IMPACT OF H5N1 INFLUENZA VIRUS INFECTION ON NATURAL KILLER CELLS AND INNATE LYMPHOID CELL POPULATIONS IN MACAQUES

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ABSTRACT

Highly pathogenic avian influenza (HPAI) H5N1 viruses are a class of emerging zoonotic viruses that present a significant threat to global health. Seasonal influenza causes an estimated 3-5 million illnesses a year, presenting a significant public health burden. Surveillance and research into the clinical and immunological mechanisms of emerging avian influenza viruses like H5N1 with pandemic potential is important to safeguarding public health worldwide. H5N1 strains are endemic in wild and domestic birds worldwide, but very rarely infect humans. When spillover into humans does occur, however, H5N1 causes severe disease, acute respiratory distress, and has a high case fatality rate. The high pathogenic potential of this virus makes a compelling argument for understanding the underlying pathological and immunological mechanisms of the disease. Our lab has demonstrated in a nonhuman primate model that aerosolized infection with H5N1 influenza virus leads to disease progression similar to that seen in human cases. This study aims to characterize some of the innate immune cells that contribute to the response to severe H5N1 infection in this macaque model. Natural killer (NK) cells are a critical cytotoxic innate responder to viral infection, and innate lymphoid cells (ILCs) are a recently discovered subset of the innate immune system that are thought to have a critical impact on early response to viral infection in the lung. These cells were characterized and quantified in lung tissue of both naïve and H5N1 infected cynomolgous macaques. I found that NK cells

showed a significant decrease in frequency in infected animals, perhaps indicating infection and subsequent loss relative to naïve animals. I was also able to identify two populations of CD45+ cells lacking lineage markers (CD3/CD20/CD163) in the macaque lung that are analogous to previously defined type 2 ILCs expressing CRTH2 but do not express CD127, and a population of type 3 ILCs that co-expressed CD127 and CD117. CRTH2+ cells accumulated non-significantly in the lungs of H5N1 infected animals in response to influenza virus, suggesting that they are stimulated and recruited by infection, and likely have a protective immune response. Further characterization of ILