: CD3, CD4, CD5, CD8, CD11b, Gr-1, CD19, B220, DX5 (or NK1.1) and TCRδ. Proliferation was assessed by Ki-67 and BrdU staining (BD Biosciences). For BrdU staining, mice were injected with 500 μl of 1 mg/ml of BrdU i.p. Six hours later, lungs were harvested and cells were strained for intranuclear BrdU incorporation. Mice that were not injected with BrdU were used for a staining control. Production of IL-33 (R&D Systems) and IL-5 (Biolegend) were detected by intracellular cytokine staining using Cytofix/Cytoperm (BD Biosciences) as per the manufacturer's instructions. CD1d-tetramer was provided by the NIH Tetramer Core Facility. Flow cytometry was performed on FACSCanto II flow cytometer (BD Biosciences), and data were analyzed using FlowJo (Tree Star, Inc.).

ILC2 cell culture *in vitro*

ILC2 were FACS sorted from lung suspensions using an iCyt Reflection sorter and kept in culture for 3 days in the presence of indicated stimuli in complete RPMI media supplemented with 10 % FCS. 30 ng/ml of PMA and 500 ng/ml of ionomycin (both from Sigma) were used to stimulate ILC2. rIL-33 (Biolegend) and rIL-2 (eBioscience) were used at 10ng/ml and 100U/ml, respectively.

***Ex vivo* intracellular cytokine assay**

Single cell suspensions from whole lung were cultured for 24 hours at 37°C (approx. 5 x 10^6 cells/ml) in culture medium containing 10 % FCS. GolgiStop (1μl/ml, BD Biosciences) was added for the last 4 hours of culture. Cells were surface stained for 15 minutes at room temperature, followed by fixation and permeabilization with Cytofix/Cytoperm (BD Biosciences). Intracellular cytokine staining for IL-5 was performed according the manufacturer's protocol. Anti-ST2 blocking antibody (Clone DJ8; MD Bioscience) was added to the culture wells at a concentration of 20 μg/ml.

Thy1.2$^+$ cell depletion *in vivo*

Rag2$^{-/-}$ mice were given 500 μg of anti-Thy1.2 depleting antibody (30H12, BioXcell) at 3 and 5 d.p.i. i.p. For ILC2 depletion during the recovery phase, wild type C57BL/6 mice were given 500 μg of 30H12 antibody at 8 d.p.i., followed by daily i.p. injections of 100 μg (or equivalent amounts of control IgG).

In vivo α-galactosylceramide (αGC) treatment.

BALB/c mice were given 2 μg of αGC (a kind gift from Dr. Tsuji, NYU) or a control volume of PBS i.p. at 3 and 5 d.p.i.. Lungs and BAL were harvested at 7 d.p.i. and assessed for IL-5$^+$ ILC2 (via *ex vivo* intracellular cytokine assay described above) and eosinophil infiltration into the lung.

Ex vivo depletion of alveolar macrophages and NKT

For *ex vivo* removal of NKT cells and alveolar macrophages (AM) from whole lung cell suspensions, cells were incubated with APC-conjugated CD1d-Tetramer (NIH tetramer facility) or PE conjugated anti-SiglecF antibody, respectively. Cells were incubated for 15 min at 4°C then washed with an excess amount of FACS buffer (PBS + 10% PBS + 10mM EDTA). Cells were then incubated with anti-APC or anti-PE magnetic beads (Miltenyi Biotec), kept at 4°C for 20 minutes, followed by washing in FACS buffer. NKT cells and AM were then removed from the suspensions by putting cells through a MACS Separation LS Colum (Miltenyi Biotec). Depletion efficiency was confirmed via FACS analyses of the collected samples. The remaining cells were resuspended in culture media for direct *ex vivo* intracellular cytokine analysis as described above.

Statistical Analysis

Statistical analyses were performed using Prism 5 (GraphPad Software). Unpaired, two-tailed Student t test; one-way ANOVA with a Tukey post test; and paired, two-

tailed t-tests were used to determine significance (individual tests are labeled in figure legends). P values <.05 were considered statistically significant.

RESULTS:

IL-5 is produced in the lungs during IAV infection

As part of a survey of cytokines produced in the respiratory tract following experimental influenza A virus (IAV) infection, we confirmed earlier reports (Baumgarth et al., 1994; Buchweitz et al., 2007; Sarawar et al., 1993) that IL-5 is released into the bronchoalveolar lavage (BAL) of IAV infected mice, in this instance mice infected with the mouse adapted IAV strain A/PR/8/ 34. IL-5 protein was first reproducibly detected in the BAL at 4 days post infection (d.p.i.), released at maximum levels in the BAL at 7 d.p.i. and detectable IL-5 in the BAL fluid subsequently declined over the next several days, concomitant with the clearance of infectious virus (Hufford et al., 2011)(Figure 2.1A). In contrast to IL-5 protein expression, IL-5 gene expression (mRNA levels) in homogenates of infected lungs, while paralleling the kinetics of release of this cytokine into the BAL fluid early during infection (Figure 2.1B), IL-5 transcripts remained readily detectable beyond 8 d.p.i in spite of the absence of detectable IL-5 protein in the BAL at this time. (Figure 2.1B). By 8 d.p.i there is a massive influx of CD45+ immune/inflammatory cells, including both adaptive immune cells (e.g. T and B cells) as well as myeloid lineage inflammatory cells (Hufford et al., 2011), thus the apparent reduction in IL-5 transcripts detectable in lung homogenates late in infection is likely an

underestimate of the actual transcript level. This would particularly be true if the IL-5 gene is expressed by a limited number of cells and the IL-5 mRNA in lung homogenate is diluted by RNA extracted from the CD45+ (IL-5 mRNA -) inflammatory cells accumulating within the lungs. Accordingly, the markedly diminished level of detectable IL-5 protein during the latter days following infection could reflect consumption of the cytokine by one or more cell types infiltrating the infected lungs.

IL-5 is the primary cytokine involved in the generation and maturation of eosinophils in the bone marrow and for the egress of eosinophils to sites of infection (Sanderson, 1992) e.g. the IAV infected lungs. Indeed, when we examined the kinetics of eosinophil accumulation in the infected lungs, we found that eosinophils accumulated progressively during the course of infection with the most pronounced accumulation of this cell type during the recovery phase of infection i.e. from 8 d.p.i. – 16 d.p.i. (Figure 2.1C). In order to establish that eosinophil accumulation in the lungs during the recovery phase was dependent on IL-5, infected mice were treated with neutralizing anti-IL-5 antibody (αIL-5) daily between 7 and 12 d.p.i. (Figure 2.1D). IL-5 neutralization resulted in a substantial reduction of eosinophils in the lung at 14 d.p.i. (Figure 2.1E and 2.1F). It is noteworthy that if αIL-5 administration were limited to the period from 7 through 10 d.p.i. and lung eosinophil numbers evaluated 4 days later, i.e. at 14 d.p.i., eosinophil numbers in the lungs were comparable to control IgG -treated animals (Figure 2.2A and 2.2B). Taken together these data suggest that IL-5 not only continues to be produced in the influenza-

infected lung during the recovery phase, but also is responsible for the progressive accumulation of eosinophils in the lungs. Therefore, our failure to demonstrate detectable levels of IL-5 protein in the BAL fluid at 8 d.p.i. and beyond most likely reflects the consumption of the cytokine by IL-5 receptor expressing cells, i.e. eosinophils.

Group 2 innate lymphoid cells are the primary producers of IL-5 during IAV infection.

We next sought to identify the cellular source of the IL-5 in the infected lungs. Since the kinetics of IL-5 release into the BAL fluid (Figure 2.1A) was comparable to the kinetics of many pro-inflammatory cytokines (e.g. IFNγ) that are released into the IAV infected lungs by responding adaptive immune IAV-specific CD8 + and CD4 + T-cells (Sun et al., 2009) we considered that one or more adaptive immune cell type was a likely source of the IL-5. To explore this possibility, we interrogated various cell types in the IAV infected lungs for expression of IL-5 transcripts. To this end, we first separated total lung cell suspensions from mice at 5 d.p.i. into Ly6G+ (primarily neutrophils), Thy1.2+ (lymphocytes), DCs (CD11c+MHC II^{hi}) and $CD45^-$ (epithelial/stromal origin). We observed that IL-5 gene expression was primarily associated with Thy1.2+ cells, with a minimal amount being detected in the $CD45^-$ fraction (Figure 2.3A). We then isolated by cell sorting of Thy1.2+ lung cells, the CD8+ T cell, CD4+ T cell, NKT cell, and NK cell fractions along with the residual lineage- Thy1.2+ cells. Somewhat unexpectedly, IL-5 mRNA was detected primarily in the Thy1.2+ LIN- cell fraction (Figure 2.3B). Consistent with these findings,

depletion of CD4, CD8 or NK cells had minimal impact on BAL IL-5 levels (Figure 2.3C). In addition, mice deficient in gamma delta T cells (TCRγδ-/-), another potential Thy1.2+ cell type, did not have significantly altered BAL IL-5 levels (Figure 2.3D). Jα18-/- mice, which are devoid of type I NKT cells, did have consistently lower BAL IL-5, however, due to the absence of IL-5 transcripts in the isolated NKT cell population, NKT cells were not considered a likely source of IL-5. These findings therefore suggested that the primary source of IL-5 in the IAV infected lungs was most likely the recently described Thy1+ Lin- cell population, the group 2 innate lymphoid cell (ILC2).

ILC2 were first identified in fat associated lymphoid clusters and have since been detected in the spleen, mesenteric LN, as well as lungs (Moro et al., 2010; Neill et al., 2010; Price et al., 2010; Spits et al., 2013) including IAV infected lungs (Chang et al., 2011; Monticelli et al., 2011). These cells produce extremely high levels of the type 2 cytokines IL-5 and IL-13 (on a per cell basis) when stimulated with IL-25 or IL-33. ILC2 have generally been described as LIN- Thy1.2+CD44+Sca-1+ and either c-kit^{+} (Moro et al., 2010) or c-kit^{-} (Price et al., 2010) depending on the tissue source or experimental conditions. We detected both c-kit^{+} and c-kit^{-} ILC2 in the IAV infected lungs (Figure 2.4A). While both c-kit^{+} and c-kit^{-} ILC2 accumulate in the lungs following IAV infection, the c-kit^{-} ILC2 subset predominates during the acute phase of infection (2- 7 d.p.i.) with the c-kit^{+} ILC2 rapidly accumulating in increasingly large numbers during the recovery phase i.e. after infectious virus clearance at 8- 9 d.p.i. and beyond (Figure 2.4B). Both ILC2 subsets appear to be morphologically

similar (Figure 2.4C) and expressed similar levels of various surface markers(Figure 2.5). In support of previously published reports, both c-kit$^+$ and c-kit$^-$ ILC2 expressed CD25, CD127, and the IL-33 receptor subunit, ST2 (Spits and Cupedo, 2012), as well as MHC II and CD69. We detected little to no FLT3, a hematopoietic growth factor receptor that is in the same family as c-kit and serves a similar function (Masson and Ronnstrand, 2009). Each subset was also equally capable of expressing amphiregulin (Figure 2.6), as this epidermal growth factor family member was recently shown to be critical for epithelial barrier protection during IAV infection (Jamieson et al., 2013; Monticelli et al., 2011). Following purification by cell sorting, both ILC2 subsets expressed comparable levels of IL-5 transcripts when analyzed directly *ex vivo* (Figure 2.4D) as well as comparable IL-5 secretion following *in vitro* stimulation of equivalent numbers of sorted cells with PMA/ionomycin (Figure 2.4E) suggesting functional as well as morphological similarity of the two ILC2 subsets.

To determine if the rapid accumulation of c-kit$^+$ ILC2 was the result of local proliferation of c-kit$^+$ ILC2 in the respiratory tract, we evaluated ongoing proliferation of this cell type by Ki67 expression at 10 d.p.i. (Figure 2.7A) and BrdU uptake at 7 d.p.i. (Figure 2.7B). We were unable to detect significant proliferation of these cells by either criteria suggesting that the c-kit$^+$ ILC2 accumulating in the IAV infected lungs were most likely being recruited from the bone marrow.

To determine whether ILC2 can produce IL-5 following influenza infection *in vivo,* we first analyzed IL-5 production and the contribution of ILC2 as a source of IL-5 in adaptive immune cell deficient, IAV infected Rag2-/- mice since the majority of Thy1.2^{+} cells in these mice are ILC2 (Hoyler et al., 2012; Moro et al., 2010; Price et al., 2010). Infected Rag2 -/- were depleted of ILC2 *in vivo* by administration of a depleting anti-Thy1.2 antibody at 3 and 5 d.p.i. (Figure 2.8A). IL-5 levels in the BAL fluid and ILC2 numbers in the IAV infected lungs were assessed at 7 d.p.i. in infected Rag2-/- and wild type (WT) mice, in order to determine the extent of antibody mediated depletion of these cells. Depletion of Thy1.2^{+} cells in both Rag2-/- and WT was >99% (Figure 2.8B and 2.8D). As Figure 2.8C demonstrates infected, untreated, Rag2 -/- mice produced dramatically higher levels of IL-5 in the BAL than their infected WT counterparts (Figure 2.8C). This finding is consistent with the higher absolute number of ILC2 accumulating in the lungs of infected Rag2 deficient mice (Figure 2.8D). Importantly, *in vivo* depletion of ILC2 in Rag2-/- (and WT) mice likewise resulted in a reduction of IL-5 in the BAL fluid to background levels (Figure 2.8C). These results support the view that ILC2 are fully capable of producing IL-5 during IAV infection, and are most likely the major *in vivo* producers of IL-5 in the respiratory tract of IAV infected mice.

To establish a direct connection between ILC2 and the IL-5 mediated accumulation of eosinophils in the lungs during the resolution phase of IAV infection, we devised an *in vivo* Thy1.2^{+} cell depletion and reconstitution strategy (Figure 2.8E). Specifically, Thy1.2+ mice were sub-lethally infected with IAV and at 8 d.p.i. the mice

were depleted of Thy1.2$^+$ cells by administration of a depleting antibody. Two hours following Thy1.2$^+$ cell depletion the infected recipient mice received an inoculum of 2x10^6 Thy1.1$^+$ cells isolated from the lungs of 8 d.p.i. congenic Thy1.1+ mice consisting of either total Thy1.1$^+$ cells (including ILC2) or CD3$^+$Thy1.1$^+$ lung cells (deficient in ILC2). To inhibit the regeneration/recruitment of endogenous Thy1.2+ ILC2 the mice received additional daily treatment with the depleting anti-Thy1.2 antibody for 3 days prior to quantification of lung infiltrating eosinophils at 12 d.p.i. (Figure 2.8E). As Figure 2.8F demonstrates, only recipients of total Thy1.1$^+$ cells (i.e. containing ILC2) had restored eosinophil numbers in the infected lungs following depletion of endogenous ILC2. These data are consistent with the view that ILC2 continue to produce IL-5 during the recovery phase and thereby facilitate eosinophil accumulation.

ILC2 are responsive to an *in vivo* source of IL-33

ILC2 have been reported to produce IL-5 following exposure to several cytokines, most notably IL-33 and IL-25 (Fallon et al., 2006; Moro et al., 2010). IL-33 is released from dead cells and infected cells including respiratory epithelial cells infected by IAV *in vivo* and *in vitro* (Le Goffic et al., 2011). Indeed, we could readily detect IL-33 (but not IL-25, Figure 2.9) in the BAL fluid from IAV infected lungs. IL-33 was detectable as early as day 1 p.i., reached a maximum on day 7 p.i., and was still detectable in the BAL fluid of mice as late as day 11 p.i. (Figure 2.10A). In order to directly assess the impact of IL-33 on IL-5 production by ILC2, we isolated c-kit$^+$ and c-kit$^-$ ILC2 via FACS from IAV infected lungs at 4, 7, and 14 d.p.i. and

stimulated them directly *ex vivo* with recombinant murine IL-33 (rIL-33) for 72 hours. Both c-kit$^+$ and c-kit$^-$ ILC2 isolated at 14 d.p.i. produced high levels of IL-5 (Figure 2.10B) in keeping with the evidence implicating IL-33 as an extremely potent stimulus for IL- 5 production by ILC2. IL- 5 production by ILC2 on a per cell basis was considerably lower (~ 10 fold) for both c-kit$^+$ and c-kit$^-$ ILC2 isolated at 7 dp.i. and was either minimal (c-kit$^-$ ILC2) or not detectable (c-kit$^+$ ILC2) when cells were isolated and stimulated at 4 d.p.i. (Figure 2.10B). A likely explanation for the difference in sensitivity of ILC2 isolated from the infected lungs at different times p.i. to rIL-33 was suggested by our analysis of the level of expression of the gene encoding the ST2 subunit of the IL-33 receptor (IL-33R, Figure 2.10C). As with IL- 5 production, ST2 expression was pronounced at 7 d.p.i. and was significantly higher on ILC2 isolated at 14 (Figure 2.10C). These findings were consistent with the concept that IL-33 serves as a regulator of IL- 5 expression by ILC2 during IAV infection and that the impact of IL-33 on IL- 5 expression was likely to be most pronounced during the later phase of IAV infection.

In order to further establish the contribution of IL-33 as a stimulus for the production of IL-5 in the IAV infected lungs we developed an *ex vivo* intracellular cytokine staining (ICS) assay to detect and quantify IL-5 producing ILC2. Total lung cell suspensions from IAV infected lungs were cultured for 24 hours in the presence or absence of blocking ST2 (IL-33R) antibody. IL-5 producing ILC2 present in infected lung cell suspensions were then enumerated by ICS (Figure 2.10D and 2.10E). As Figures 2.10D and 2.10E demonstrate, blockade of the IL-33 receptor ST2

subunit during *in vitro* culture of lung cell suspensions reduced the frequency of IL-5 producing ILC2 for both the c-kit$^+$ and c-kit$^-$ lung ILC2 subsets. This finding further implicates IL-33 produced *in vivo* during IAV infection as an important regulator of IL-5 production by ILC2

Sources of IL-33 in the IAV infected lungs

As noted above, IL-33 has been reported to be produced by a variety of cell types (Mirchandani et al., 2012) and its expression in and release from injured/stressed cells –including epithelial cells–suggests the role of the cytokine as an "alarm" signal released during cell death (Cayrol and Girard, 2009; Moussion et al., 2008; Talabot-Ayer et al., 2009). To identify potential sources of IL-33 in the IAV infected lungs we initially analyzed IL-33 gene expression in various cell types isolated from the infected lungs at 12 d.p.i. IL-33 gene expression was prominent both in the CD45$^-$ fraction of whole lung cell suspensions (which includes infected respiratory epithelial cells undergoing virus induced as well as immune mediated cell death(Hufford et al., 2011)), as well as, surprisingly, NKT cells (Figure 2.11A). Both CD45$^-$ and NKT cells were also prominent sources of IL-33 transcript at 7 d.p.i. (data not shown). NKT expressed and secreted IL-33 protein both at 7 and 12 d.p.i. (Figure 2.12 and Figure 2.11B). Although IL-33 has documented effects on NKT cells (Bourgeois et al., 2011; Smithgall et al., 2008), NKT had not heretofore been implicated as a source of IL-33, particularly during IAV infection. Lung infiltrating NKT cells increased in numbers over the course of infection reaching a maximum at 7 d.p.i. and only gradually decreased in numbers up to 12 d.p.i. (Figure 2.11C).

NKT cells and the regulation of ILC2 IL-5 production.

The above observations raised the possibility that NKT cells may regulate IL-5 production by ILC2. To explore this possibility, we first analyzed IL-5 release into the BAL fluid of control and invariant NKT cell deficient (Jα18-/-) and global NKT deficient (CD1d-/-) mice undergoing IAV infection. As Figure 2.11D demonstrates, IL-5 production was significantly decreased at 7 d.p.i. in Jα18-/- and CD1d-/- mice relative to infected WT mice. This suggested that NKT cells, because of their absence of IL-5 transcript were not producers of IL-5, but instead were most likely regulating IL-5 production, at least partially, by ILC2.

To more directly assess the impact of NKT cell deficiency on IL-5 production by ILC2 during infection, we examined ILC2 IL-5 production in total lung cell suspensions from WT and CD1d-/- mice at 7 and 12 d.p.i. using our *ex vivo* ICS assay. As Figures 2.13A and 2.13B demonstrate, the frequency of IL-5 producing ILC2 was significantly diminished at 7 d.p.i. in both c-kit^{+} and c-kit^{-} ILC2 from the infected NKT cell deficient donors. A corresponding decrease in the absolute number of IL-5+ ILC2 from lungs of infected CD1d-/- mice was likewise observed (Figure 2.13C). There was, similarly, a significant decrease in the frequency and number of IL-5 producing ILC2 among the more abundant c-kit^{+} ILC2 isolated from lungs of infected NKT cell deficient CD1d-/- mice at 12 d.p.i. (Figure 2.13D, 2.13E, and 2.13F). Although there was also a diminution in the frequency of IL-5+ c-kit^{-} ILC2, this did not achieve statistical significance (Figure 2.13E).

In view of these findings it was of interest to determine if addition of IL-33 to the lung cell cultures from NKT cell deficient donors could reverse the deficit in the frequency of IL-5 producing ILC2. As Figures 2.13G and 2.13H demonstrate, addition of rIL-33 to 12 d.p.i. lung cell suspensions at the start of the 24-hour *ex vivo* culture system resulted in an increase in the frequency of IL-5+ ILC2 among the more abundant c-kit^{+} ILC2 to levels comparable to that of NKT cell sufficient WT mice, indicating that IL-33 can compensate for the decrease in the frequency of IL-5+ ILC2 resulting from NKT cell deficiency. Of note, IL-33 supplementation had no effect on the frequency of IL-5+ ILC2 in lung cell suspensions from control WT mice, suggesting that endogenous IL-33 may be at a saturating level in WT lung suspensions.

Alpha-galactosylceramide (αGC) is an extremely potent and specific activator of NKT cells (Kawano et al., 1997). If NKT cells are an important contributor to the control of IL-5 production by ILC2 then augmenting NKT cell numbers and function by αGC administration would be expected to enhance ILC2-dependent IL-5 production during IAV infection. αGC administration to IAV infected WT mice at 3 and 5 d.p.i., resulted in a 2- 3 fold increase of NKT cells in the infected lungs at 7 d.p.i. (Figure 2.14A). Consistent with the above findings, we observed an increase in the frequency of IL-5 producing ILC2 in 7 d.p.i. *ex vivo* lung cultures from αGC treated infected WT mice (Figure 2.14B and 2.14C) but no increase in the absolute number of ILC2 (Figure 2.14C). The increase in IL-5 producing ILC2 following αGC

administration coincided with an increase in both the frequency and absolute numbers of eosinophils in the treated IAV infected lungs (Figure 2.14D).

NKT and alveolar macrophages support ILC2-derived IL-5 production.

Alveolar macrophages (AM) were recently shown to be a likely source of IL-33 that regulates IL-13 production by ILC2 during IAV (Chang et al., 2011). IL-33 gene expression is induced in both NKT and AM following IAV infection (Figure 2.15). AM isolated at 7 d.p.i. indeed expressed IL-33 transcripts at levels comparable to that of NKT cells, however, AM isolated from 12 d.p.i. lungs expressed approximately one fifth the level of IL-33 mRNA as NKT cells (Figure 2.16A). It was recently noted that NKT cells can influence the level of IL-33 in AM (Kim et al., 2012), however, the expression level (MFI) of IL-33 protein in 12 d.p.i. AM from WT and NKT cell deficient (CD1d-/- or Jα18) mice was comparable (Figure 2.16B) suggesting that the diminished IL-5 response in NKT cell deficient mice was not linked to decreased IL-33 expression by AM.

To further assess the contribution of NKT cells and AM in the production of IL-5 by c-kit + ILC2 in the IAV infected lungs we analyzed the impact of selective depletion of NKT cells and/or AM from 12 d.p.i. total lung cell suspensions prior to 24-hour *ex vivo* culture on the frequency of IL-5 + c-kit +ILC2 (the predominant ILC2 type at this time after infection). Depletion of either cell type from the lung cell suspension resulted in ~ 40% - 50% reduction in IL-5 producing ILC2 (Figure 2.16C and 2.16D). Of note, simultaneous depletion of both cell types did not result in a further

significant reduction in the frequency of IL-5 + ILC2, suggesting that other factors may also regulate IL-5 production by ILC2.

DISCUSSION

IL-5 has previously been reported to be produced in the lungs during experimental IAV infection (Baumgarth et al., 1994; Buchweitz et al., 2007; Sarawar et al., 1993). In the current study we focused on defining the cellular source of IL-5 in the influenza-infected lungs and the tempo of IL-5 protein and gene expression. We observed that the peak of IL-5 release into the airways (as detected in BAL fluid) corresponded to the peak of effector T cell dependent pro-inflammatory cytokine release into the BAL i.e. 6–8 d.p.i., while IL-5 gene expression persisted for a longer time frame i.e. 11–13 d.p.i.. IL-5 production was associated with the progressive increase in eosinophils infiltrating the infected lungs particularly during the recovery phase, that is following infectious virus clearance (8–10 d.p.i.; (Hufford et al., 2011)). We went on to demonstrate that the source of this IL-5 produced in the lungs during IAV infection is primarily the recently described group 2 innate lymphoid cell (ILC2) (Spits et al., 2013). We found that both c-kit^{+} and c-kit^{-} ILC2 accumulate in the lung throughout the course of infection, however, these two ILC2 subsets appear to be recruited in a biphasic manner, that is c-kit^{-} ILC2 are predominant during the acute phase of infection (0-7 d.p.i.) while the c-kit^{+} dramatically increase during the recovery phase (Figure 2.4B). As has been

previously reported (Moro et al., 2010), ILC2 IL-5 production is regulated by IL-33, one source of which we now identify as the NKT cell. In support of this conclusion we found that NKT cell deficient mice have less IL-5 present in the BAL at 7 d.p.i. as well as significantly diminished IL-5 production from ILC2 during the recovery phase at 12 d.p.i.. Conversely, treatment of wild type mice with the NKT cell activating ligand αGC significantly increased IL-5 production from ILC2 and subsequently increased eosinophil numbers by 2-fold.

Our finding that ILC2 are the major source of the type 2 cytokine IL-5 (as well as IL-13, data not shown) and that ILC2 produce extremely high levels of this cytokine on a per cell basis, are in keeping with recent findings on type 2 cytokine production by ILC2 in a model of experimental helminth infection (Moro et al., 2010; Price et al., 2010). Similarly, ILC2 were implicated as the primary source of the type 2 cytokine IL-13 in a model of experimental IAV infection (Chang et al., 2011), as well as major producers of the epidermal growth factor family member, amphiregulin (Monticelli et al., 2011). We detected elevated levels of amphiregulin in ILC2 during the recovery phase, consistent with the role of ILC2 in maintaining epithelial barrier integrity (Figure 2.6). In the current study, we also analyzed the tempo of accumulation of c-kit^{+} and c-kit^{-} ILC2 in the IAV infected lungs. As noted above, c-kit^{+} ILC2 are the predominant IL-5 producing cell type during the late, “recovery” phase of infection, which follows virus clearance. Although there is abundant expression of the c-kit ligand, stem cell factor, in the IAV infected lungs (data not shown) we could not demonstrate by several criteria (Ki67 expression and BrdU uptake) local

proliferation of the c-kit^{+} ILC2 in the infected respiratory tract. Therefore, the marked accumulation of this ILC2 subset in the infected lungs during the recovery phase of infection most likely reflects the recruitment of the cell type from bone marrow stores (Hoyler et al., 2012; Yang et al., 2011). At present, it is not clear why the tempo of accumulation of c-kit + and c-kit - ILC2 differs during the course of infection as we detect no difference in the intrinsic capacity of these two ILC2 subsets to produce IL-5. Since current evidence suggests that this novel lymphoid like cell type arises from progenitors in the bone marrow (Hoyler et al., 2012; Yang et al., 2011), differences in the type or magnitude of cytokines produced within the IAV infected lungs at different points in the course of infection may regulate the preferential recruitment and accumulation of one or the other ILC2 subset.

IL-33 (along with IL-25) has been reported to be a regulator of type 2 cytokine production by ILC2 (Fallon et al., 2006; Moro et al., 2010; Price et al., 2010). Indeed in a recent analysis of the role of IL-33 in the regulation of type 2 cytokine production by ILC2, alveolar macrophages (AM) were implicated as the source of IL-33 regulating ILC2 IL-13 production (Chang et al., 2011). In the current report, we demonstrate that NKT cells infiltrating the IAV infected lungs are another source of IL-33 influencing the magnitude of type 2 cytokine, in this case IL-5, production. While NKT cells and AM expressed comparable levels of IL-33 mRNA at 7 d.p.i., IL-33 transcript levels were up to four-fold higher in NKT cells at 12 d.p.i.. However, analysis of the contribution of each of these innate immune cell subsets in the regulation of IL-5 production by ILC2 in our *in vitro* lung cell suspension culture

system suggests that, at 12 d.p.i. at least, NKT cells and AM may have a similar impact on ILC2 cytokine production. It is noteworthy that simultaneous depletion of both innate immune cell subsets from lung cell suspensions did not have an additive effect on the frequency of IL-5 + ILC2. Since multiple cell types, including as noted in this report $CD45^-$ cells, from infected lungs can produce IL-33 (Mirchandani et al., 2012), it is likely that IL-33 produced by one or more other cell types can in part compensate for the loss of IL-33 resulting from the elimination of NKT cells (and/or AM) *in vitro*. Thus, our data favors the view that multiple cell types within the IAV infected lungs act in concert to regulate type 2 cytokine production by ILC2 through release of IL-33.

Pulmonary eosinophilia is not usually considered to be a feature of the strong type 1 immune response elicited by infection with potent respiratory viral pathogens like IAV. Nevertheless, other reports (Wareing et al., 2004) as well as data presented here have documented the influx of eosinophils into the lungs during experimental IAV infection as well as peripheral eosinophilia under certain circumstances in human IAV infection (Terai et al., 2011). Our analysis of the impact of IL-5 neutralization *in vivo* on eosinophil accumulation in the infected lungs implicates pulmonary IL-5 as a critical controller of eosinophil influx during IAV infection. Our IL-5 neutralization experiments also suggest a critical role for the cytokine in orchestrating the progressive accumulation of eosinophils in the lungs following virus clearance when IL-5 is no longer detectable in BAL fluid. It is also likely that other mediators (or lack thereof), such as eotaxins (along with the precipitous drop

in the production of pro-inflammatory, eosinophilia suppressing cytokines such as IFNγ following IAV clearance in the lungs), work in concert with IL-5 to enhance eosinophil accumulation in the lung during the recovery phase (Rothenberg and Hogan, 2006). Several factors likely account for our inability to detect IL-5 in the airways at later time points during the resolution phase of infection. Following virus clearance there is regeneration/restoration of the airway basement membrane and respiratory epithelial cell barrier integrity. This would certainly limit the extent to which soluble mediators like IL-5 could "leak" into the airways. A more likely possibility is that IL-5 produced by ILC2 is taken up and consumed by IL-5 receptor expressing cells, most notably eosinophils. Whether other cell types express a functional IL-5 receptor in the IAV infected lungs and therefore also contribute to the consumption of this cytokine during the resolution phase of infection remains to be determined.

In the setting of underlying reactive airway disease, respiratory virus infections are strongly linked to subsequent asthma exacerbation (Al-Garawi et al., 2009; Marsland et al., 2004). The proinflammatory cytokines produced in response to respiratory viral infection can enhance airway hyperreactivity and damage the sensitized respiratory epithelial barrier. Type 2 cytokines e.g. IL-4, IL 13, if produced during respiratory viral infection, are capable of promoting airway smooth muscle contraction and increased mucus secretion. Indeed, Chang et al. in their recent analysis of IL-13 production by ILC2 during IAV infection have suggested that this ILC2 derived IL-13 triggers airway hyperreactivity and therefore

may represent an important mechanism resulting in asthma exacerbation during IAV infections. They did not, however, find any eosinophil accumulation in their model of influenza infection (using the reassortant Mem/71/Bel strain of IAV). On the other hand, in this report we demonstrate that ILC2 derived IL-5 is critical for eosinophil accumulation during IAV infection with A/PR8/34. The reason for the difference in eosinophil accumulation in these two reports is uncertain but differences in the properties of the two IAV strains employed for infection likely contributes.

Allergic airway disease is characterized by respiratory tract eosinophilia and eosinophils are proposed to play a role in asthma exacerbation by stimulating mucus hyper secretion and enhanced airway hyperreactivity (Lee et al., 2004). At present, the precise role of eosinophils in the host response to IAV infection is uncertain. Thus this cell type may contribute to enhanced airway disease as proposed for IL-13 (Chang et al., 2011). On the other hand eosinophils have been reported to have an antiviral role through release of secreted products (Domachowske et al., 1998; Phipps et al., 2007). Such a role for the cell type in experimental IAV infection seems unlikely since maximum accumulation of eosinophils in the pulmonary interstitium occurs after infectious virus clearance. On the other hand, the tempo of eosinophil accumulation and increased IL-5 production during the resolution phase of infection raises the possibility that this type 2 response could have a positive role in restoration of pulmonary architecture and function by promoting tissue repair (Chen et al., 2012b). Additional studies will

need to be undertaken to better understand the contribution of eosinophils to the host response to IAV infection.

In conclusion, we have demonstrated that ILC2 are the cellular source of IL-5 produced during experimental IAV infection. The production of IL-5 by ILC2 is essential for the gradual, progressive infiltration and accumulation of eosinophils into the infected lungs. We demonstrate that one of the important sources of IL- 33, an essential regulator of IL-5 production by ILC2, is the NKT cell. The precise role of eosinophils in the host response to IV infection is currently being evaluated.

Figure 2.1: IL-5 is produced in the influenza-infected lung and results in eosinophil accumulation in the lung. (A) BAL fluid from C57BL/6 mice was collected and IL-5 levels were measured via ELISA. (B) cDNA was derived from whole lung homogenate and analyzed for IL-5 transcript as described in material and methods. (C) Total eosinophils present in the lungs as identified by flow cytometry. (D) BALB/c mice were given 100 μg of neutralizing anti-IL-5 antibody (αIL-5) i.p. daily from 7-12 d.p.i. and lung cell suspensions analyzed at 14 d.p.i.. (E) Eosinophils were identified in the lung as $CD45^{+}SiglecF^{+}CD11c^{lo}$. Plots represent analysis of lung cell suspensions first gated on CD45+ cells. (F) Total number of eosinophils present in the lung at 14 d.p.i. following αIL-5 treatment. Bars represent SEM of a representative experiment repeated at least three times. n=3-5 mice per experiment. *$p<0.05$ (Student's t-test)

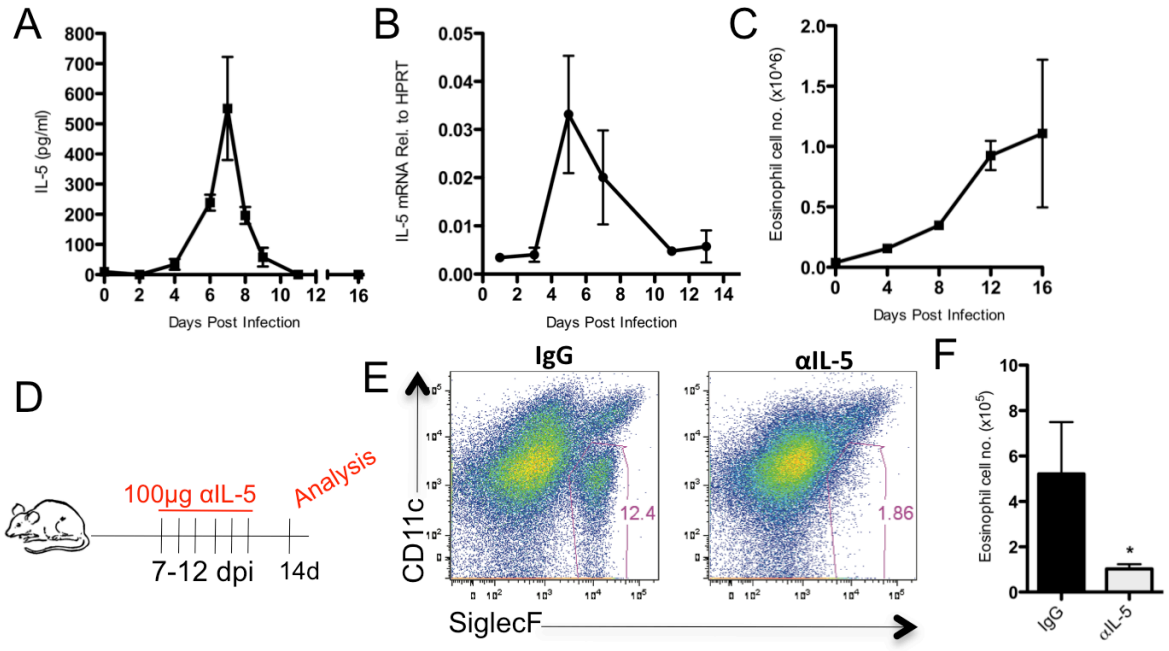

A
IL-5 (pg/ml)
Days Post Infection
B
IL-5 mRNA Rel. to HPRT
Days Post Infection
C
Eosinophil cell no. (x10^6)
Days Post Infection
D
100µg αIL-5
Analysis
7-12 dpi
14d
E
IgG
αIL-5
CD11c
SiglecF
12.4
1.86
F
Eosinophil cell no. (x10^5)
IgG
αIL-5

Figure 2.2: Eosinophil numbers rebound following early cessation of αIL-5 treatment. (A) C57BL/6 mice were given 100μg of neutralizing anti-IL-5 antibody (αIL-5) i.p. daily from 7-10 d.p.i. with lungs being analyzed at 14 d.p.i.. (B) Representative flow plots of 14 d.p.i. lungs of mice given control IgG or αIL-5 as described in (A). Eosinophils were identified as $CD45^{+}SiglecF^{+}CD11c^{lo}$.

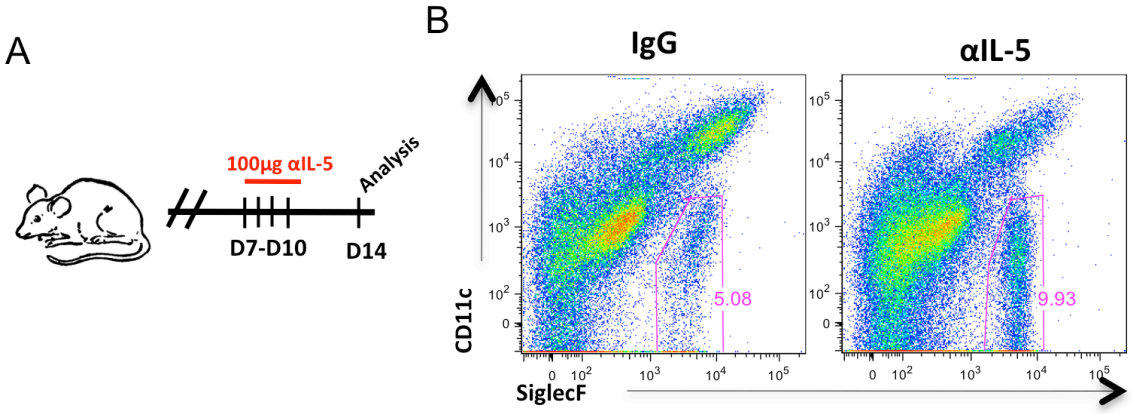

A
100μg αIL-5
Analysis
D7-D10
D14
B
IgG
αIL-5
CD11c
SiglecF
5.08
9.93

Figure 2.3: IL-5 transcripts are present in a Thy1.2+ non-canonical cell population (A) Indicated cell populations were sorted from the lungs at 5 d.p.i. and analyzed for IL-5 transcript via RT-PCR. (B) Lymphocytes thought to make up the Thy1.2^{+} population were FACS sorted from 7 d.p.i. lung. ND = Not detected. (C) C57BL/6 mice were given indicated depleting antibodies as described in Materials and Methods and BAL was collected at 7 d.p.i. for measurement of IL-5 protein by ELISA. (D) BAL from indicated knockout mouse strains was collected at 7 d.p.i. and analyzed for IL-5 protein. (A-B) from pooled mice, n=5, (C-D) n=3-5 per group. Error bars are +/- SEM.

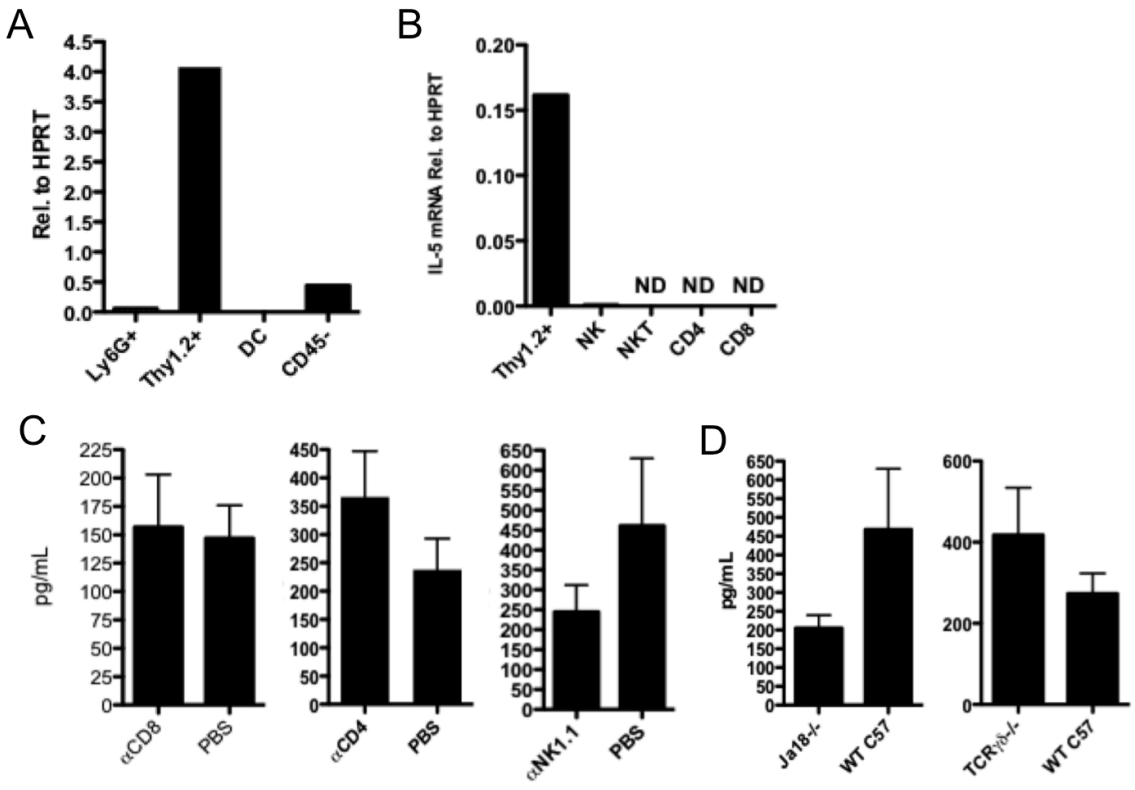

A
Rel. to HPRT
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Ly6G+
Thy1.2+
DC
CD45-
B
IL-5 mRNA Rel. to HPRT
0.20
0.15
0.10
0.05
0.00
ND ND ND
Thy1.2+
NK
NKT
CD4
CD8
C
pg/mL
αCD8
PBS
αCD4
PBS
αNK1.1
PBS
D
pg/mL
Ja18-/-
WT C57
TCRβδ-/-
WT C57

Figure 2.4: Group 2 innate lymphoid cells (ILC2) expand and are more potent producers of IL-5 during the resolution phase of infection. (A) Representative FACS plots identifying ILC2 in C57BL/6 mice. LIN antibody cocktail included: CD3, CD4, CD5, CD8, CD11b, Gr-1, CD19, B220, DX5 (or NK1.1) and TCRδ. (B) Absolute number of both c-kit^{+} and c-kit^{-} ILC2 following infection. (C) c-kit^{+} and c-kit^{-} ILC2 were FACS sorted from the lung at 7 d.p.i. and stained with hematoxylin and eosin. (D-E) c-kit^{+} and c-kit^{-} ILC2 were FACS sorted from the lung at indicated times and (D) directly analyzed for IL-5 transcript or (E) cultured for three days in the presence PMA/ionomycin. (E) Supernatants were collected and IL-5 levels analyzed via ELISA. Data is representative of two experiments with $\geq$3 mice per group (A-C) or pooled from 15-20 mice (D-E). Bars = +/- SEM

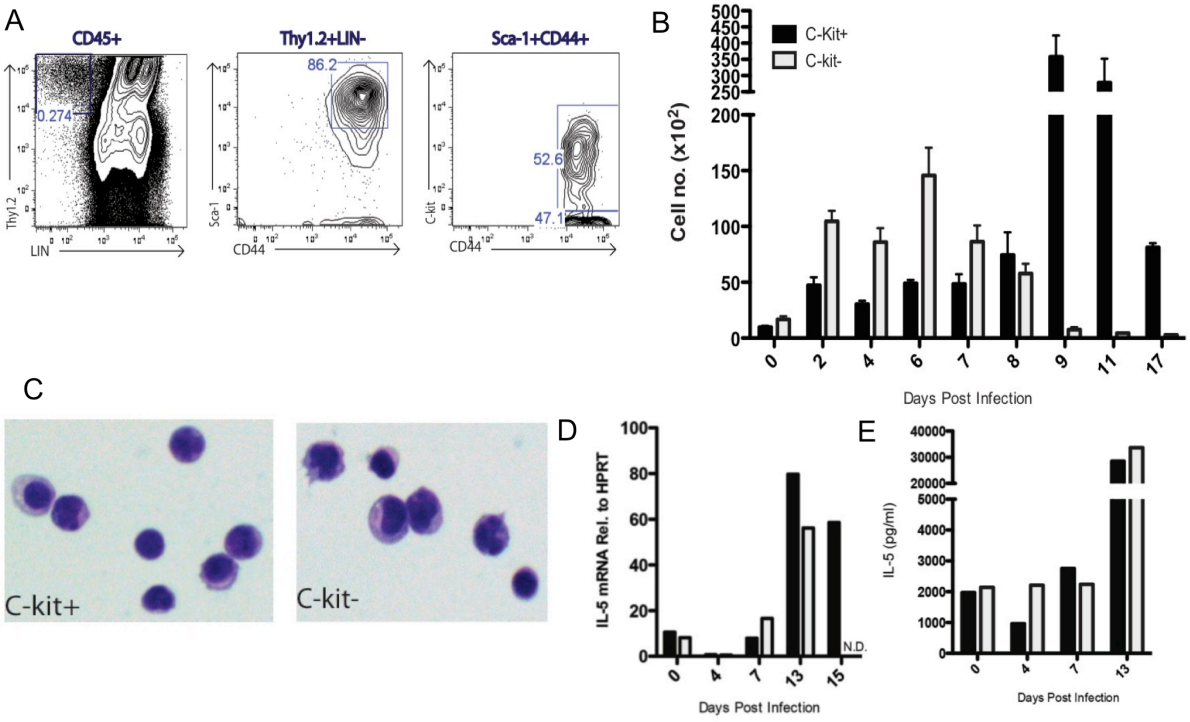

A
CD45+
Thy1.2+LIN-
Sca-1+CD44+
0.274
86.2
52.6
47.1
Thy1.2
LIN
Sca-1
CD44
C-kit
CD44
B
C-Kit+
C-kit-
Cell no. (x10²)
Days Post Infection
C
C-kit+
C-kit-
D
IL-5 mRNA Rel. to HPRT
N.D.
Days Post Infection
E
IL-5 (pg/ml)
Days Post Infection

Figure 2.5: Surface marker expression of ILC2 subsets. Lung c-kit^{+} (black line) and c-kit^{-} (blue line) ILC2 subsets were analyzed for indicated surface markers between 10-12 d.p.i.. Isotype controls are represented as shaded histrograms.

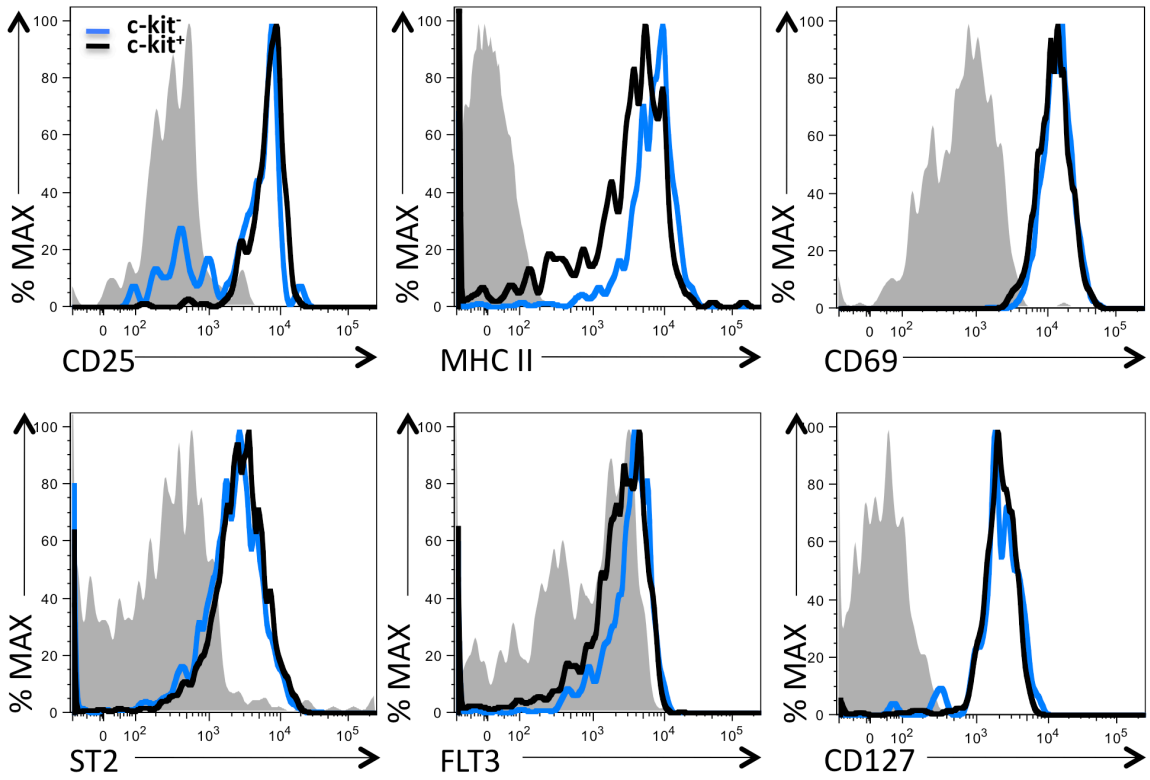

c-kit-
c-kit+
% MAX
CD25
MHC II
CD69
ST2
FLT3
CD127

Figure 2.6: ILC2 express amphiregulin. ILC2 subsets were FACS sorted from the lung and analyzed for amphiregulin (AREG) transcripts at indicated d.p.i.. N.D. = not determined.

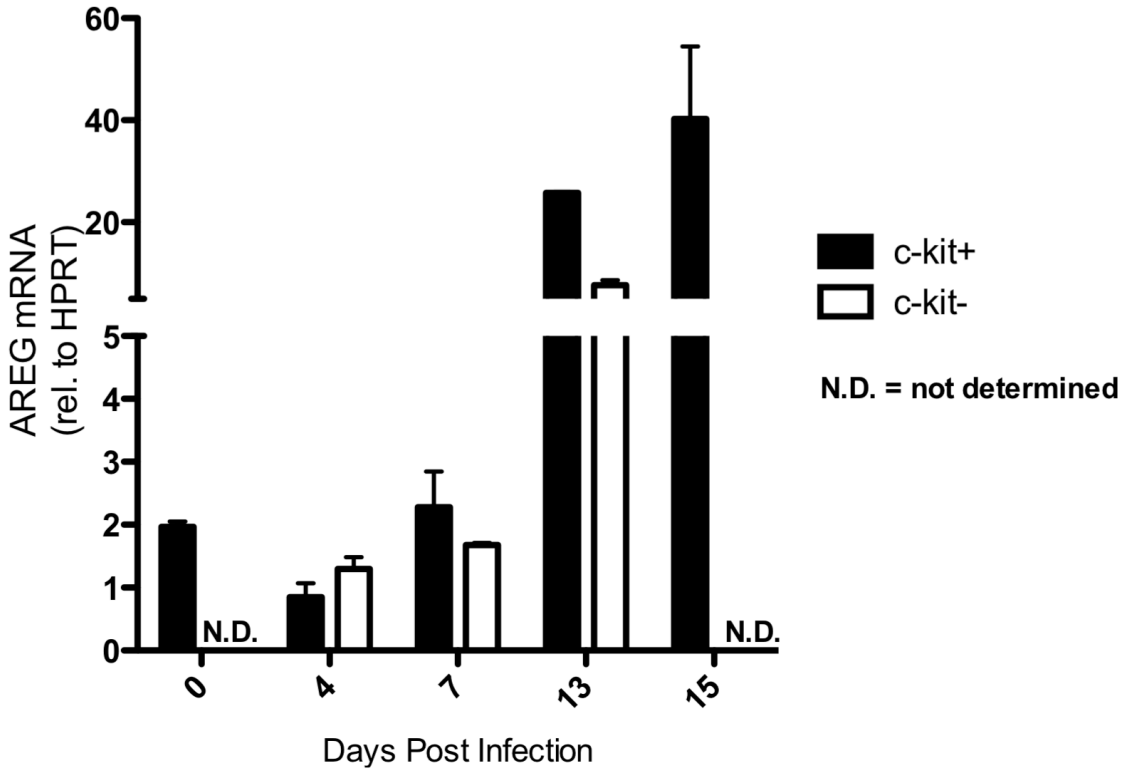

60
40
20
5
4
3
2
1
0
AREG mRNA
(rel. to HPRT)
N.D.
N.D.
0
4
7
13
15
Days Post Infection
c-kit+
c-kit-
N.D. = not determined

Figure 2.7: ILC2 do not proliferate in the respiratory tract. (A) ILC2 subsets from 10 d.p.i. lung were intracellularly stained for the proliferation marker Ki67. (B) 7 d.p.i. mice were injected with BrdU 4 hours before harvesting the lungs and staining for BrdU.

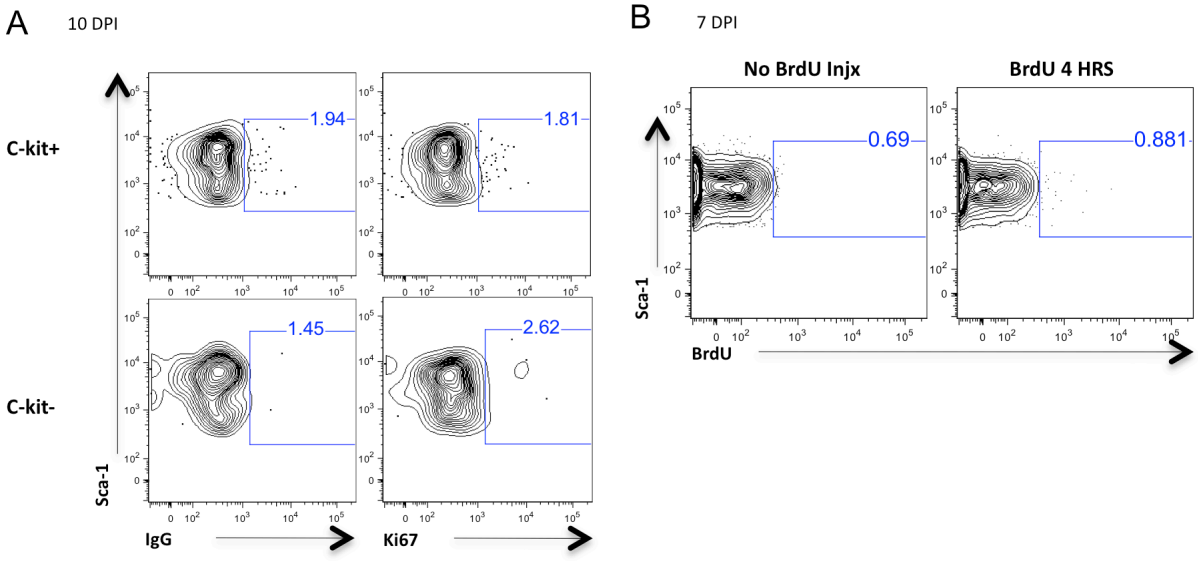

A
10 DPI
C-kit+
C-kit-
1.94
1.81
1.45
2.62
Sca-1
IgG
Ki67
B
7 DPI
No BrdU Injx
BrdU 4 HRS
0.69
0.881
Sca-1
BrdU

Figure 2.8: ILC2 are responsible for IL-5 production and eosinophil accumulation. (A-D) $Rag2^{-/-}$ or C57BL/6 mice were infected with PR8 followed by i.p. injection of 500μg of αThy1.2 depleting antibody at 3 and 5 d.p.i.. Lungs and BAL were analyzed at 7 d.p.i.. (B) Representative FACS plots of lung ILC2 and total $Thy1.2^{+}$ cell depletion. (C) BAL IL-5 and (D) absolute number of ILC2 following protocol described in (A). (E) Strategy of depleting ILC2 during the resolution phase via administration of αThy1.2 at 8 d.p.i., followed by i.v. transferring $2x10^{6}$ total $Thy1.1^{+}$ cells (inclusive of ILC2) or $CD3^{+}Thy1.1^{+}$ (excluding ILC2) from the lungs of 8 d.p.i. congenic Thy1.1+ mice. 100μg of αThy1.2 was given 9-11 d.p.i. in order to continuously deplete endogenous ILC2. Lungs were harvested at 12 d.p.i.. (F) Total eosinophil numbers in the lung at 12 d.p.i. following (E). Data are representative of 2-3 independent experiments with $n \geq 4$ per group. Error bars = +/- SEM. *$p<.05$ (Student's t-test)

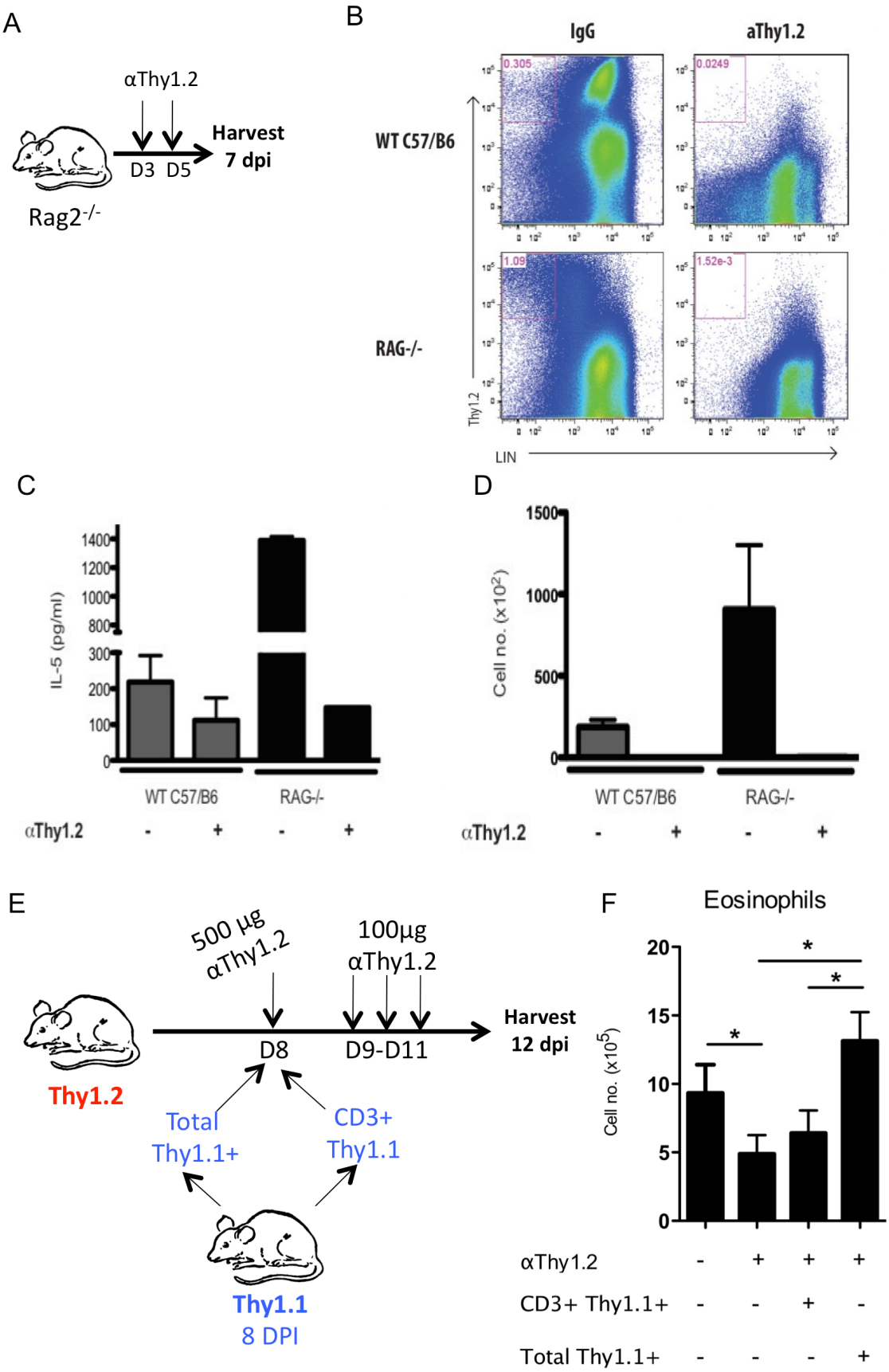

A
αThy1.2
Harvest
7 dpi
D3 D5
Rag2-/-
B
IgG
aThy1.2
WT C57/B6
RAG-/-
0.305
0.0249
1.09
1.52e-3
Thy1.2
LIN
C
IL-5 (pg/ml)
1400
1200
1000
800
300
200
100
0
WT C57/B6
RAG-/-
αThy1.2 - + - +
D
Cell no. (x10²)
1500
1000
500
0
WT C57/B6
RAG-/-
αThy1.2 - + - +
E
500 µg
αThy1.2
100µg
αThy1.2
D8
D9-D11
Harvest
12 dpi
Thy1.2
Total
Thy1.1+
CD3+
Thy1.1
Thy1.1
8 DPI
F
Eosinophils
Cell no. (x10⁵)
20
15
10
5
0
*
*
*
αThy1.2 - + + +
CD3+ Thy1.1+ - - + -
Total Thy1.1+ - - - +

Figure 2.9: IL-25 is not detectable in the BAL during IAV infection. C57BL/6 mice were infected with PR8 and BAL fluid harvested at the indicated d.p.i.. Protein analyzed via Luminex. Limit of detection = .08 pg/ml

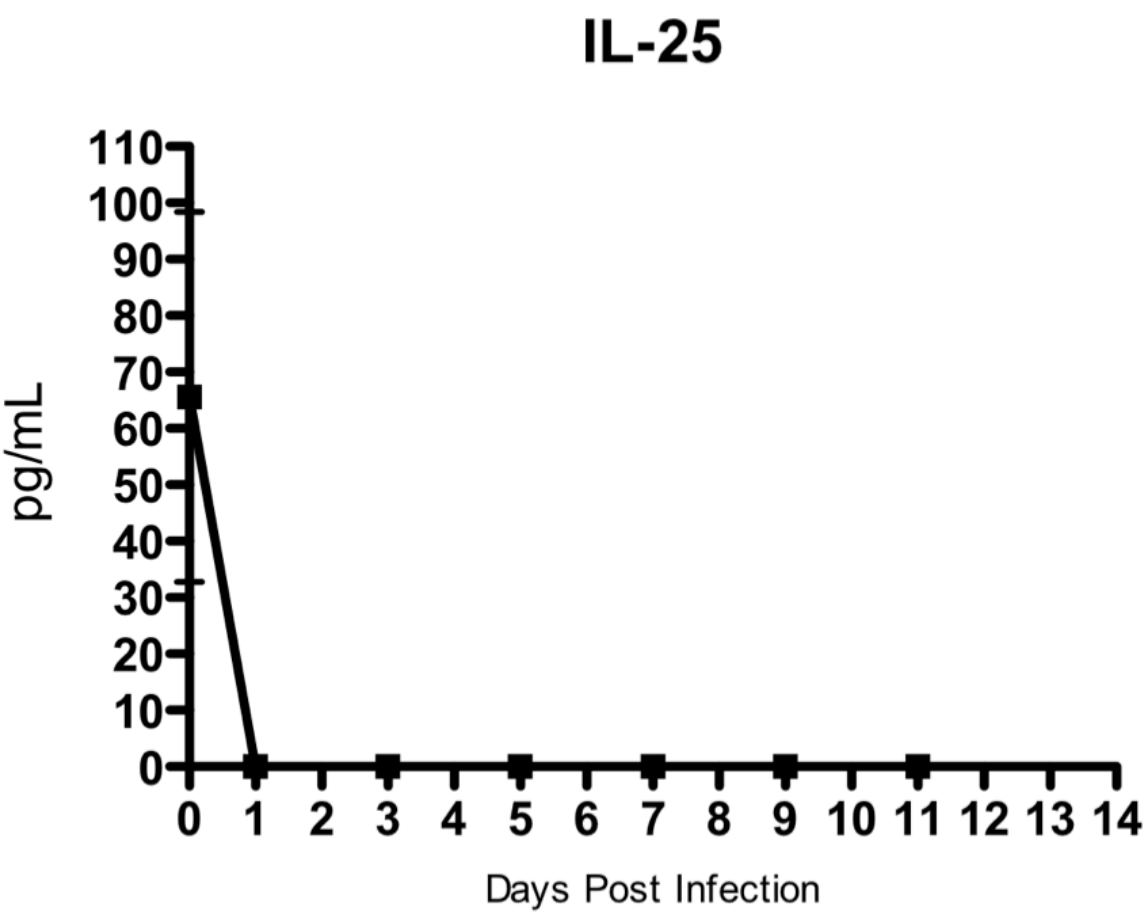

IL-25
pg/mL
110
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days Post Infection

Figure 2.10: ILC2 are responsive to IL-33 present in the lung. (A) IL-33 present in the BAL was determined by Luminex at indicated d.p.i.. (B) c-kit^{+} and c-kit^{-} ILC2 were FACS sorted from the lung at the indicated d.p.i. and cultured in the presence of 10ng/ml rIL-33 and 4U rmIL-2 for 72 hr (ILC2 stimulated with IL-33 alone produced no detectable IL-5 protein – data not shown). Supernatants were collected and analyzed for IL-5 by ELISA. N.D. = Not detected. (C) c-kit^{+} and c-kit^{-} ILC2 were FASC sorted from the lung at 7 and 14 d.p.i. (4 d.p.i. was not analyzed) and stored in trizol at -80 C. cDNA was made as described in Materials and Methods and ST2 gene expression was measured by RT-PCR. (D) Whole lung cell suspensions were cultured for 24 hours with or without 20μg of ST2 blocking antibody. Golgi Stop was added for last fours hour of culture and intracellular IL-5 was assessed in ILC2 subsets. (E) Frequency of IL-5+ c-kit^{+} and c-kit^{-} ILC2 after blocking ST2 as in (D). Error bars are +/- SEM. n= 3-5 per group. (B-C) cells were FACS sorted from pooled lungs of 10-15 mice per day. Data are representative of 3 independent experiments. **p <.01 (Student's t-test)

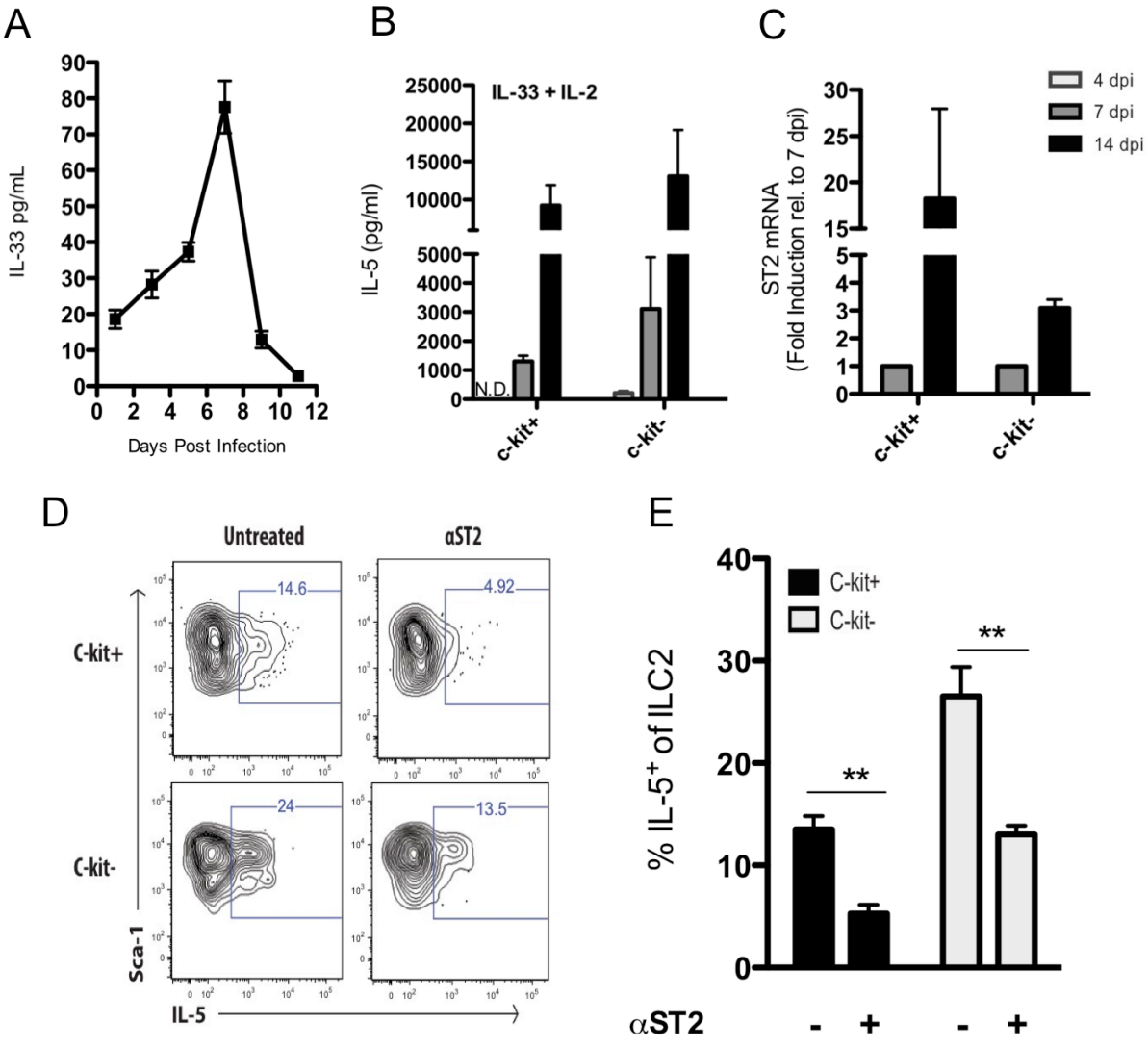

A
IL-33 pg/mL
Days Post Infection
B
IL-33 + IL-2
IL-5 (pg/ml)
N.D.
c-kit+
c-kit-
C
4 dpi
7 dpi
14 dpi
ST2 mRNA
(Fold Induction rel. to 7 dpi)
c-kit+
c-kit-
D
Untreated
αST2
14.6
4.92
24
13.5
C-kit+
C-kit-
Sca-1
IL-5
E
C-kit+
C-kit-
% IL-5+ of ILC2
**
**
αST2
- + - +

Figure 2.11: NKT are an *in vivo* source of IL-33. (A) IL-33 mRNA found in cell populations FACS sorted from the lung at 12 d.p.i.. (B) IL-33 protein in NKT as detected by intracellular staining (filled) compared to FMO control (shaded gray) at 12 d.p.i. (C) Total NKT cells identified in the influenza-infected lung as defined by CD3 and CD1d tetramer staining. (D) BAL IL-5 levels at 7 d.p.i. in indicated mouse strains. Data is representative of at least 2 independent experiments with 2-3 mice each. mRNA was collected from pooled, sorted cell populations from >5 mice. Bars = +/- SEM. *p < .05 (ANOVA)

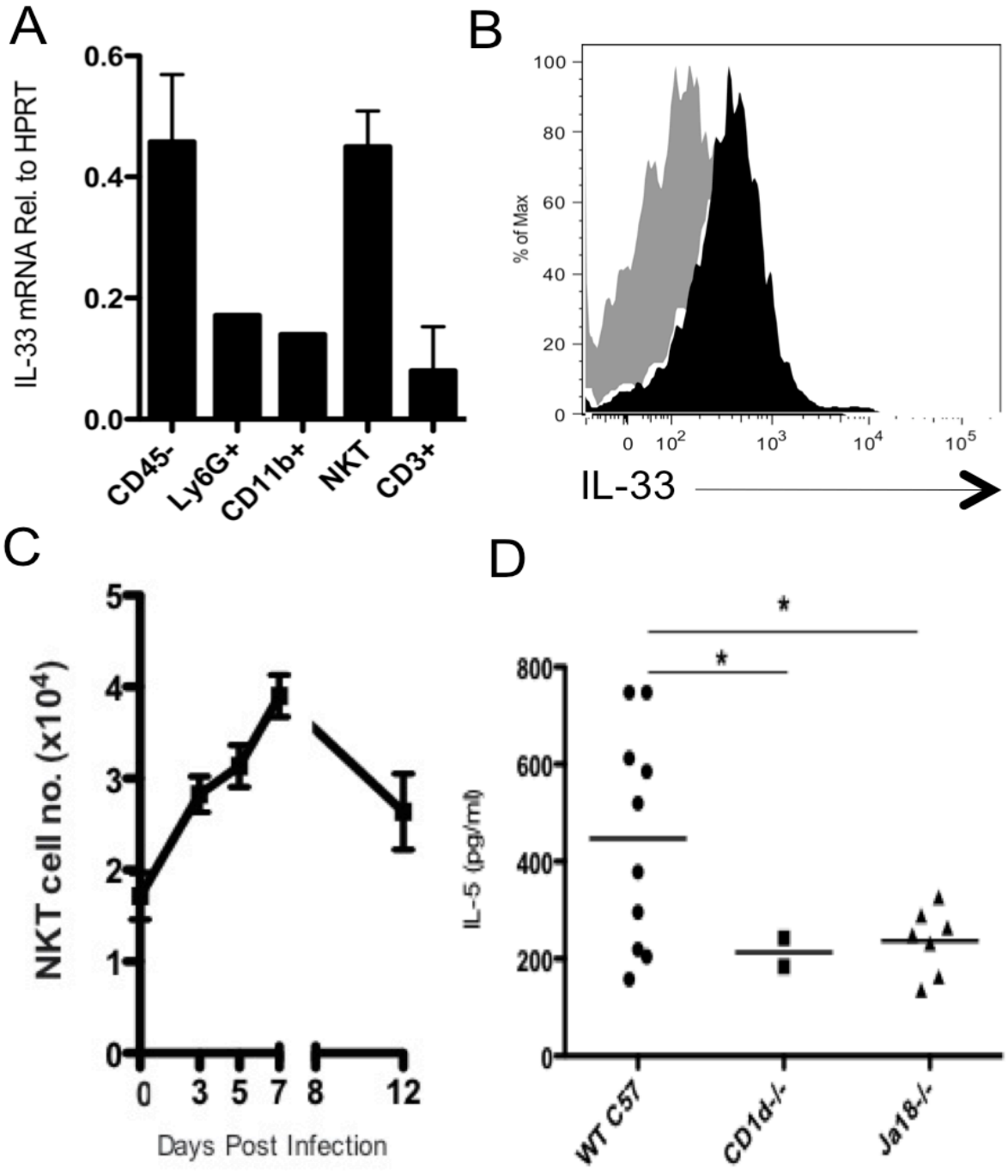

A
IL-33 mRNA Rel. to HPRT
0.6
0.4
0.2
0.0
CD45-
Ly6G+
CD11b+
NKT
CD3+
B
% of Max
100
80
60
40
20
0
0
10^2
10^3
10^4
10^5
IL-33
C
NKT cell no. (x10^4)
5
4
3
2
1
0
0
3
5
7
8
12
Days Post Infection
D
IL-5 (pg/ml)
800
600
400
200
0
*
*
WT C57
CD1d-/-
Ja18-/-

Figure 2.12: NKT cells secrete IL-33 protein. (A) NKT cells were MACS enriched from 7 d.p.i. lung cell suspensions (purity >92%) and cultured (2 x 10^5 cells/well) with or without BMDC and/or 10ng/ml αGalCer for 24-48 hours. Supernatants were analyzed for IL-33 via ELISA (Biolegend). ***p<.001 (ANOVA: compared to BMDC alone). (B) Intracellular IL-33 was analyzed in NKT cells from 12 d.p.i. lung cell suspensions cultured for 24 hours *ex vivo* with or without GolgiSTOP added for the last 4 hours of culture. n= 5-6 per group. Bars = +/- SEM. BMDC = bone marrow dendritic cell, n.s. non-significant. **p<.01 (Paired t-test)

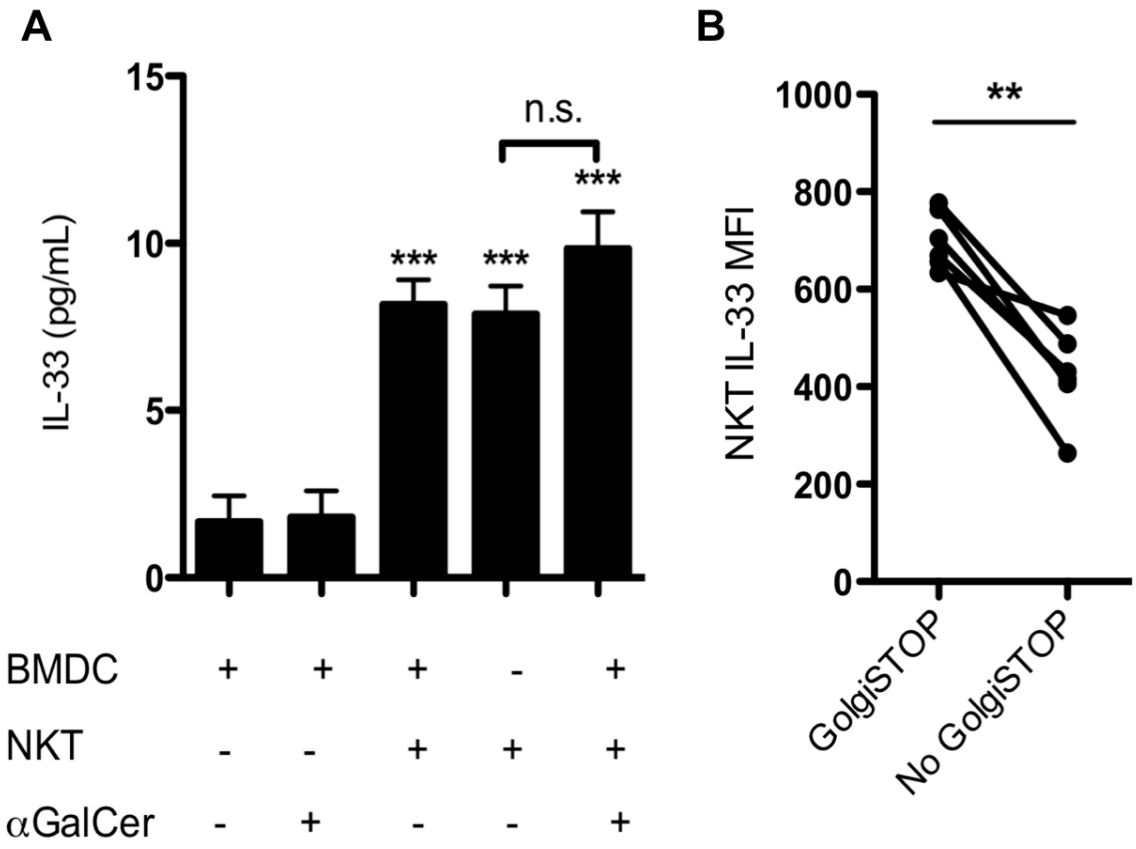

A
B
IL-33 (pg/mL)
15
10
5
0
n.s.
***
***
***
BMDC + + + - +
NKT - - + + +
αGalCer - + - - +
NKT IL-33 MFI
1000
800
600
400
200
0
**
GolgiSTOP
No GolgiSTOP

Figure 2.13: NKT cell deficient mice have fewer IL-5 producing ILC2. Lungs from infected CD1d$^{-/-}$ or C57BL/6 (WT C57) were harvested at 7 (A-C) and 12 d.p.i. (D-E). (A) IL-5 production was measured in each ILC2 subset via the *ex vivo* intracellular cytokine assay (B) Total frequency and (C) number of IL-5+ c-kit^{+} or c-kit^{-} ILC2 at 7 d.p.i.. (D) ILC2 subsets from 12 d.p.i. lungs were examined as in (A). (E) Frequency and (F) total number of IL-5+ ILC2 subsets at 12 d.p.i.. (G) Whole lung cell suspensions from WT or CD1d$^{-/-}$ mice at 12 d.p.i. were treated with 20ng/ml rIL-33 for 24 hours in culture and analyzed for intracellular IL-5 in ILC2. (H) Percentage of IL-5+ c-kit^{+} ILC2 after treatment with rIL-33. Data is representative of 2 (G-H) and 4 (A-F) independent experiments with 2-4 mice per group. Bars = +/- SEM. *$p < 0.05$, **$p < 0.01$ (Student t-tests)

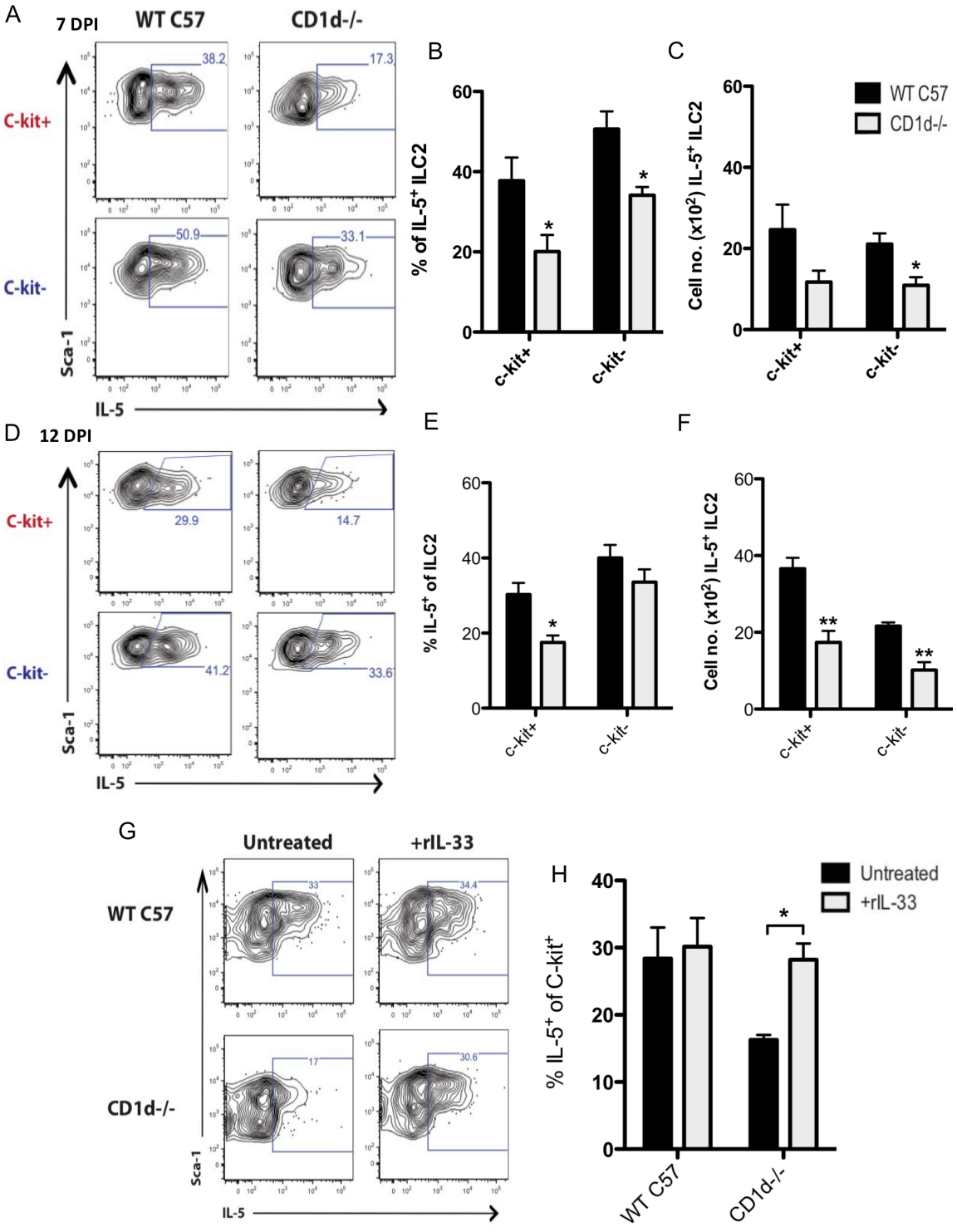

A
7 DPI
WT C57
CD1d-/-
C-kit+
C-kit-
38.2
17.3
50.9
33.1
Sca-1
IL-5
B
% of IL-5+ ILC2
C-kit+
C-kit-
C
Cell no. (x10²) IL-5+ ILC2
WT C57
CD1d-/-
D
12 DPI
29.9
14.7
41.2
33.6
E
% IL-5+ of ILC2
F
Cell no. (x10²) IL-5+ ILC2
G
Untreated
+rIL-33
WT C57
CD1d-/-
33
34.4
17
30.6
H
% IL-5+ of C-kit+
Untreated
+rIL-33

Figure 2.14: αGC administration increases both IL-5+ ILC2 and eosinophil numbers in the lung. (A) 1μg/ml αGC was injected i.p. at 3 and 5 d.p.i. Lungs were harvested at 7 d.p.i. and total NKT cells were quantified by use of CD3 and CD1d tetramer staining. (B) Representative FACS plots of intracellular IL-5 after 24 hours of *ex vivo* culture. (C) Frequency (left panel) of IL-5+ ILC2 and absolute number (right panel) of ILC2 at 7 d.p.i. (D) Frequency (left panel) and absolute number (right panel) of eosinophils detected in the lung at 7 d.p.i. Data is representative of 2 independent experiments with 3 mice each. Error bars = +/- SEM. *$p < 0.05$, **$p < 0.01$ (Student t-test)

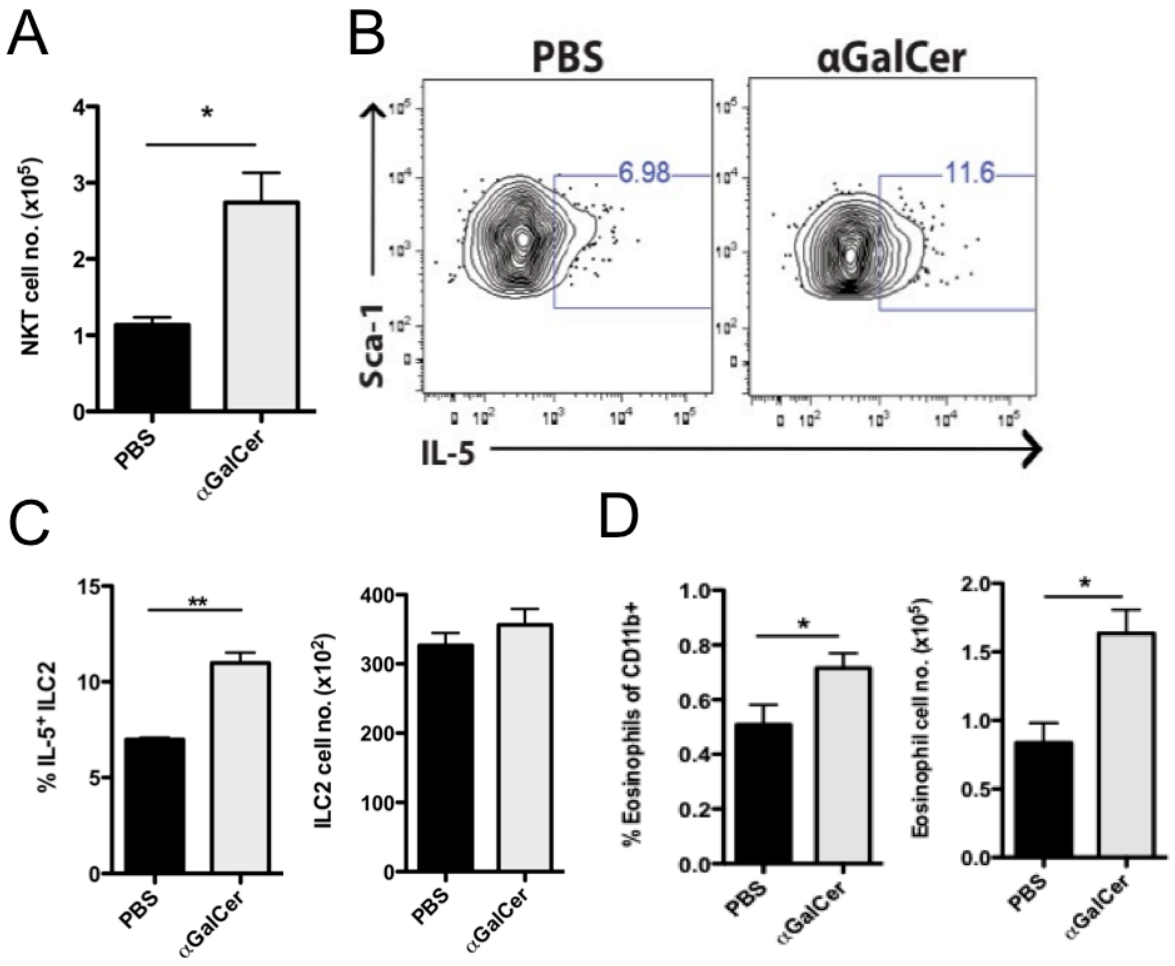
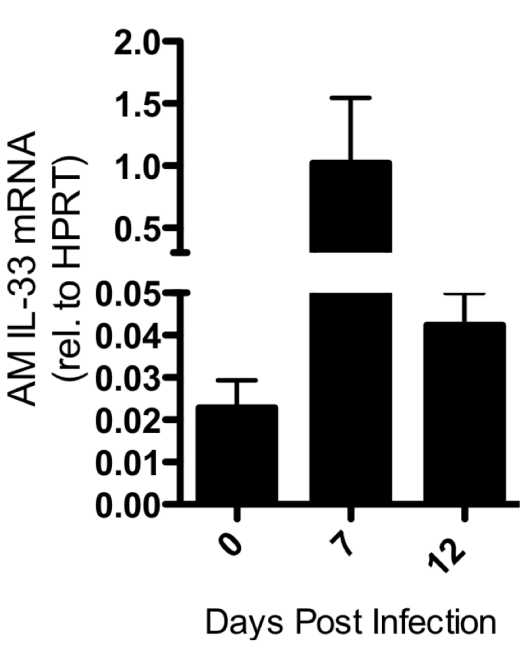

A
B
PBS
αGalCer
NKT cell no. (x10^5)
*
PBS
αGalCer
6.98
11.6
Sca-1
IL-5
C
D
% IL-5+ ILC2
**
ILC2 cell no. (x10^2)
% Eosinophils of CD11b+
*
Eosinophil cell no. (x10^5)
*

Figure 2.15: IAV infection induces IL-33 expression in alveolar macrophages and NKT cells. Alveolar macrophages (AM) and NKT cells were FACS sorted from the lung at indicated d.p.i. and analyzed for IL-33 transcript levels. Cells from n= 5-15 pooled lungs per day.


AM IL-33 mRNA
(rel. to HPRT)
2.0
1.5
1.0
0.5
0.05
0.04
0.03
0.02
0.01
0.00
0
7
12
Days Post Infection

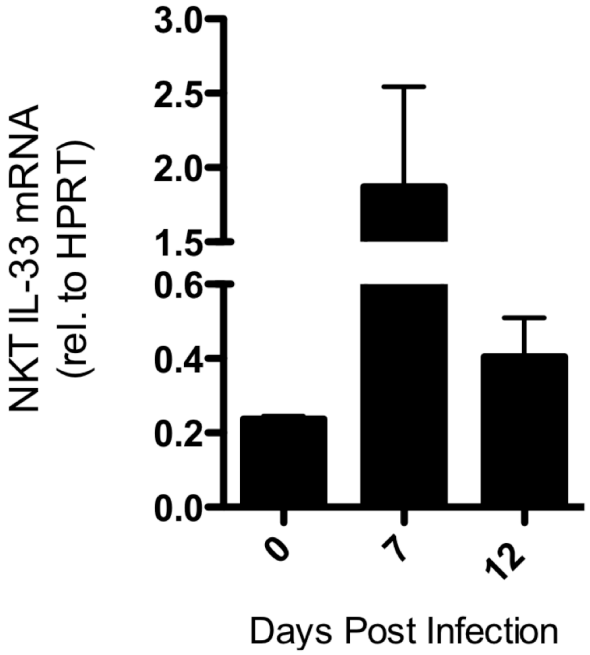
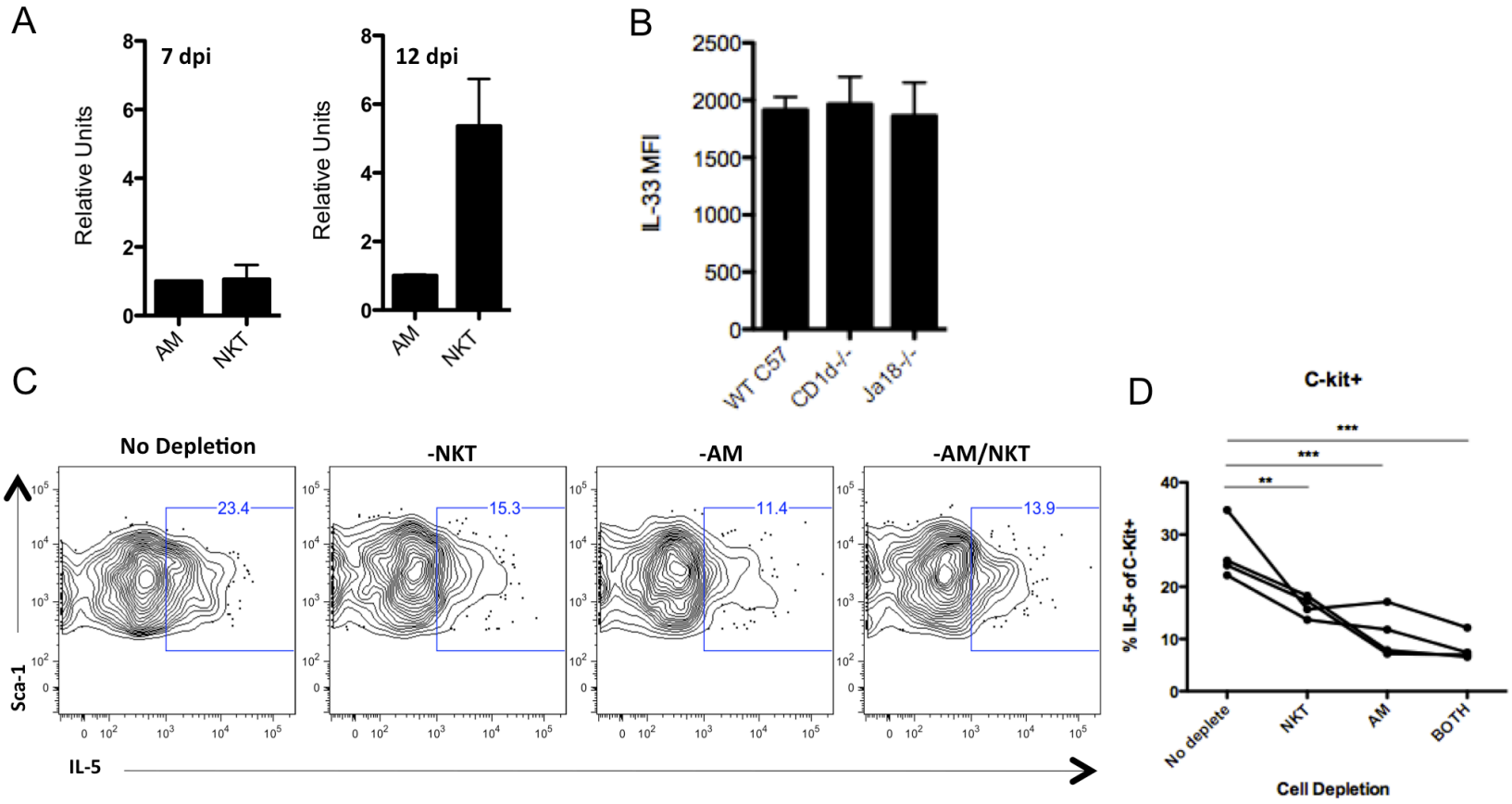

NKT IL-33 mRNA
(rel. to HPRT)
3.0
2.5
2.0
1.5
0.6
0.4
0.2
0.0
0
7
12
Days Post Infection

Figure 2.16: NKT cells regulate ILC2 derived IL-5 production independently of alveolar macrophages. (A) Alveolar macrophages (AM) and NKT were FACS sorted from the lungs at the indicated days and analyzed for IL-33 mRNA via RT-PCR. Cells were pooled from >5 mice for each group at each day. (B) AM were analyzed for intracellular IL-33 protein at 12 d.p.i. in indicated mouse strains. (C) Representative FACS plots of intracellular IL-5 in c-kit^{+} ILC2 at 12 d.p.i. after 24 hour *ex vivo* culture with or without indicated cell depletion. (D) Frequency of IL-5+ c-kit^{+} ILC2 at 12 d.p.i. following *ex vivo* cell depletion after 24 hour culture. Data is representative of 3 experiments with 4-10 mice each. Bars = +/- SEM. **p < .01, ***p < .001 (ANOVA)


A
7 dpi
12 dpi
Relative Units
AM
NKT
B
IL-33 MFI
WT C57
CD1d-/-
Ja18-/-
C
No Depletion
-NKT
-AM
-AM/NKT
23.4
15.3
11.4
13.9
Sca-1
IL-5
D
C-kit+
% IL-5+ of C-Kit+
No deplete
NKT
AM
BOTH
Cell Depletion

CHAPTER 3: ROLE OF IL-5 IN PULMONARY REPAIR FOLLOWING INFLUENZA INFECTION.

INTRODUCTION

Illnesses associated with seasonal influenza infections account for over 200,000 hospitalizations each year in the United States (Thompson et al., 2004). Much of this morbidity is due to an over exuberant host immune response, in addition to the direct cytopathic effects of the virus itself. The induction of a robust immune response, including pro-inflammatory cytokines, chemokines, and cytotoxicity, results in a substantial amount of bystander tissue damage, which must be rapidly repaired if the host is to maintain respiratory function. Type 2 immune responses, which are characterized by the production of interleukin (IL)-4, -5, and -13, have been postulated to be a type of tissue protective and reparative response during tissue injury (Allen and Wynn, 2011).

In chapter 2 we confirmed previous reports that demonstrated that IL-5 is produced during influenza infection (Baumgarth et al., 1994; Buchweitz et al., 2007; Gorski, 2013). IL-5 production peaks in the bronchoalveolar lavage (BAL) at day 7 post infection with influenza, but continues to be produced within the lung interstitium throughout the recovery phase (Figure 2.1A). Production of IL-5 in the influenza-infected lung is mediated by group 2 innate lymphoid cells (ILC2). ILC2 are present in the lung throughout infection, but dramatically increase in number immediately

following clearance of infectious virus from the lung (Hufford et al., 2011) and at the beginning of the recovery phase. Interestingly, not only do ILC2 increase in number during the recovery phase, but ILC2 also produce 10-fold more IL-5 on a per cell basis during this time. IL-5 is highly lineage restricted to eosinophils, as well as murine B-1 cells and we demonstrated that continued production of IL-5 during influenza infection is necessary for accumulation of eosinophils into the lung; however, the role of IL-5 and/or eosinophils during the recovery phase remains unclear.

Eosinophils have been shown in numerous models to contribute to virus-induced airway hyperreativity (Foster et al., 1996; Skappak C, 2010). The mechanisms proposed for this include hyper mucus secretion, smooth muscle activation and direct cytopathic effects of eosinophil degranulation products (Gleich, 2000). However, eosinophils can also be beneficial, as they have been shown to have antiviral activity during respiratory synctial virus infection (Domachowske et al., 1998; Phipps et al., 2007). Eosinophils can also contribute to tissue repair through their production of IL-4 and the pro-fibrogenic cytokine, IL-13 (Heredia et al., 2013).

In this chapter, we examine the roles of IL-5 and eosinophils during the recovery phase following influenza infection by utilizing IL-5 neutralizing antibody and eosinophil deficient mice. We show that in the absence of IL-5, mice are significantly delayed in their recovery and this is independent of any effects on virus clearance. Surprisingly, IL-5 dependent recovery was also independent of

eosinophils, but instead was acting to suppress neutrophil inflammation and subsequently promote epithelial regeneration.

MATERIALS AND METHODS

Mice and infection

BALB/c and C57BL/6 mice were purchased from the National Cancer Institute and maintained at the University of Virginia in a pathogen-free environment. PHIL mice were a kind gift from Drs. J.J. Lee and Nancy Lee (Mayo Clinic). All mice used in experiments were between the ages of 8-12 weeks and matched for age and sex. Type A influenza virus A/PR/8/34 (H1N1) was grown in day 10 chicken embryo allantoic cavities as described previously (Lawrence and Braciale, 2004). Mice were infected with 300 egg infectious doses (EID_{50}) of A/PR/8/34 i.n. (corresponding to a 0.1 LD_{50} dose) unless otherwise stated.

Preparation of lung tissue

Lungs were perfused with 5 mL of PBS via the right ventricle of the heart to remove cells from the vasculature. To prepare a single cell suspension, lungs were minced and digested in media containing 183 U/ml type II collagenase (Worthington) or 2 U/ml dispase II (Invitrogen) for 45 minutes at 37°C. Lungs digested in dispase had a greater fraction of epithelial cells, particularly alveolar epithelial cells, as measured

by $CD45^{-}CD31^{-}$T1α staining. Lung tissues were then pushed through a steel screen and red blood cells were lysed with ammonium chloride buffer (e.g. ACK lysis buffer). Cells counts were performed with a hemocytometer and trypan blue was used to exclude dead cells.

In vivo antibody administration

Mice were treated with 100μg of IL-5 neutralizing antibody (Bio X Cell; clone TRFK5) i.p. daily, beginning at 7 d.p.i. Mice were simultaneously treated with αIL-5 and αIL-6 (Bio X Cell; clone MP5-20F3) by administering αIL-5 as described above, followed by 500μg of αIL-6 i.p. daily beginning at 8 d.p.i. Mice were simultaneously treated with αIL-5 and depleted of neutrophils by administering αIL-5 as described above, followed by 300μg of αLy6G (Bio X Cell; clone 1A8) given at 7, 9, 11, and 13 d.p.i. i.p.

Flow cytometry and antibodies

Single cell suspensions were suspended in FACS buffer containing PBS, 2% FBS, 10 mM EDTA, and 0.01% NaN . Cell suspensions were blocked with anti–mouse CD16/32, followed by incubation with either specific mAbs or isotype-matched control Igs for 20 min at 4°C. The following antibodies were used: CD11c, CD11b, CD45, EpCAM, T1α (podoplanin), MHC II, CD74, CD31 (Biolegend); Ly6G, SiglecF, CD49b, CD3, CD4, CD8, CD19, NK1.1 (BD Biosciences); and Thy1.2 (eBioscience). Ki-67 was assessed by intracellular staining using Cytofix/Cytoperm, Perm/Wash Buffer and FACS Permeabilizing Solution 2 (all BD Bioscience).

Virus titer

BAL fluid viral titers were determined via an endpoint dilution assay on Madin-Darby canine kidney cells (MDCK; American Type Culture Collection) and expressed as tissue culture infectious dose$_{50}$ (TCID$_{50}$). MDCK cells were incubated with 10-fold dilutions of BAL fluid for 4 days in serum-free DMEM culture at 37°C with 5% $C0_2$. Supernatants were then collected and added at 1:1 to a solution of 1.5% chicken red blood cells (Charles River Laboratories, Inc.). Hemagglutination was determined one half hour later.

Antibody titer

BAL and serum αPR8 antibody titers were determined via ELISA. Split virion PR8 was coated onto 96 well plates followed by incubation with BAL fluids or serum overnight at 4°C. Plates were washed and αPR8 IgG was detected with HPR-conjugated anti-mouse IgG (Jakcson ImmunoResearch Laboratories, Inc.). Plates were developed with TMB substrate (eBioscience, Inc.) and read at 450 nm.

Evans blue dye

Mice were injected i.v. with Evans blue dye (Sigma-Aldrich Co., LLC) diluted in PBS at a concentration of 20mg/kg. One hour later BAL fluids were harvested and samples were read on a spectrophotometer at 620nm and compared to a standard curve. OD readings were corrected for hemoglobin content by subtracting the OD values read at 740.

Pulse oximetry

BALB/c mice that had been treated with αIL-5 or IgG were anesthetized with bupivacaine. Blood oxygen saturation levels were measured using Mouse Ox Plus (Life Star Technologies). The oximeter clip was placed on the right thigh and blood oxygen saturation (SpO_2) was recorded every .3 seconds. Overall SpO_2 was determined by taking the average of all measurements once values reached a plateau and immediately prior to mice recovering from anesthesia.

ROS production

Whole lung cell suspensions were kept in culture for 1 hour prior to the addition of 10μM 2′,7′-dichlorofluorescein diacetate (DCFA-DA; Sigma-Aldrich). Cells were kept at 37°C for 30 minutes before being surface stained for 10 minutes on ice and then immediately analyzed via flow cytometry. For *in vitro* experiments, bone marrow cells were kept in culture with 10μM DCFA-DA for 60 minutes before adding indicated concentrations of rIL-5 or PMA (100ng/ml; positive control). Cells were kept at 37°C for 15 minutes before being surface stained on ice for 10 minutes and then immediately analyzed via flow cytometry.

HL-60 differentiation

HL-60 cells were kept in RPMI 1640 media (supplemented with 10% FCS) with 1.5% DMSO (Sigma-Aldrich Co., LLC) for the indicated number of days.

pSTAT Analysis

HL-60 cells or lung cell suspensions were stimulated with rIL-5 for 3 or 10 min, respectively, and were analyzed for pSTAT5 and/or pSTAT1 (Cell Signaling Technology, Inc) via intracellular staining. Briefly, cells were fixed with BD Phosflow lyse/fix buffer followed by permeabilization with Phosflow Perm Buffer III. GM-CSF and IFNγ were used as positive controls for pSTAT5 and pSTAT1, respectively. Lung cell suspensions were also pre-treated with 1 ng/ml LPS before the addition of indicated concentrations of rIL-5. Cells were then left for 10 minutes at 37°C before staining for pSTAT5 and pSTAT1 (described above).

Statistical Analysis

Statistical analyses were performed using Prism 5 (GraphPad Software). Unpaired, two-tailed Student t test or one-way ANOVA with a Tukey post test were used to determine significance (individual tests used are labeled in figure legends). P values <.05 were considered statistically significant.

RESULTS

IL-5 is required for optimal recovery from IAV infection and is independent of virus clearance

We reported in Chapter 2 that ILC2 accumulate in the lung and synthesize and secrete abundant IL-5 following experimental infection with the mouse adapted

H1N1 strain A/PR8/34 (PR8). IL-5 production and ILC2 accumulation in the lungs increased progressively following IAV infection in parallel with a progressive increase of eosinophil accumulation in lungs. Both IL-5 production and ILC2 accumulation as well as increased eosinophil numbers were particularly evident following infectious virus clearance. These data suggested that ILC2-derived IL-5 (and potentially eosinophils) might have a role in recovery from IAV infection. In order to understand the role of IL-5 specifically during the recovery phase, we treated infected mice with neutralizing IL-5 monoclonal antibody (αIL-5 mAb) daily, beginning at day 7 post infection, (e.g. 1 day prior to complete clearance of infectious virions from the BAL (Hufford et al., 2011)). Mice treated with αIL-5 mAb displayed a marked delay in recovery (Figure 3.1A). Of note, there was no significant difference in virus titer between treated and control IAV infected recipients at day 8 (i.e. one day following αIL-5 treatment) and infectious virions were undetectable by 14 d.p.i. in both groups (Figure 3.1B). Consistent with this, αIL-5 treated mice had equivalent numbers of adaptive cells infiltrating the lung, including B cells, CD4+ and CD8+ T cells, as well as flu-specific, NP-tetramer positive CD8+ T cells (Figure 3.1C). IL-5 has also been implicated in driving maturation of activated B cells from IgM- to IgG1 expressing plasma cells (Mizoguchi et al., 1999). Therefore to ensure that IL-5 neutralization had no impact on the humoral response during the recovery phase, we examined the anti-PR8 IgG responses in the BAL and in the serum of control and αIL-5 mAb treated mice. Antibody titers were comparable between the IgG-treated control group and the αIL-5 group (Figure 3.1D and 3.1E). These data suggested that the delay in recovery observed following

IL- 5 neutralization was independent of anti-viral antibody generation and virus clearance.

Lack of IL-5 during the recovery phase results in delayed epithelial regeneration.

In the absence of any clear defect in virus clearance, we began to examine the innate response during the recovery phase. We found that αIL-5 treated mice had significantly higher levels of many innate cytokines in the BAL at 12 d.p.i., including IL-6 and MIG, suggesting low levels of sustained inflammation and/or enhanced leakage of these proteins into the airspaces (Figure 3.2A). Eotaxin levels were also elevated, presumably due to lack of consumption by eosinophils. IFNγ levels were not different between the two groups, consistent with an intact adaptive response in the αIL-5 treated animals. ILC2 were shown to be integral for epithelial barrier protection during influenza infection via their production of the epidermal growth factor family member, amphiregulin (Monticelli et al., 2011). Epithelial integrity and regeneration following influenza infection is particularly important as IAV is a lytic virus and therefore infection results in large numbers of necrotic and apoptotic cells in the airways, which subsequently impedes respiratory function. However, we observed no difference in the level of amphiregulin expression in the lungs of αIL-5 treated mice (Figure 3.2B). Thus we reasoned that the increased production of IL-5 itself by ILC2 during the recovery phase might also play a role in epithelial repair since αIL-5 treatment had no effect on virus clearance, the adaptive immune

response, or amphiregulin production. Injection of mice with Evans blue dye revealed that the αIL-5 treated mice had significantly higher levels of vascular leak at 10 d.p.i. (Figure 3.2C). Consistent with this, we found that αIL-5 treated mice also had a significant reduction in epithelial regeneration as measured by the proliferation marker, Ki67 at 10 d.p.i. (Figure 3.2D).

In order to understand how αIL-5 treatment was affecting epithelial regeneration, we analyzed the kinetics of Ki67 expression in the lung epithelia using dispase digestion to enhance epithelial cell, particularly alveolar epithelial cell, liberation (Messier et al., 2012). We were able to analyze the various $CD45^-$ epithelial cell subsets within the lung by first excluding any cell of hematopoietic origin ($CD45^+$) or endothelial cells ($CD31^+$). We then divided the populations into subsets based on T1α and epithelial cell adhesion molecule (EpCAM) expression (Figure 3.3A). T1α expression is restricted to AEC I in the lung (Rishi et al., 1995) and EpCAM is a pan-epithelial marker expressed on the basolateral membrane of non-squamous epithelial cells (Litvinov et al., 1994). The $T1\alpha^+EpCAM^-$ population was accordingly identified as the AEC I and the double positive $T1\alpha^+EpCAM^+$ were identified as AEC II, as these cells were also MHC II positive and expressed CD74 – consistent with previous reports describing AEC II (Cunningham et al., 1994; Harbeck et al., 1988; Marsh et al., 2009; Messier et al., 2012). $EpCAM^+$ cells lacking T1α expression were designated as upper airways epithelial cells and the remaining double negative populations encompassed non-epithelial, non-endothelial subsets including fibroblasts, progenitor cells, and remaining stromal cells. αIL-5 treatment did not

affect proliferation of the AEC II nor upper airway epithelial cells (Figure 3.3B and 3.3C).

αIL-5-treated mice had a significant delay in the frequency of proliferating epithelial cells at 8 d.p.i. (Figure 3.4A) and also a significant diminution of the overall magnitude of the repair response up until 12 d.p.i. (Figure 3.4B). This overall diminished repair response in the αIL-5 treated mice appeared to be, at least in part, due to a delay in regeneration of the type I alveolar epithelial cells (AEC I) (Figure 3.4C) and cells within the non-alveolar, non-conducting airway epithelia group (EpCAM-T1α-) between 8 and 10 d.p.i. (Figure 3.4D). Consistent with a delay in the regeneration of AEC I between 8-12 d.p.i. αIL-5 treated mice had a pronounced drop in lung function at 8 and 10 d.p.i. as measured by pulse oximetry (Figure 3.4E).

Delayed recovery in αIL-5-treated mice is independent of eosinophils.

IL-5 is essential for survival, proliferation, and differentiation of murine B-1 cells and eosinophils (Palacios et al., 1987; Yamaguchi et al., 1988). In humans, only eosinophils have been shown to express the high affinity IL-5 receptor (Lopez et al., 1992). Since αIL-5 treatment did not appear to affect B cell numbers or antibody production, we hypothesized that the delayed recovery observed in the αIL-5-treated mice was due to some role of eosinophils in the repair process. We utilized the eosinophil-deficient mouse strain, PHIL, to determine how the loss of

eosinophils would influence the outcome of infection. PHIL mice express the diptheria toxin alpha chain (DTα) under the eosinophil-specific *Eosinophil peroxidase* promoter and thus are selectively deficient in eosinophils (Lee et al., 2004), which we confirmed via FACS analysis (data not shown). Recovery of PHIL mice that were positive for the DTα transgene (PHIL Tg+) was comparable to that of littermate, wildtype, controls (PHIL Tg-) (Figure 3.5A). Furthermore, there was no difference in the amount of vascular leak, suggesting normal repair in the PHIL Tg+ mice (Figure 3.5B). Importantly, αIL-5 treatment of PHIL Tg+ mice resulted in delayed recovery (Figure 3.5C) and significantly reduced Ki67+ epithelial cells (Figure 3.5D). These results suggested that IL-5 is acting independently of eosinophils during the recovery phase. In order to confirm these findings, as well as confirm delayed epithelial proliferation and decreased lung function from 8-12 d.p.i. following treatment with αIL-5, an independent mouse model of eosinophil deficiency (dblGATA1 mice (Humbles et al., 2004)) is currently being investigated.

Neutrophils constitutively express the IL-5 receptor

The surprising finding that IL-5 was acting independently of eosinophils to promote recovery and epithelial regeneration raised the possibility that another cell type was responsive to IL-5 signaling and was capable of mediating pulmonary repair. We examined cell types in the lung for expression of the IL-5 receptor alpha chain (IL-5Rα) and found that in addition to eosinophils, surprisingly >95% of neutrophils also expressed IL-5Rα (Figure 3.6A). Neutrophils are also responsive to GM-CSF

(Lopez et al., 1986) and thus express the common beta chain, which when combined with IL-5Rα, forms the high-affinity IL-5 receptor (Kitamura et al., 1991). The expression of IL-5Rα was not unique to lung neutrophils as neutrophils analyzed in the naïve and infected BAL, blood, bone marrow and spleen were found to be virtually 100% positive (Figure 3.6B). Furthermore, neutrophils FACS sorted from a 10 d.p.i. lung demonstrated equivalent IL-5Rα mRNA expression as eosinophils (Figure 3.6C). Importantly, very little IL5Rα mRNA was detected in endothelial ($CD45^{+}CD31^{+}$) or epithelial cell ($CD45^{-}$ $CD31^{-}$) fractions. Cell surface expression of IL-5Rα was also not found on epithelial cells during dispase or collagenase digest of the lung (Figure 3.7 and data not shown, respectively). These data suggest that IL-5 is unlikely to be acting directly on epithelial cells to promote regeneration, but instead may be acting via neutrophils.

Specific neutrophil pro-inflammatory functions are enhanced in the absence of IL-5

The expression of IL-5R on neutrophils suggested that neutrophils may be responsive to IL-5 signaling and thus may behave differently during IL-5 neutralization. The total frequency of neutrophils infiltrating the lung was slightly, but significantly, elevated in the αIL-5 treated mice (Figure 3.8A). Whether this represents a direct effect of IL-5 on neutrophil chemotaxis or is simply an indicator of increased inflammation in the lung of the αIL-5 treated mice is not yet clear. One of the primary effector activities of neutrophils is to produce reactive oxygen

species (ROS) (Cowburn et al., 2008). While ROS is important for host defense, it can also cause significant collateral tissue damage (de Groot, 1994). We found that neutrophils from the lungs of 10 d.p.i. αIL-5 treated mice produced significantly more ROS as measured by the ROS indicator dye 2′,7′-dichlorofluorescein diacetate (DCFA-DA) (Figure 3.8B). Importantly, ROS production in monocytes or lymphocytes was comparable between αIL-5 and IgG treated mice, suggesting that IL-5 is acting directly on neutrophils. In support of IL-5 acting to suppress neutrophil effector activity, FACS sorted neutrophils from 10 d.p.i. αIL-5 treated mice also had slightly higher gene expression for *Inducible nitric oxide synthase* (*iNOS*), an enzyme essential for ROS production. Furthermore, neutrophils from the αIL-5 treated mice had higher expression of IL-6, although there was a high degree of variability among different mice. Levels of TNFα and IL-17, other notable inflammatory neutrophil products, were comparable between αIL-5 and IgG treated mice (Figure 3.8C).

Neutrophils are responsive to IL-5 signaling

In order to determine if IL-5 was acting directly on neutrophils to suppress their function, we treated lung-derived neutrophils with increasing concentrations of recombinant murine IL-5 (rIL-5). Neutrophils treated with rIL-5 had significantly less ROS production compared to unstimulated neutrophils (Figure 3.9A). The relatively high amount of ROS production in the unstimulated cells most likely reflects activation due to isolation and handling, or possibly their elevated activation state in the recovering lungs

in vivo. Neutrophils treated with 10ng/ml rIL-5 showed no difference in ROS production compared to unstimulated cells, possibly due to down-regulation of the high affinity IL-5Rα. Treatment of lung neutrophils with rIL-5 resulted in no change to the expression levels of activation markers such as CD11b (Figure 3.9B) or CD62L (data not shown) – consistent with our findings *in vivo*.

Engagement of the IL- 5 receptor in eosinophils primarily results in signaling through a STAT5 dependent pathway resulting in transient phosphorylation of STAT5, with a possible minor contribution from STAT1 dependent signaling (Alam et al., 1995; Horikawa et al., 2001; Kagami et al., 2000). The ability of neutrophils to respond to IL-5 was surprising, and therefore we sought to determine if IL-5 was capable of inducing phosphorylation of STAT5 or STAT1 directly in neurtrophils. The HL-60 cell line is derived from human promyelocytic leukemia and can be differentiated into neutrophil-like cells upon incubation with DMSO (Collins, 1987; Collins et al., 1977; Collins et al., 1978). Incubation of HL-60 cells in the presence of DMSO resulted in a steady increase of IL-5Rα expression out to day 4 (Figure 3.10A and 3.10B). Treatment of these cells with rIL-5 also resulted in a transient increase of pSTAT5 following 3 minutes of stimulation, which was rapidly lost within 5 minutes post treatment (Figure 3.10C), presumably due to phosphates activity. Interestingly, treatment of naïve, murine bone marrow neutrophils with rIL-5 did not result in a significant change in pSTAT5 (Figure 3.11A). This may be due to longer incubation times with rIL-5 i.e. approximately 10 minutes before assessment of pSTAT5 levels compared to HL-60 cells, in which case the transient phosphorylation event would not be observed. However, treatment of

neutrophils that were pre-activated with LPS did result in a modest, but significant decrease in pSTAT5 at all concentrations of rIL-5 tested after 10 minutes (Figure 3.11B). In light of the HL-60 data where pSTAT5 was only observed at 3 minutes post-exposure to rIL-5 and was rapidly decreased by 5 minutes, this decrease in pSTAT5 levels following LPS activation may reflect an overall inhibitory signal by IL-5 on cellular pSTAT5 levels or may be a rebound effect of phosphatase activation by 10 minutes (e.g. following high levels of phosphorylation between 3-5 minutes). There were no significant changes in pSTAT1 in all conditions tested (data not shown). Taken together, these data suggest that IL-5 is capable of signaling in neutrophils through STAT5 and may serve as an inhibitory signal to neutrophil oxidative burst.

Elimination of neutrophils or IL-6 partially restores recovery in αIL-5 treated mice

The above observations raised the possibility that neutrophils were contributing to sustained inflammation and subsequently delaying recovery in the absence of IL-5. To evaluate the contribution of neutrophils in the αIL-5 treated mice, we depleted neutrophils using the anti-Ly6G (αLy6G) depleting antibody, which has been shown to be neutrophil specific in the influenza-infected lung (Hufford et al., 2012; Tate et al., 2011). Mice given αLy6G at the same time as αIL-5 treatment had a significant, although not complete, recovery of weight gain (Figure. 3.12A). Although neutrophil depletion did not reduce IL-6 levels in the BAL (Figure 3.12B), it did significantly reduce systemic IL-6 levels detected in the serum (Figure 3.12C).

There was no reversal of the reduced epithelial regeneration following neutrophil depletion (Figure 3.12D), however, this may be due to the sustained IL-6 levels seen in the BAL fluid as IL-6 has been shown to inhibit epithelial proliferation and has known effects on pulmonary remodeling (Kishimoto, 2010; Pantelidis et al., 2001). To examine the possibility that sustained levels of pulmonary IL-6 were preventing normal epithelial regeneration, we neutralized IL-6 concomitant with IL-5 neutralization. IL-6 neutralization slightly increased weight gain compared to αIL-5 alone (Figure 3.12E), however, there was significant improvement in the frequency of proliferating AEC I at 10 d.p.i. (Figure 3.11F).

DISCUSSION

IL-5 has been extensively studied in the context of allergic disease and asthma. Eosinophils, as well as murine B-1 cells, require IL-5 for proliferation and differentiation (Palacios et al., 1987; Yamaguchi et al., 1988). Eosinophils also require IL-5 for egress out of the bone marrow and into the circulation (Collins et al., 1995). Despite the prominent roles of eosinophils in virus-induced asthma exacerbation, the role of IL-5 during respiratory virus infection has not heretofore been examined (Foster et al., 1996; Skappak C, 2010). Here we present data suggesting that one of the functions of IL-5 produced during influenza virus infection is to suppress pro-inflammatory functions of neutrophils and in turn, to promote epithelial regeneration. Unexpectedly, this anti-inflammatory effect of IL-5

was independent of eosinophils and instead was the result of IL-5 signaling directly through IL-5 receptors expressed on neutrophils.

We demonstrated (see Chapter 2) that IL-5 is produced in the influenza-infected lung by the novel cell type, group 2 innate lymphoid cells (ILC2) (Gorski, 2013). ILC2 are dramatically increased in their capacity to make IL-5 on a per cell basis during the recovery phase; however, the purpose of this significant enhancement of IL-5 production during the recovery phase was not clear. In the absence of any defect in virus clearance or in the adaptive immune response, it appeared that ILC2 were producing IL-5 to promote repair processes in the lung, particularly regeneration of type I alveolar epithelial cells (AEC I) and various stromal cells. Neutralization of IL-5 beginning at 7 d.p.i. appeared to delay the kinetics of AEC I proliferation (i.e. regeneration following infection) as well as the regeneration of a cell type(s) that lack both T1α and EpCAM expression (e.g. fibroblasts, progenitors, stromal cells). Concomitant with a delay in regeneration, αIL-5 treated mice had striking deficits in their respiratory function and exhibited enhanced vascular leak into the airspaces.

IL-5 is required for B-1 cell survival and proliferation and B-1 cells are responsible for >80% of circulating "natural IgM." This B-1 cell derived IgM non-specifically binds to pathogens, and due to its polymeric structure, can be transported to mucosal surfaces where it could potentially mediate antiviral immunity. This raised the possibility that IL-5 neutralization could interfere with the B-1 cell response.

However, the role of B-1 cells and their production of IgM during influenza infection is controversial. Palladino et al. showed that IgM could be protective against influenza, but only if administered prophylactically (Palladino et al., 1995), while Baumgarth et al. suggested that natural IgM is required during the acute phase of infection for optimal protection (Baumgarth et al., 2000). Regardless of the specific role of B-1 derived IgM during influenza infection, we specifically limited IL-5 neutralization to day 7 post infection and later so as to not interfere with the acute anti-viral immune response, including B-1 cells. Indeed, we saw no delay in virus clearance in mice treated with αIL-5 (Figure 3.1B). The delay in epithelial regeneration in the αIL-5 treated mice was therefore not the result of sustained virus replication.

In addition to B-1 cells, eosinophils are considered to be the other main cellular target of IL-5. Eosinophil progenitors require IL-5 for their survival and differentiation and IL-5 is required to mobilize mature eosinophils out of the bone marrow (Collins et al., 1995; Foster et al., 1996; Mori et al., 2009; Sher et al., 1990; Yamaguchi et al., 1988). Indeed, we previously demonstrated that IL-5 production during influenza infection leads to a mild pulmonary eosinophilia during the recovery stage (Figure 2.1C). Eosinophils, and by extension IL-5, have long been implicated in contributing to asthma and the allergic phenotype largely due to the initial observation of a large frequency of eosinophils in the sputum of asthmatics (Fulkerson and Rothenberg, 2013). Later work demonstrated that degranulation of eosinophil-derived products, such as major basic protein or eosinophil perioxidases,

can be toxic to airway epithelial cells and contribute to the allergic phenotype, particularly during respiratory virus infection (Brottman et al., 1996; Gleich et al., 1979; Jacobsen et al., 2012). However, more recent work has demonstrated that eosinophils can also be beneficial, particularly in promoting tissue repair (Heredia et al., 2013).

Eosinophils constitutively express IL-4 mRNA and thus are capable of producing large amounts of IL-4 protein (Gessner et al., 2005). IL-4 is essential to alternatively activated macrophage (AAM) differentiation, and AAMs have been shown in numerous models to participate in tissue repair processes (Chen et al., 2012a; Gordon and Martinez, 2010; Loke et al., 2007). Eosinophils have also been shown to produce TGF-β, a known stimulator of fibroblasts which is also essential for epithelial progenitor proliferation (Halwani et al., 2011; Jacobsen et al., 2012). Thus the accumulation of eosinophils during the recovery phase of influenza infection might suggest their role in the pulmonary repair process. However, infection of PHIL mice, which are selectively deficient in eosinophils, surprisingly demonstrated a recovery process that was independent of eosinophils (Figure 3.5). One caveat to this model, however, is that the PHIL mice are deficient in eosinophils from birth, and the contribution of eosinophils during the acute stage of infection or even prior to infection is not known. Importantly, however, αIL-5 treatment of PHIL Tg+ mice demonstrated that IL-5 is specifically needed during the recovery phase and that eosinophils are not (Figure 3.5C and 3.5D).

In order to determine how IL-5 was mediating recovery independently of eosinophils, we analyzed all cell types present in the lung for IL-5Rα expression and found that eosinophils and, unexpectedly, neutrophils were the major IL-5Rα+ cell types (Figure 3.6A). The α subunit of the IL-5R specifically binds IL-5 and upon binding will recruit the common beta chain, which ultimately forms the high affinity IL-5R (Kitamura et al., 1991). Kinetic analysis of IL-5Rα expression by neutrophils showed that they constitutively express IL-5Rα throughout infection and in all tissues analyzed (i.e. blood, lung and bone marrow). Because of the delay in epithelial regeneration, CD45 negative cells, which comprise most cells of non-hematopoietic origin, were closely examined for IL-5Rα expression to explore the possibility that IL-5 could act directly on these cell types. By using both collagenase and dispase digestions of the lung, no IL-5Rα surface expression was detected (Figure 3.7), and minimal levels of IL-5Rα mRNA was detected in FACS sorted $CD45^-$ endothelial and non-endothelial cells (Figure 3.6B). This suggested that IL-5 was promoting epithelial regeneration indirectly, and possibly through a neutrophil-dependent manner.

IL-5Rα expression on neutrophils and macrophages was recently reported in a sepsis-model, where IL-5 was also shown to be protective in an eosinophil independent manner (Linch et al., 2012). That report also demonstrated enhanced pulmonary capillary leak in IL-5 deficient animals, which was consistent with our findings. However, it was also reported that neutrophils do not express IL-5Rα in the naïve state, but rather, its expression is induced upon stimulation with LPS.

Furthermore, while the authors demonstrate that the IL-5R is functional on macrophages, they reported no impact on the function of neutrophils treated with rIL-5; however, calcium mobilization was detected, demonstrating that the receptor was functional on neutrophils. Here we present data that neutrophils constitutively express IL-5Rα and demonstrate that neutrophils exhibit more inflammatory activity in the absence of IL-5, as evidenced by increased ROS production and enhanced IL-6 and iNOS gene expression (Figure 3.8). Conversely, neutrophils treated with rIL-5 *ex vivo* showed decreased ROS production (Figure 3.9A). Furthermore, depletion of neutrophils in αIL-5 treated mice partially enhanced recovery (Figure 3.12A) demonstrating the detrimental effect of neutrophils in the face of IL-5 signaling blockade. These data suggest that IL-5 acts to dampen inflammatory effector functions of neutrophils in the inflamed lung during the recovery phase of influenza infection.

In order to demonstrate direct activity of IL-5 on neutrophils, bone marrow neutrophils were treated with rIL-5 *ex vivo*. IL-5 signaling through the high affinity IL-5 receptor has been shown to induce STAT5 phosphorylation in eosinophils, as well as STAT1 phosphorylation in some cases (Takatsu, 2011). We were able to demonstrate that human neutrophil-like cells derived from the HL-60 cell line were capable of directly responding to rIL-5 as evidenced by the transient increase in pSTAT5 levels after only 3 minutes of rIL-5 treatment *in vitro*. Preliminary studies also suggest this event is JAK2 dependent (data not shown), consistent with previous reports demonstrating JAK2 is constitutively associated with IL-5Rα

(Ogata et al., 1998; Takaki et al., 1994). In contrast, no differences in phosphorylation of STAT5 (pSTAT5) or pSTAT1 were observed in murine bone marrow neutrophils within 10 minutes of rIL-5 stimulation (Figure 3.11A and data not shown). This may be due to the transient appearance of pSTAT5, which is undetectable by 10 minutes post treatment with rIL-5 as evidenced by the treatment of HL-60 neutrophils. Direct signaling of IL-5 in murine neutrophils will need to be more closely examined at shorter time periods to fully implicate pSTAT5 or pSTAT1 signaling in this cell type. Interestingly, pretreatment of neutrophils with LPS, followed by incubation with rIL-5 for 10 minutes led to significantly decreased levels of pSTAT5 (Figure 3.11B), which may reflect a rebound effect following phosphorylation of STAT5 in activated neutrophils at times earlier than 10 minutes. Again, a more in-depth kinetic analysis of signaling events at shorter time periods will help to clarify this issue.

Elevated levels of the proinflammatory cytokine, IL-6, were consistently observed in the BALs of mice treated with αIL-5. IL-6 is a pleiotropic cytokine involved in a variety of inflammatory and autoimmune diseases (Kishimoto, 2010) and thus was assumed to be playing a pathological role in our model of IL-5 neutralization. IL-6 can be produced by any number of cell types, including neutrophils (Ericson et al., 1998; Wright et al., 2010)(Figure 3.8C). Indeed when we depleted neutrophils in the αIL-5 treated mice, systemic levels of IL-6 were significantly decreased, but remained elevated in the BAL (Figure 3.12B and 3.12C), demonstrating that IL-6 was also being produced locally by cell(s) other than neutrophils. The sustained

levels of IL-6 specifically in the BAL in the neutrophil depleted mice may explain the lower levels of epithelial regeneration, which were comparable to the αIL-5 treated alone cohort (Figure 3.12D). IL-6 has been implicated in many models to inhibit epithelial proliferation and its signaling in the lung can cause dysregulated repair and remodeling (Kishimoto, 2010; Pechkovsky et al., 2012; Rincon and Irvin, 2012). Thus we neutralized IL-6 in the αIL-5 treated mice to determine if epithelial regeneration could be rescued. We found that IL-6 neutralization did, in fact, result in significantly enhanced AEC I proliferation at 10 d.p.i. Taken together, these data suggest that in the absence of IL-5, sustained levels of basal inflammation, including that from neutrophils and IL-6, contribute to a delay in pulmonary recovery.

In conclusion, we have demonstrated that IL-5 produced during the recovery phase following influenza virus infection is a necessary component of the pulmonary repair process. The requirement for IL-5 was unexpectedly independent of eosinophils, and was, at least in part, dependent on dampening the hyper-state of neutrophil activation. These data reveal a novel mechanism of IL-5 dependent repair in the lung and identifies neutrophils as being a previously unrecognized IL-5 target cell type.

Figure 3.1: IL-5 is required for optimal recovery from IAV infection and is independent of virus clearance. (A) BALB/c mice were infected with 0.05 LD_{50} PR8 and given 100 μg of αIL-5 i.p. daily beginning at 7 d.p.i. (denoted by arrow). (B) Virus titer from BAL fluid taken at indicated days post infection (d.p.i.). Red line denotes limit of detection. (C) Number of indicated cell populations present in the lung at 12 d.p.i.. (D) PR8-specific IgG antibodies present in the BAL at 14 d.p.i.. Positive control (cntrl) was BAL taken from wildtype BAL from 30 d.p.i.. (E) PR8-specific IgG detected in the serum at 14 d.p.i.. Data are represented as mean +/- SEM (n=5-15). Student t-test was used to compare αIL-5 and IgG treated groups. Data are representative of at least two independent experiments.

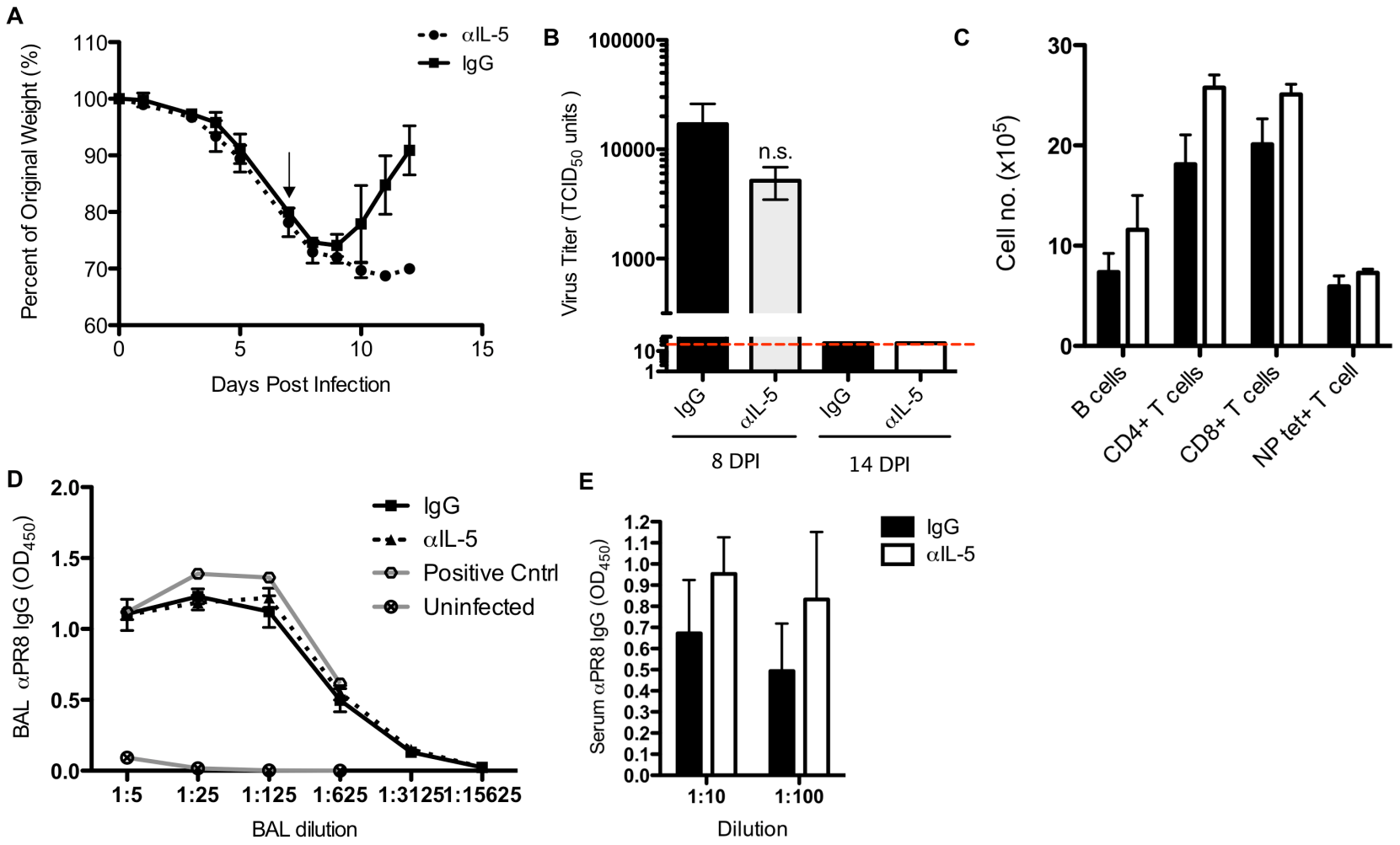

A
αIL-5
IgG
Percent of Original Weight (%)
110
100
90
80
70
60
0
5
10
15
Days Post Infection
B
Virus Titer (TCID50 units)
100000
10000
1000
10
1
n.s.
IgG
αIL-5
IgG
αIL-5
8 DPI
14 DPI
C
Cell no. (x10^5)
30
20
10
0
B cells
CD4+ T cells
CD8+ T cells
NP tet+ T cell
D
BAL αPR8 IgG (OD450)
2.0
1.5
1.0
0.5
0.0
IgG
αIL-5
Positive Cntrl
Uninfected
1:5
1:25
1:125
1:625
1:3125
1:15625
BAL dilution
E
Serum αPR8 IgG (OD450)
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
IgG
αIL-5
1:10
1:100
Dilution

Figure 3.2: IL-5 neutralization during the recovery phase results in sustained inflammation and decreased epithelial proliferation. (A) Cytokines present in the BAL at 12 d.p.i. (B) Amphiregulin gene expression present in whole lung homogenates at 14 d.p.i. as measured by qPCR. (C) C57BL/6 mice were injected with 20 mg/kg of Evans blue dye i.v. 1 hour prior to collection of BAL. BAL was harvested on 10 d.p.i. (D) Number of Ki67+ epithelial cells at 10 d.p.i. with representative FACS plots. Data are represented as mean +/- SEM (n=4-10/group). Data are representative of at least two independent experiments. * $p<0.05$, ***$p<0.001$ (Student t-test)

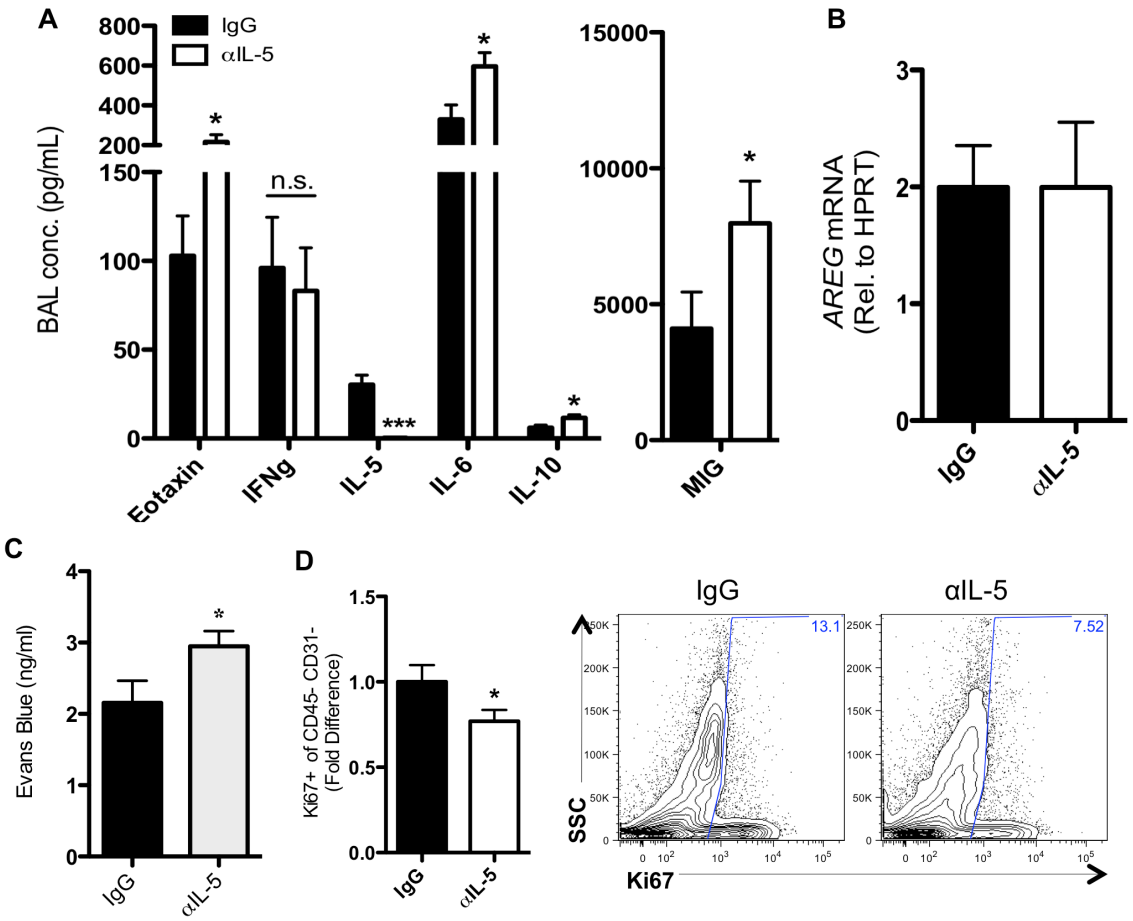
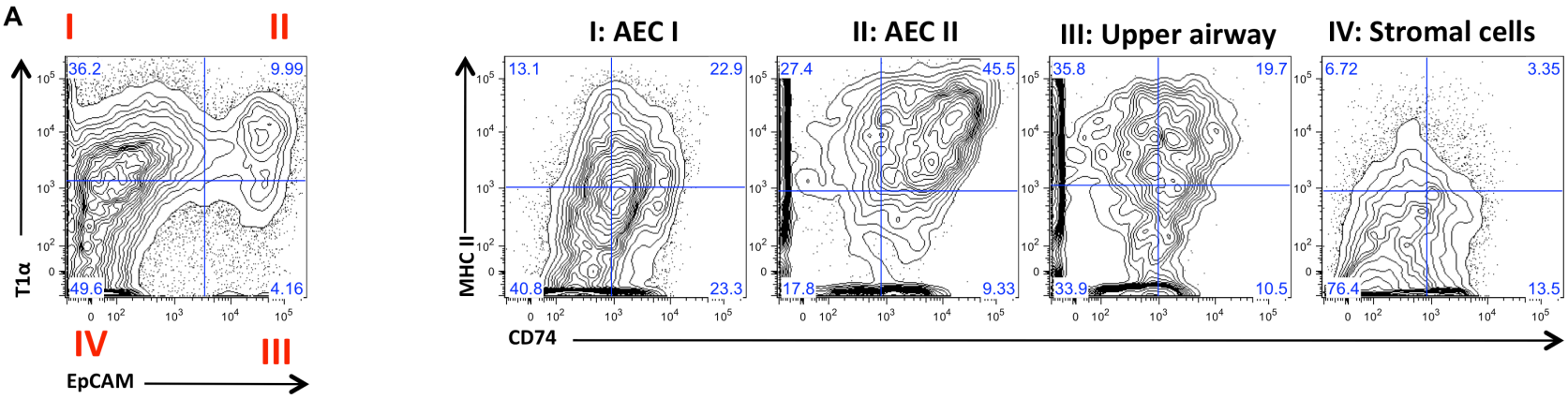
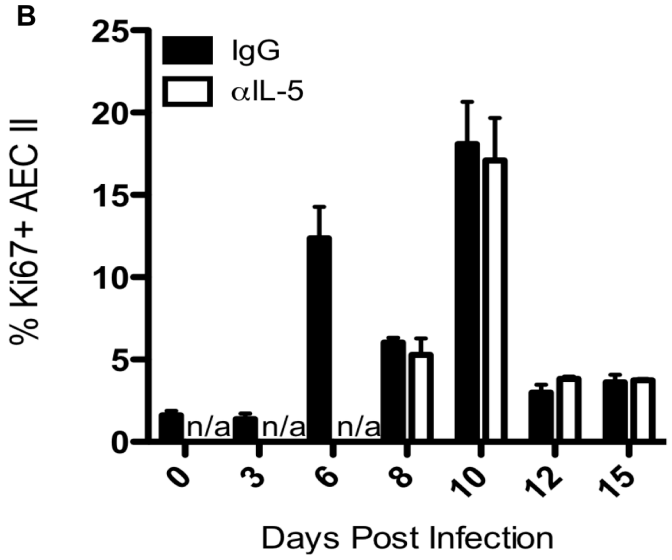

A
IgG
αIL-5
BAL conc. (pg/mL)
800
600
400
200
150
100
50
0
15000
10000
5000
0
n.s.
***
*
Eotaxin
IFNg
IL-5
IL-6
IL-10
MIG
B
AREG mRNA
(Rel. to HPRT)
3
2
1
0
IgG
αIL-5
C
Evans Blue (ng/ml)
4
3
2
1
0
IgG
αIL-5
D
Ki67+ of CD45- CD31-
(Fold Difference)
1.5
1.0
0.5
0.0
IgG
αIL-5
IgG
αIL-5
13.1
7.52
SSC
Ki67

Figure 3.3: Identification of lung epithelial subsets. (A) Cells were gated on $CD45^-$ (non-hematopoietic) and $CD31^-$ (non-endothelial). Expression of T1α and/or EpCAM denoted four separate subsets. (B) Effect of αIL-5 treatment on proliferation of type II alveolar epithelial cells (AEC II) and (C) upper airway epithelial cells. n/a = not applicable as αIL-5 treatment was not commenced until 7 d.p.i. Student t-tests were used to determine statistical significance at each d.p.i. (no data points were found to have significance). Data are represented as mean +/- SEM (n=3-5).


A
I
II
IV
III
T1α
EpCAM
36.2
9.99
49.6
4.16
I: AEC I
II: AEC II
III: Upper airway
IV: Stromal cells
MHC II
CD74
13.1
22.9
40.8
23.3
27.4
45.5
17.8
9.33
35.8
19.7
33.9
10.5
6.72
3.35
76.4
13.5


B
% Ki67+ AEC II
IgG
αIL-5
n/a
n/a
n/a
0
3
6
8
10
12
15
Days Post Infection

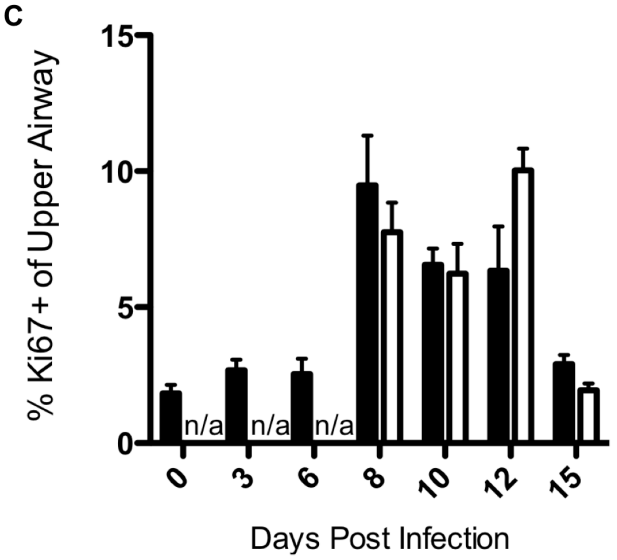

C
% Ki67+ of Upper Airway
n/a
n/a
n/a
0
3
6
8
10
12
15
Days Post Infection

Figure 3.4: IL-5 neutralization delays epithelial regeneration and impairs lung function. (A) Kinetics and (B) absolute number of Ki67+ epithelial cells at the indicated times post infection. (C) Frequency of Ki67+ type I alveolar epithelial cells (AEC I) and (D) EpCAM-T1α- cells. (E) BALB/c mice were given 100μg of αIL-5 beginning at 7 d.p.i. and blood oxygen saturation levels (as a correlate of lung function) were measured by pulse oximetry. n/a is not applicable as αIL-5 treatment was not commenced until 7 d.p.i.. Data are represented as mean +/- SEM (n=3-6 per group). *$p<0.05$ (Student t-test used to compared αIL-5 and IgG treated groups at each d.p.i.).

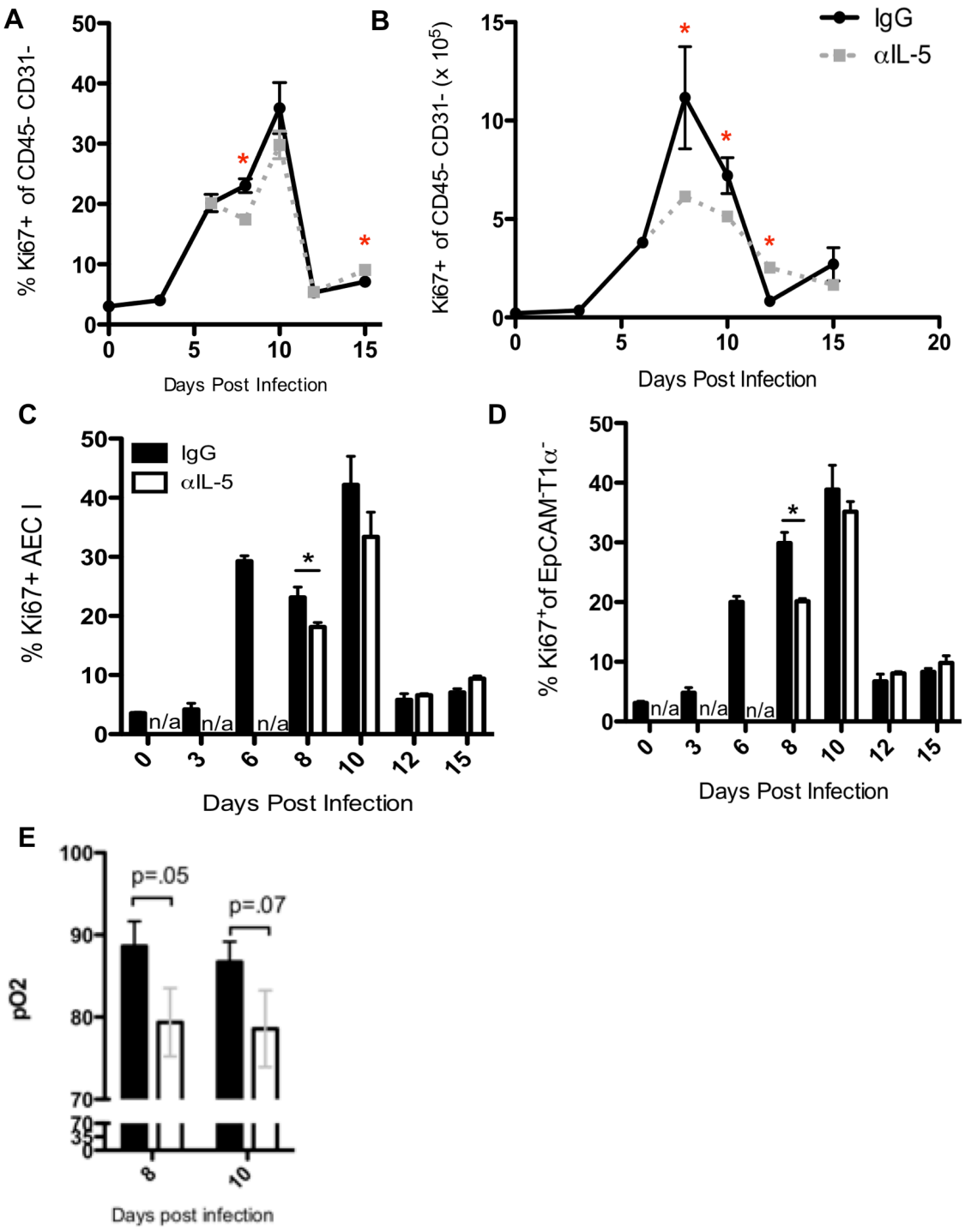

A
% Ki67+ of CD45- CD31-
Days Post Infection
B
Ki67+ of CD45- CD31- (x 10^5)
IgG
αIL-5
Days Post Infection
C
% Ki67+ AEC I
IgG
αIL-5
n/a
Days Post Infection
D
% Ki67+of EpCAM-T1α-
n/a
Days Post Infection
E
pO2
p=.05
p=.07
Days post infection

Figure 3.5: Delayed recovery in αIL-5-treated mice is independent of eosinophils. (A) PHIL mice on the B6 background were infected with 0.1 LD_{50} PR8 and monitored for weight loss. (B) PHIL mice were i.v. injected with 20 mg/kg of Evans blue dye one hour prior to collection of BAL. BAL was harvested at 12 d.p.i.. (C) PHIL mice positive for the diptheria toxin alpha chain (i.e. eosinophil deficient; PHIL Tg+) were treated with αIL-5 or control IgG antibody and monitored for weight loss. (D) PHIL mice were assessed for Ki67+ epithelial cells ($CD45^-$ $CD31^-$). Data are represented as mean +/- SEM (n=3-21). Data are representative of at least two independent experiments. *$p<0.05$ (ANOVA)

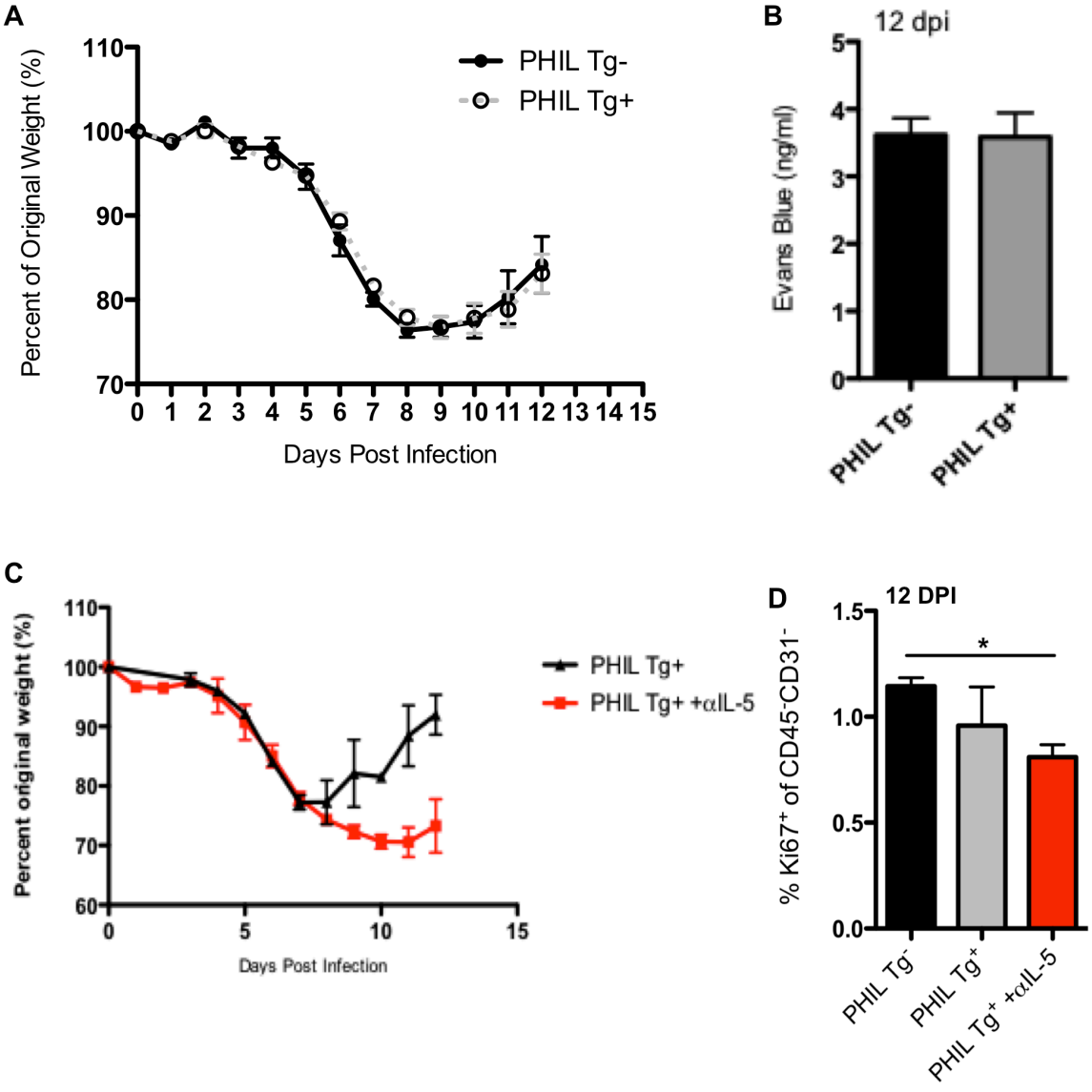

A
Percent of Original Weight (%)
110
100
90
80
70
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days Post Infection
PHIL Tg-
PHIL Tg+
B
12 dpi
Evans Blue (ng/ml)
5
4
3
2
1
0
PHIL Tg-
PHIL Tg+
C
Percent original weight (%)
110
100
90
80
70
60
0
5
10
15
Days Post Infection
PHIL Tg+
PHIL Tg+ +αIL-5
D
12 DPI
% Ki67+ of CD45-CD31-
1.5
1.0
0.5
0.0
*
PHIL Tg-
PHIL Tg+
PHIL Tg+ +αIL-5

Figure 3.6: Neutrophils express the IL-5 receptor. (A) Indicated cell populations from 10 d.p.i. lungs were interrogated for surface IL-5Rα expression. (B) Neutrophils (defined as $CD45^{+}CD11b^{+}Ly6G^{+}$) were examined in indicated tissues in naïve mice for IL-5Rα expression. Shaded histogram = isotype control. (C) Cell populations were FACS sorted from the lung at 12 d.p.i. and analyzed for IL-Rα gene expression by real time PCR. Data are represented as mean +/- SEM (n=6-10). Data are representative of at least three independent experiments.

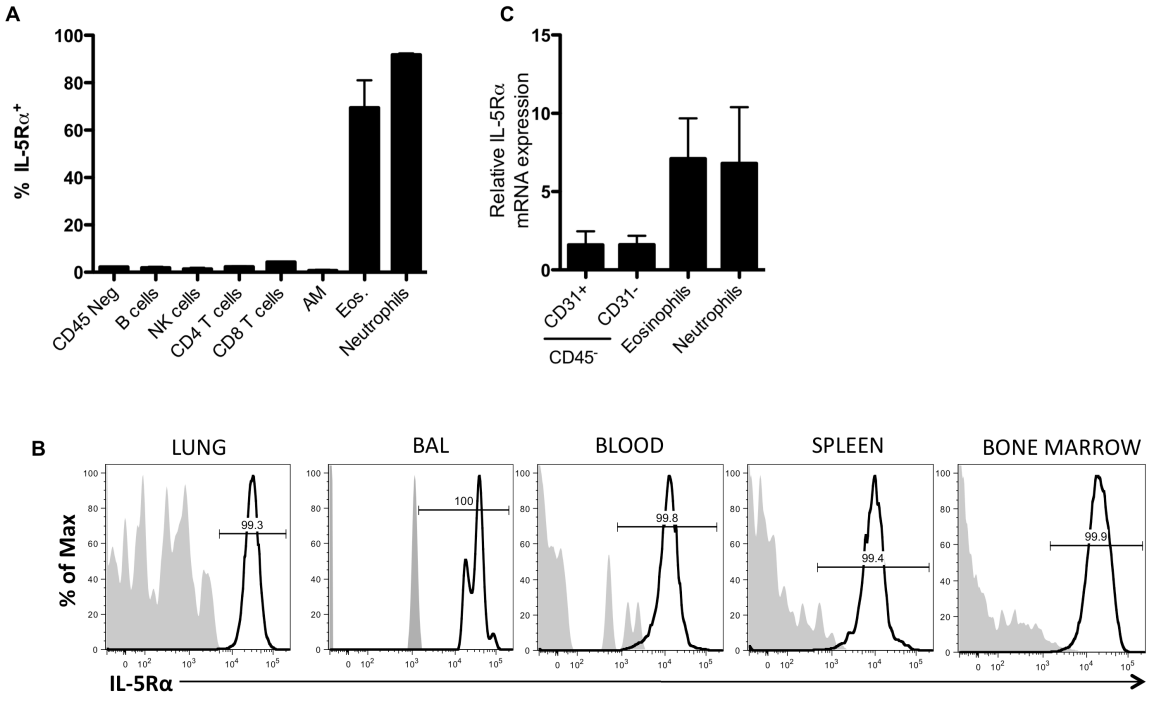

A
% IL-5Rα+
100
80
60
40
20
0
CD45 Neg
B cells
NK cells
CD4 T cells
CD8 T cells
AM
Eos.
Neutrophils
C
Relative IL-5Rα mRNA expression
15
10
5
0
CD31+ CD45-
CD31-
Eosinophils
Neutrophils
B
LUNG
BAL
BLOOD
SPLEEN
BONE MARROW
% of Max
99.3
100
99.8
99.4
99.9
IL-5Rα

Figure 3.7: Epithelial cells do not express the IL-5 receptor. Lungs from indicated days post infection were digested in dispase and examined for IL-5Rα expression (top row) compared to an FMO control (bottom row). Shown are representative FACS plots from n=4/d.p.i..

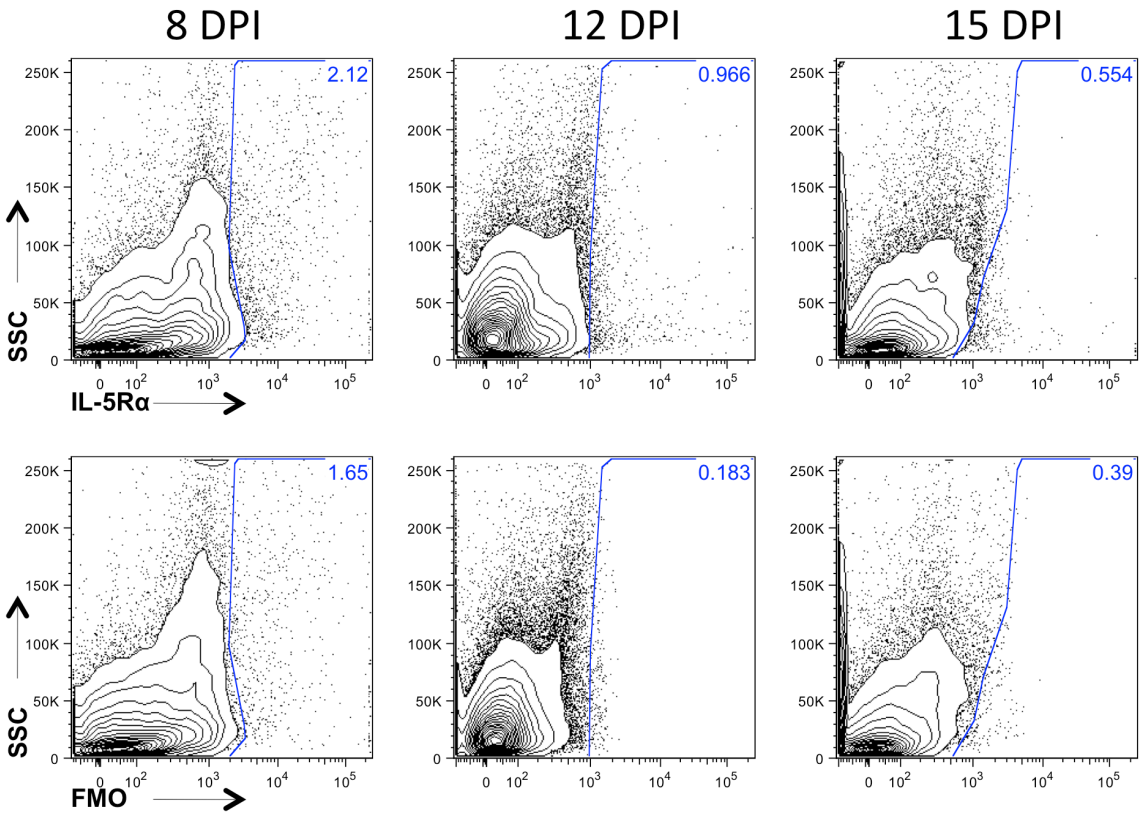

8 DPI
12 DPI
15 DPI
2.12
0.966
0.554
1.65
0.183
0.39
SSC
IL-5Rα
FMO
250K
200K
150K
100K
50K
0
0 10^2 10^3 10^4 10^5

Figure 3.8: Neutrophils have enhanced pro-inflammatory activity during IL-5 neutralization. (A) Frequency of neutrophils in the lungs at 14 d.p.i. Individual mice are represented by each dot. (B) MFI of ROS indicator dye DCFA-DA in indicated populations from 12 d.p.i. lung. Lymphocytes were identified as $CD11b^-$ and low FSC/SSC; monocytes were identified as $CD11b^+Ly6G^-$(C) Neutrophils were FACS sorted from the lungs at 12 d.p.i. and analyzed for gene expression via real time PCR. Data are represented as mean +/- SEM (n=3-13). Data are representative of at least two independent experiments. *$p<0.05$ (Student t-test)

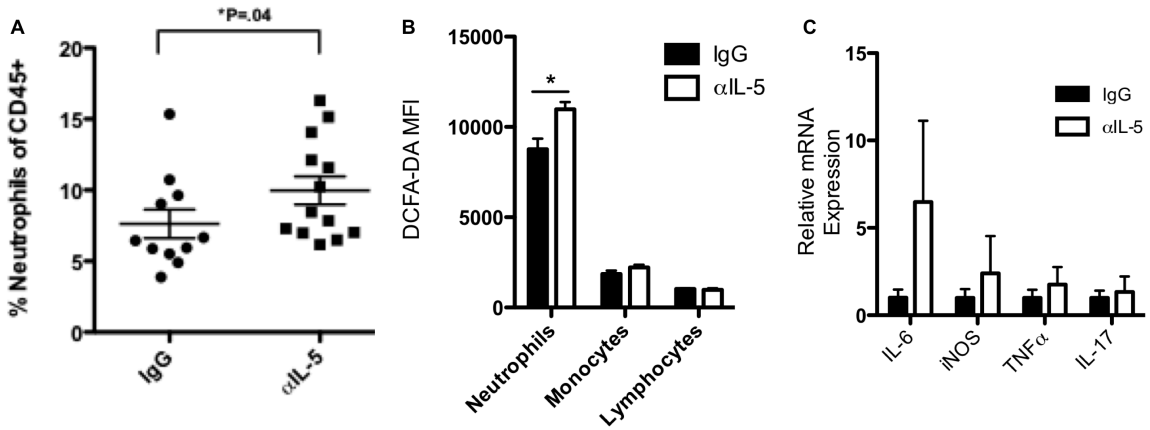

A
*P=.04
% Neutrophils of CD45+
20
15
10
5
0
IgG
αIL-5
B
15000
10000
5000
0
DCFA-DA MFI
*
IgG
αIL-5
Neutrophils
Monocytes
Lymphocytes
C
15
10
5
0
Relative mRNA Expression
IgG
αIL-5
IL-6
iNOS
TNFα
IL-17

Figure 3.9: IL-5 suppresses neutrophil activity *in vitro*. (A) Whole lung cell suspensions were kept in culture with indicated stimuli for 1 hour prior to addition of the ROS indicator dye DCFA-DA. Neutrophil DCFA-DA and (B) CD11b MFI were analyzed 30 minutes following the addition of DCFA-DA to whole lung cell suspensions. Data are represented as mean +/- SEM (n=4 per group). Data are representative of four independent experiments. **p<0.01, ***p<0.001 (ANOVA)

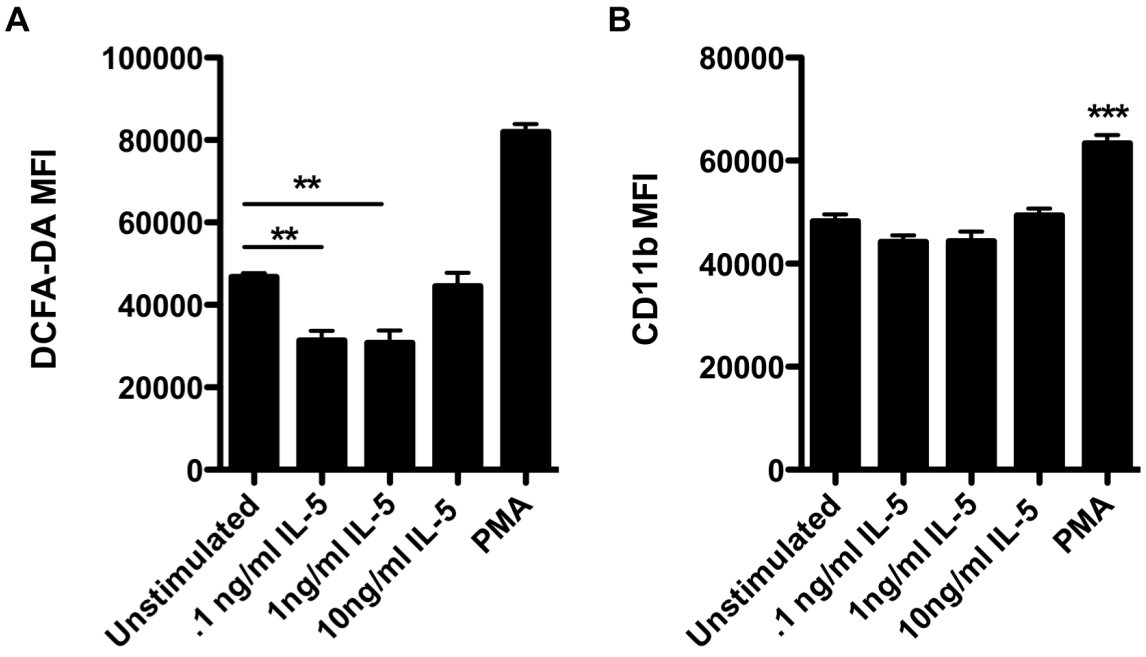

A
DCFA-DA MFI
100000
80000
60000
40000
20000
0
**
**
Unstimulated
.1 ng/ml IL-5
1ng/ml IL-5
10ng/ml IL-5
PMA
B
CD11b MFI
80000
60000
40000
20000
0
***
Unstimulated
.1 ng/ml IL-5
1ng/ml IL-5
10ng/ml IL-5
PMA

Figure 3.10: Human HL-60 neutrophils are responsive to IL-5. (A) HL-60 cells were treated with 1.5% DMSO over the period of 4 days and analyzed for IL-5Rα (CD125) expression. Shaded histrogram = isotype. (B) MFI levels of IL-5Rα on HL-60 relative to MFI of isotype control. (C) HL-60 cells treated with DMSO for 4 days were stimulated with 0.1ng/ml rIL-5 and analyzed for pSTAT5 at indicated times. Data are representative of two independent experiments. Data are represented as mean +/- SEM (n=3/group) and are representative of two independent experiments.

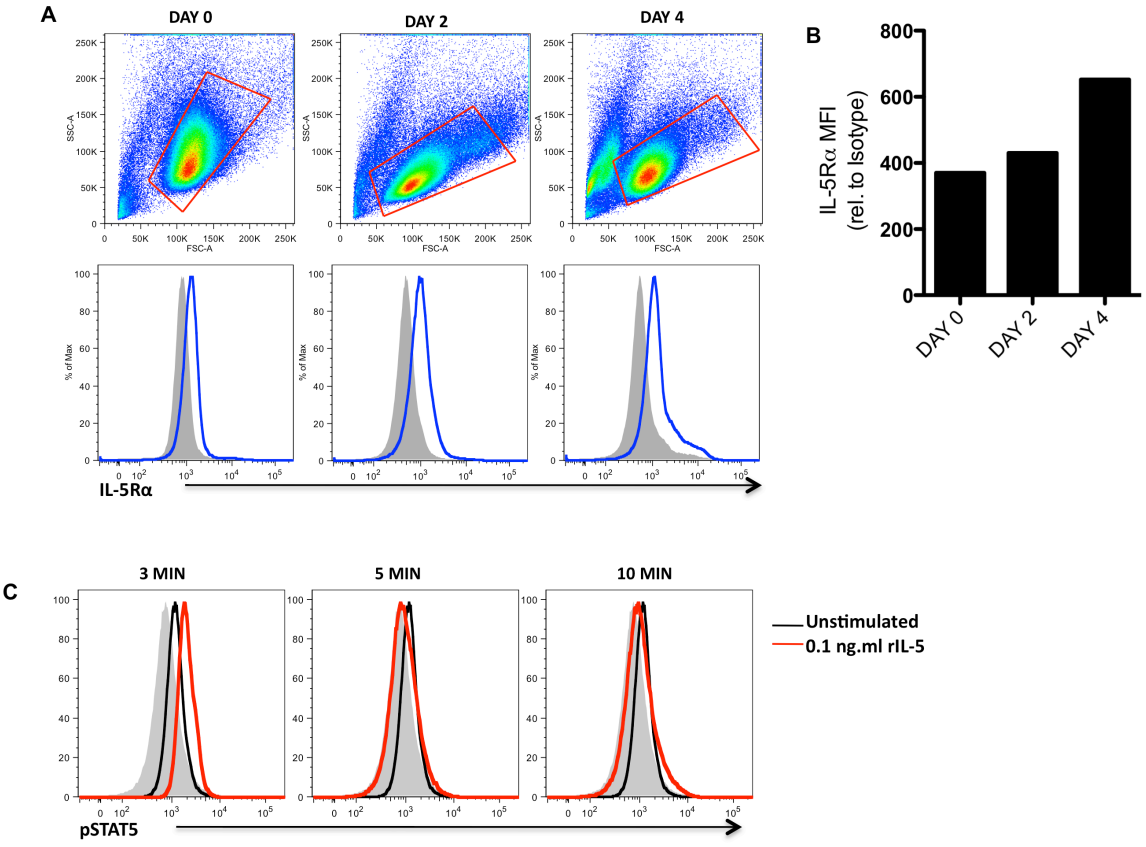

A
DAY 0
DAY 2
DAY 4
SSC-A
FSC-A
% of Max
IL-5Rα
B
IL-5Rα MFI
(rel. to Isotype)
DAY 0
DAY 2
DAY 4
C
3 MIN
5 MIN
10 MIN
pSTAT5
Unstimulated
0.1 ng.ml rIL-5

Figure 3.11: IL-5 does not enhance pSTAT5 in murine bone marrow neutrophils. (A) Bone marrow neutrophils were treated with rIL-5 for 10 minutes and examined for pSTAT5 via FACS. GM-CSF was used as a positive control (*p<.02 comparing GM-CSF and unstimulated using a Student t-test). (B) Bone marrow neutrophils were primed with 1ng/ml LPS for 30 minutes before adding rIL-5. Cells were assessed for pSTAT5 10 minutes following addition of rIL-5 via FACS. *p<.05 (ANOVA).

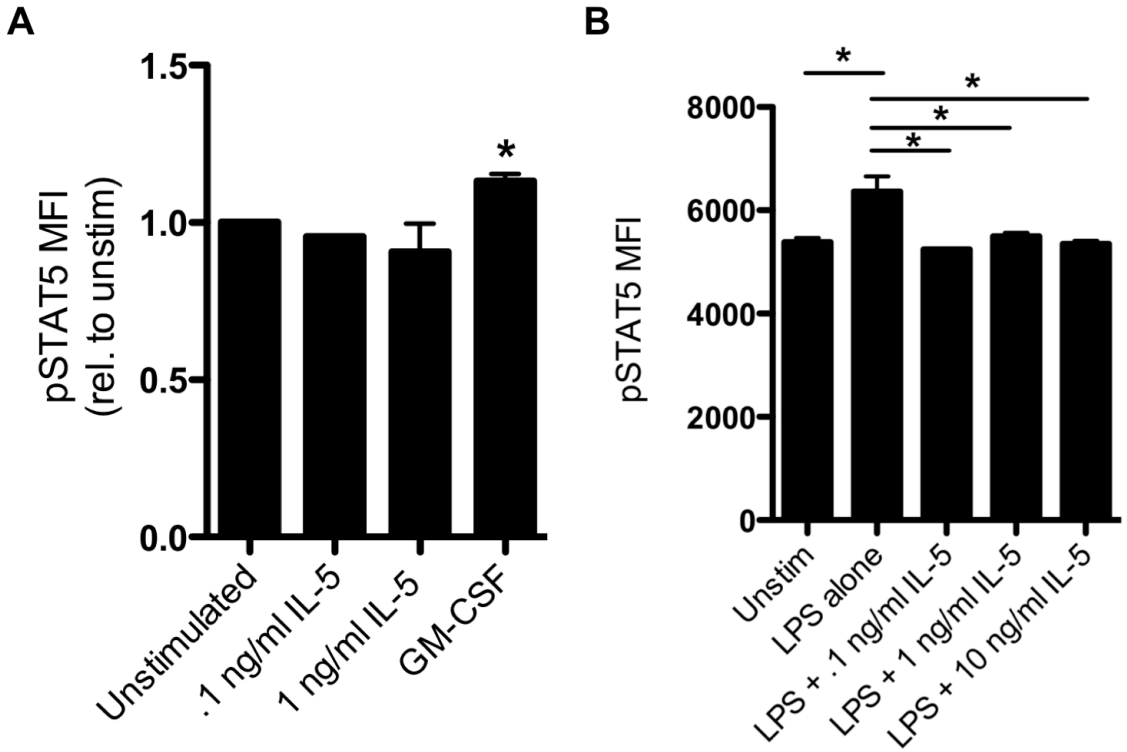
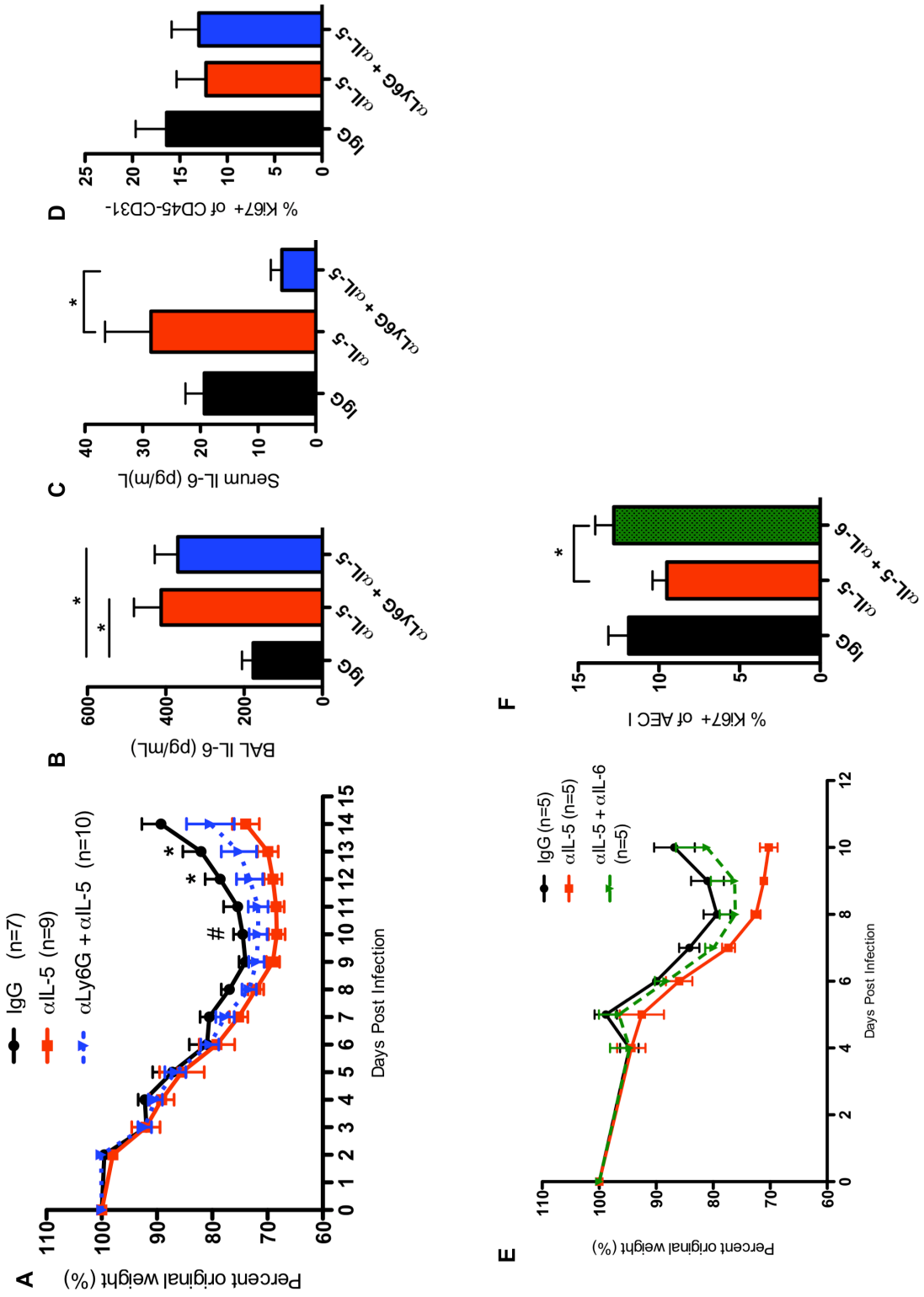

A
pSTAT5 MFI
(rel. to unstim)
1.5
1.0
0.5
0.0
*
Unstimulated
.1 ng/ml IL-5
1 ng/ml IL-5
GM-CSF
B
pSTAT5 MFI
8000
6000
4000
2000
0
*
*
*
*
Unstim
LPS alone
LPS + .1 ng/ml IL-5
LPS + 1 ng/ml IL-5
LPS + 10 ng/ml IL-5

Figure 3.12: Elimination of neutrophils or IL-6 partially restores recovery in IL-5 neutralized mice. (A) BALB/c mice were given αIL-5 daily beginning at 7 d.p.i., with or without 300μg of αLy6G i.p. at 7, 9, 11, and 13 d.p.i. $p<0.05$, $\#p<0.07$ (Student t-test comparing αIL-5 and αLy6G + αIL-5) (B) BAL and (C) serum levels of IL-6 were measured at 10 d.p.i. ($*p<0.05$, ANOVA) (D) Frequency of Ki67+ epithelial cells at 10 d.p.i.. (E) BALB/c mice were given IgG or αIL-5 beginning at 7 d.p.i., and in indicated groups, this was followed by 500 μg of αIL-6 given i.p. at 8, 9 and 10 d.p.i.. (F) Frequency of Ki67+ alveolar type I cells at 10 d.p.i. following treatment as described in (E). $*p<0.05$ (Student t-test). Data are represented as mean +/- SEM (n=5-10 per group). Data are representative of 1-3 independent experiments. ,


A
Percent original weight (%)
IgG (n=7)
αIL-5 (n=9)
αLy6G + αIL-5 (n=10)
Days Post Infection
B
BAL IL-6 (pg/mL)
C
Serum IL-6 (pg/m)L
D
% Ki67+ of CD45-CD31-
IgG
αIL-5
αLy6G + αIL-5
E
Percent original weight (%)
IgG (n=5)
αIL-5 (n=5)
αIL-5 + αIL-6 (n=5)
Days Post Infection
F
% Ki67+ of AEC I
αIL-5 + αIL-6

CHAPTER 4: CONCLUDING REMARKS AND FUTURE DIRECTIONS

Influenza A virus (IAV) is a significant human pathogen that has the potential to cause devastating pandemics. Morbidity associated with infection is caused by the virus itself, as well as the host immune response (i.e. host-mediated immunopathology), both of which can trigger significant lung damage and as a consequence, a decrease in respiratory function. This thesis contributes to our understanding of how the immune system utilizes the cytokine interleukin (IL)-5 to regulate and promote pulmonary repair following IAV infection. We used several mouse models to: 1) identify the cell type responsible for IL-5 production, 2) detail how IL-5 production is regulated 3) demonstrate how IL-5 participates in pulmonary repair and 4) identify those cells that are capable of responding to IL-5 during the recovery phase. We identified the newly discovered innate immune cell type, group 2 innate lymphoid cells (ILC2) as the major producer of IL-5 during and following IAV infection, as well as how IL-5 production is regulated by IL-33 produced by NKT cells. We also demonstrate that IL-5 is required for optimal recovery following IAV infection and that this effect is independent of the archetypical IL-5-responsive cell type, the eosinophil. In contrast, we identify neutrophils as a novel cellular target of IL-5 and show that IL-5 signaling in neutrophils acts to suppress their inflammation.

In chapter 2, we observed a relatively high level of IL-5 present in the BAL of IAV-infected mice. IL-5 is a prototypical type 2 cytokine that is commonly produced in

response to allergens and during parasitic infection or, in pathological conditions, during asthma. However, respiratory viruses such as IAV typically elicit a classic type 1 immune response, which includes production of pro-inflammatory cytokines such as IL-12 and interferon (IFN)-γ. Thus the high levels of IL-5 seen during IAV infection were surprising. IL-5 is highly lineage-restricted to eosinophils (a classic type 2 effector cell), in humans and it is also important for B-1 B cell survival in mice. This led us to investigate if eosinophils are a prominent cell type that responds to influenza in the lung. Indeed, we found that eosinophils accumulate in the lung during the recovery phase of influenza infection and their accumulation was dependent on continued IL-5 production from ILC2. Although IL-5 levels peak in the bronchoalveolar lavage (BAL) at 7 d.p.i., we showed that ILC2 continue to produce substantial levels of IL-5 during the recovery phase. Using Thy1.2 depletion in both Rag1-/- and wildtype mice to selectively deplete ILC2, we were able to demonstrate that ILC2 produce essentially all of the IL-5 that is produced throughout infection. This is consistent with earlier reports that ILC2 make up the majority of Thy1.2+ cells in the Rag deficient animal (Monticelli et al., 2011). However, this is the first report of selective depletion of ILC2 in wildtype animals, whereby all Thy1.2+ cells are depleted and, with the exception of ILC2, are restored using Thy1.1+CD3+ cells (i.e. T and NKT cells) from a congenic donor. This model allowed us to assess the contribution of ILC2 specifically during the recovery phase. Thus we were able to demonstrate a substantial decrease in eosinophil accumulation in the lung in the absence of ILC2, further illustrating that ILC2-

derived IL-5 is necessary for eosinophil accumulation in the lung following virus clearance.

In chapter 2, we also provide compelling evidence that NKT cells produce IL-33 during the recovery phase of IAV infection, which promotes/enhances IL-5 production by ILC2. IL-33 has been described as an "alarmin," that is released during necrotic cell death (Luthi et al., 2009). It is constitutively present in the nucleus of endothelial and epithelial cells, and is highly expressed in these cell types at mucosal surfaces (Carriere et al., 2007; Moussion et al., 2008). We found this to be true in the influenza-infected lung as well, with high IL-33 mRNA detected in the $CD45^-$ cell fraction. However, recent data suggests that IL-33 can also be present in the cytoplasm of both hematopoietic and non-hematopoietic cell types (Kakkar et al., 2012; Le et al., 2013). Interestingly, Chang et al. recently demonstrated that alveolar macrophages secrete IL-33 during influenza infection, and that this helps to promote IL-13 production by ILC2 (Chang et al., 2011). We were able to corroborate this finding by showing both IL-33 protein and mRNA in alveolar macrophages and furthermore, depletion of alveolar macrophages from whole lung cell suspensions resulted in less IL-5 produced in ILC2. Interestingly, we found that NKT cells also contained IL-33 protein and mRNA, and that the level of expression was comparable to alveolar macrophages at 7 d.p.i.. However, by 12 d.p.i. NKT cells expressed >5 fold more IL-33 compared to alveolar macrophages and are actively secreting it at this time. In addition, NKT cell deficient mouse models, including $J\alpha18^{-/-}$ and $CD1d^{-/-}$ mice, had significantly less ILC2-derived IL-5. These mice are

deficient in type I (Jα18$^{-/-}$) as well as type II (CD1d$^{-/-}$) NKT cells, which correspond to the invariant and semi-invariant NKT cells, respectively (Seino and Taniguchi, 2005). A recent report suggested that NKT cells enhance IL-33 secretion from alveolar macrophages, rather than producing IL-33 directly, in a model of airway hyperreactivity (Kim et al., 2012). In order to rule out this possibility in our IAV model, we showed that there is no decrease in alveolar macrophage IL-33 levels in the NKT cell deficient mice, and furthermore, that NKT cells produced and secrete IL-33 protein. This shows that while NKT and alveolar macrophages may interact, a direct interaction between these two cell types is unnecessary for either cell type to produce IL-33 during IAV infection. We went on to show that ILC2 also dramatically upregulate the expression of the IL-33 receptor subunit, ST2, during the recovery phase. This potentially provides an explanation for the substantially higher levels of IL-5 that we detected in ILC2 during this phase, as these cells become more responsive to IL-33 signaling. This is also the first report, to our knowledge, of IL-33 being produced by NKT cells.

Another important finding presented in chapter 2 was the biphasic response of c-kit^{+} and c-kit^{-} ILC2. ILC2 were originally described as c-kit^{+} in fat associated lymphoid clusters (Moro et al., 2010), however, later reports suggested variability in c-kit expression on ILC2 (Neill et al., 2010; Price et al., 2010). C-kit is a receptor tyrosine kinase that has been shown to be essential for general hematopoiesis (Lennartsson and Ronnstrand, 2012). Mice with a mutant form of c-kit (W/W^{v}) were originally described as having mast cell deficiency (Bernstein et al., 1990;

Grimbaldeston et al., 2005), but ILC2 numbers were also found to be significantly reduced in these mice. Furthermore, mice deficient for the c-kit ligand, stem cell factor (SCF), have significantly less ILC2 (Moro et al., 2010). These data suggested that the c-kit/SCF signaling pathway is involved in the differentiation and/or survival of ILC2. ILC2 are c-kit$^-$ in the bone marrow and up-regulate c-kit expression upon release into the circulation (Gorski, 2013; Hoyler et al., 2012; Yang et al., 2011). This led us to hypothesize that the relatively large expansion of c-kit$^+$ ILC2 seen in the lung at 9 d.p.i. was the result of recruited, mature ILC2 released from the bone marrow. In support of this, proliferation analyses using Ki67 and a 4-hour pulse of BrdU resulted in no detectable ILC2 proliferation in the lung. We also detected both soluble and membrane-bound SCF in the lung, however, its expression was ubiquitous and did not correlate with the kinetics of c-kit$^+$ ILC2 in the lung (data not shown). Thus we propose that at the initiation of the recovery phase c-kit$^-$ ILC2, either resident in the lung and/or recruited from the circulation, receive a maturation signal (which may be related to SCF production) that ultimately results in a large number of c-kit$^+$ ILC2 in the lung.

Important issues raised by our findings regarding NKT cell regulation of ILC2 cytokine production which warrant future evaluation include: 1) how NKT cells become activated to produce IL-33 during IAV infection, 2) confirmation that ILC2 are actively secreting IL-5 *in vivo* during the recovery phase and 3) a better definition of the relationship between c-kit$^-$ and c-kit$^+$ ILC2. Although it is not yet clear how NKT cells become activated during influenza infection, it has been

suggested that they become activated through interactions with antigen-bearing dendritic cells present within the lung (Kamijuku et al., 2008; Paget et al., 2012). NKT cells are thought to be protective during IAV infection through their production of cytokines such as IL-4, IL-22 and IFNγ (Exley, 2012; Juno et al., 2012), and now deeper understanding is required of their ability to produce IL-33 and how this activity is regulated. In addition, it would be of interest to formally demonstrate that ILC2 secrete much higher quantities of IL-5 *in vivo* during the recovery phase of IAV infection. Although we have overwhelming circumstantial evidence to suggest that ILC2 dramatically increase their rate of IL-5 production on a per cell basis during the recovery phase *in vivo*, we were unable to show elevated levels of IL-5 protein in the BAL or lung homogenate. This is most likely due to consumption of IL-5 by eosinophils as well as decreased vascular permeability that precludes cytokine leak into the airspaces. However, the definitive demonstration of IL-5 production by ILC2 *in vivo* can be achieved by using the newly generated IL-5 reporter mouse (Ikutani et al., 2012). These mice would also be beneficial in the analysis of the anatomical location of both the c-kit$^+$ and c-kit$^-$ ILC2 during infection in order to observe what cell types ILC2 associate with *in vivo*. Finally, the relationship between the c-kit$^+$ and c-kit$^-$ ILC2 present in the IAV-infected lung should be investigated. Although recent data suggests that c-kit is a maturation marker, and that a progenitor-descendent relationship could be shown between c-kit$^-$ ILC2 in the bone marrow and c-kit$^+$ ILC2 in the circulation (Hoyler et al., 2012), it remains to be determined if this progenitor-descendent relationship is true in the lung. If indeed c-kit$^-$ ILC2 represent an immature precursor to c-kit$^+$ ILC2 in the

lung, the signals that recruit c-kit$^-$ ILC2 to the lung, and then induce maturation into c-kit$^+$ ILC2 should be definable. KLRG1 expression has been reported as a marker for mature ILC2 present in the gut (Hoyler et al., 2012), thus it would be of interest to determine if c-kit$^+$ and c-kit$^-$ ILC2 in the lung exhibit differential KLRG1 expression.

In chapter 3 we went on to examine the role of IL-5 and eosinophils during the recovery phase of IAV infection. Although eosinophils have previously been detected in the lung following IAV infection, they have only been investigated for their contribution to virus-induced asthma exacerbation and airway hyperreactivity (Yamaya, 2012). Furthermore, the role for IL-5 specifically during IAV infection (i.e. independent of eosinophils) has not yet been reported. We specifically limited our examination of the role of IL-5 to the recovery phase by administering IL-5 neutralizaing antibody (αIL-5) beginning at 7 d.p.i. and administering the antibody daily thereafter. This allowed us to investigate the effects of IL-5 neutralization independent of virus clearance, as infectious virions are cleared from the lung between 8-10 d.p.i. (Hufford et al., 2011). IL-5 was found to be required for optimal recovery as evidenced by the delay in weight gain of mice receiving αIL-5 as compared to mice treated with IgG. As expected, this was not due to any defect in virus clearance or in the adaptive immune response to the virus. In contrast, we found sustained levels of tissue injury, including elevated levels of vascular leak and decreased levels of epithelial regeneration and respiratory function. Lytic viral infections such as influenza can cause significant tissue injury by directly killing

epithelial cells and these cells must be replaced in order to maintain respiratory function (Gorski et al., 2012; La Gruta et al., 2007). The epithelial proliferation and differentiation phase of repair requires the presence of a pulmonary progenitor cell that is either resident in the lung or recruited following IAV infection. These progenitor cells give rise to the specialized epithelial cells that will regenerate on the denuded areas of the lung. The lack of proliferation observed specifically in the AEC I and stromal cell fractions suggested that in the absence of IL-5, epithelial regeneration was delayed, and this may be due to direct or indirect effects of IL-5 on progenitor populations. The factors that regulate the activation and/or recruitment of pulmonary stem cells are not well understood. Recent studies show that distinct progenitor cells can exist for the bronchiolar and alveolar regions as well as the trachea (Kumar et al., 2011; Rawlins et al., 2009; Rock et al., 2009). Kumar et al. found that following H1N1 influenza infection in mice, p63+ progenitor cells, which are thought to mark basal cells in the trachea (Daniely et al., 2004; Senoo et al., 2007), begin forming clusters around damaged foci into distinct keratin 5+ (Krt5+) cellular pods. Within these pods, distal airway stem cells (DASCs), distinct from the upper airway stem cell populations, are capable of differentiating into cells that appear to be of alveolar lineage. What factors and mediators control the DASC differentiation event are not known. This was of particular importance following infection with the 2009 pandemic H1N1 influenza strain, where loss of epithelial repair mechanisms was shown to be a major contributor to pathogenesis, as opposed to a heightened inflammatory response (Kash et al., 2011). Thus understanding whether these progenitor cells express the receptor for IL-5 and are

responsive to it will be critical for understanding how the lack of IL-5 signaling impacts pulmonary epithelial regeneration.

In chapter 3 we also provide the surprising data that indicate that the protective effect of IL-5 is independent of eosinophils. Using the PHIL mouse model, whereby the diptheria toxin alpha chain is constitutively expressed under the *eosinophil peroxidase* promoter, we were able to assess the contribution of eosinophils during IAV infection. These mice are selectively deficient in eosinophils and have no known defects in other cellular compartments (Lee et al., 2004). Indeed, infection of transgene expressing-PHIL mice (PHIL Tg+) with IAV resulted in no differences in cellular infiltrates into the lung (with the exception of eosinophils) and this coincided with no differences in morbidity as compared to littermate controls (PHIL Tg-). Confirmation of the eosinophil-independent effects of IL-5 was demonstrated by αIL-5 treatment of PHIL Tg+ mice. Treatment of PHIL Tg+ mice with daily αIL-5 administration, beginning at 7 d.p.i., resulted in their delayed recovery and decrease in epithelial regeneration. The protective effects of IL-5 in an eosinophil-independent manner were also recently reported in a model of polymicrobial sepsis (Linch et al., 2012). PHIL Tg+ mice transgenically overexpressing IL-5 were significantly protected from sepsis-induced death and the effects were attributed to IL-5 acting on macrophages to enhance bacterial killing; however, the authors of that report also presented data of IL-5Rα expression on neutrophils. Although we did not detect IL-5Rα expression on macrophages, we do corroborate the finding

presented in that study that neutrophils are capable of IL-5Rα expression and furthermore, are capable of responding to IL-5/IL-5R ligation.

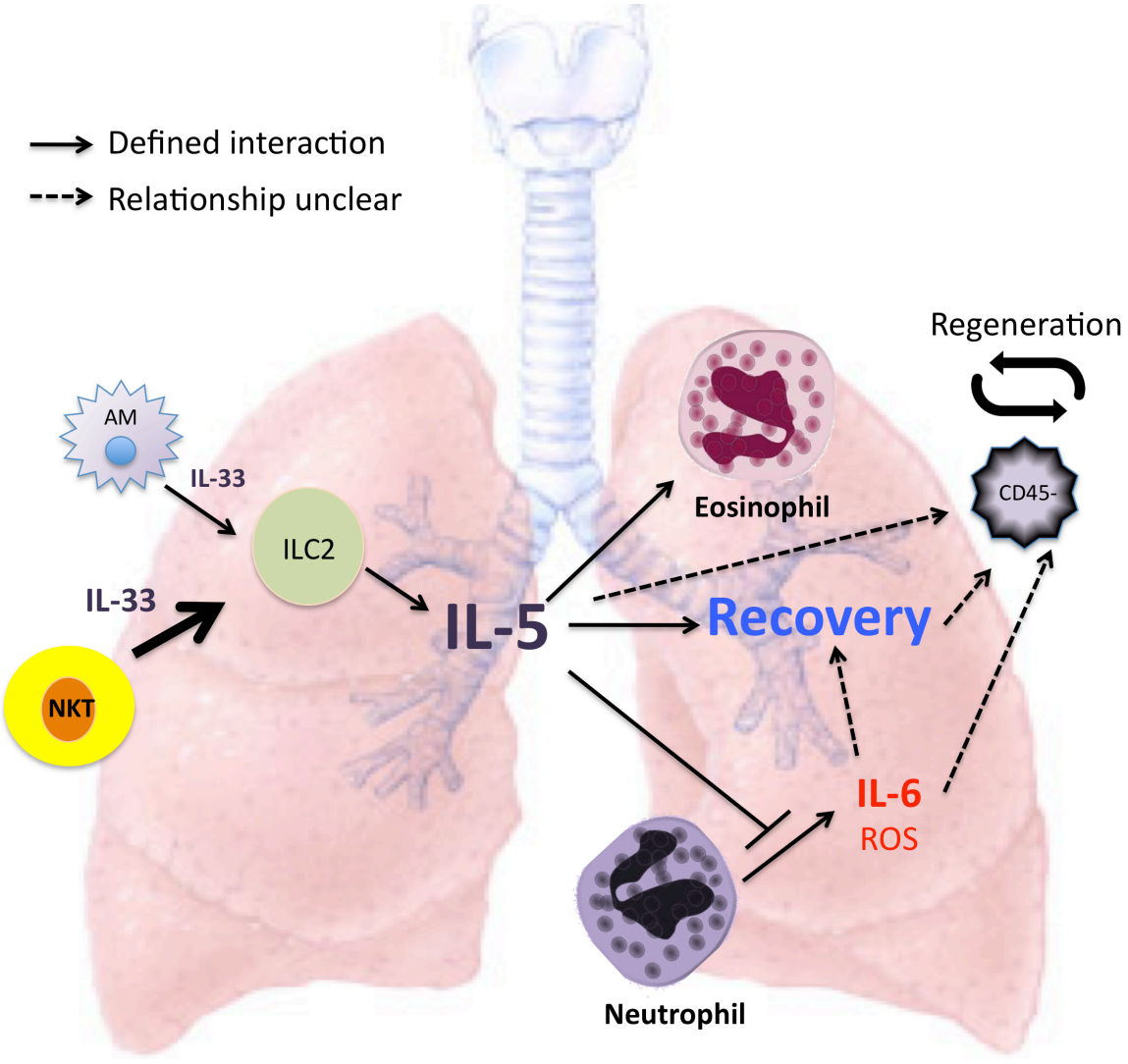
IL-5Rα expression has traditionally been thought to be restricted to eosinophils, B-1 B cells, and a small number of activated B-2 b cells during class switch recombination (Takatsu and Nakajima, 2008). In chapter 3 we show that neutrophils constitutively express surface IL-5Rα in all tissues examined, including lung, blood, bone marrow, and spleen. Furthermore, FACS sorted neutrophils from 10 d.p.i. mice had IL-5Rα transcript levels that were comparable to eosinophils. We did not detect any IL-5Rα expression on macrophages as was reported by Linch et. al (Linch et al., 2012), however, this may be due to differences in the infection model used (i.e. systemic inflammation in polymicrobial sepsis vs. localized inflammation during IAV infection). We demonstrate that the IL-5R present on neutrophils is functional using *in vitro* and *ex vivo* analysis. Lung and bone marrow derived neutrophils treated *in vitro* with rIL-5 exhibited reduced levels of ROS production and diminished signaling through phosphorylation of STAT5 (pSTAT5) following stimulation with LPS. The Jak2/STAT5 signaling cascade is the major pathway induced upon IL-5/IL-5R ligation in eosinophils (Ogata et al., 1998; Takatsu et al., 2009). We also investigated signaling through pSTAT1, as it has been reported to be induced in some circumstances following IL-5 stimulation of eosinophils (Pazdrak et al., 1995); however, we detected no changes in pSTAT1 in rIL-5 treated neutrophils (data not shown). This *in vitro* data was supported by our *ex vivo* analysis of neutrophils from αIL-5 treated mice. Neutrophils from the αIL-5 treated mice

expressed significantly more ROS and had a trend of higher gene expression for IL-6 and iNOS at 12 d.p.i (although these data are not statistically significant). Higher ROS production was only observed in neutrophils, as ROS levels in monocytes/macrophages and lymphocytes were comparable between αIL-5 and IgG treated animals, suggesting a specific effect of IL-5 on neutrophils. Furthermore, depleting neutrophils in the αIL-5 treated mice significantly improved recovery, albeit not fully, demonstrating that neutrophils were contributing to the delayed recovery in the αIL-5 treated mice. Further studies are needed to address the specific signaling pathway that IL-5 utilizes in neutrophils and identify the downstream molecular targets. In addition to pSTAT5 analysis, other strategies, such as calcium mobilization, can be used to demonstrate that the cells are responsive to IL-5 and gene arrays may be used to identify genes that are differentially expressed upon IL-5R ligation. More in depth functional analyses are also needed to address the functional consequences of IL-5 signaling in neutrophils, such as phagocytosis and degranulation assays (van Eeden et al., 1999; Vander Top et al., 2006). Overall, the data presented in this thesis suggest that during the recovery phase of IAV infection, IL-5 acts as an inhibitory signal to suppress neutrophil inflammatory activity.

Therefore, two plausible, non-mutually exclusive models that could explain how ILC2-derived IL-5 promotes recovery include 1) IL-5 signals through neutrophils to suppress their effector activity and this indirectly promotes epithelial regeneration or 2) in addition to acting on neutrophils, IL-5 can independently signal through an

as yet unidentified lung progenitor and/or epithelial cell to directly promote epithelial barrier regeneration (Figure 4.1). Our current data support the former, as we were unable to detect IL-5R expression on $CD45^-$ cells, using two methods of lung digestion to assess surface staining as well as qPCR to quantitate transcript levels. However, it is formally possible that there is a low level of IL-5R expression on a small population of progenitor cells that was undetectable using our methods or that the receptor is inducible under certain conditions. IL-5R expression can be induced on human bronchial epithelia following metal-induced toxicity and minimal expression has been detected in endothelial cells (Andrew et al., 2003; Colotta et al., 1993). In addition, a soluble IL-5R has been identified in both mice and humans, however, its functional significance has yet to be determined (Takaki et al., 1990; Tavernier et al., 1992). The relevance of any possible IL-5R expression on non-hematopoietic cells could be easily assessed by utilizing the $IL\text{-}5R^{-/-}$ mouse model (Yoshida et al., 1996). By generating a bone marrow chimera of wildtype bone marrow in IL-5R-/- donors, the importance of any $CD45^-$ cell expression of IL5R during recovery from IAV infection could be determined. This and other previously discussed future research strategies provide several examples of possible extensions of the findings presented in this thesis that could further our understanding of the regulation of IL-5 and its functional role during influenza infection.

Figure 4.1: Model for the role of IL-5 during influenza infection. ILC2 produce IL-5 during IAV infection. Alveolar macrophages (AM) and NKT cells producing IL-33 enhance the production of IL-5 from ILC2. Eosinophil accumulation in the lung is dependent on IL-5 production throughout the recovery phase. IL-5 is also produced during the recovery phase to suppress neutrophil effector activity (e.g. IL-6 and ROS production) and, directly or indirectly, promote epithelial regeneration.


Defined interaction
Relationship unclear
Regeneration
AM
IL-33
ILC2
IL-33
NKT
IL-5
Eosinophil
CD45-
Recovery
IL-6
ROS
Neutrophil

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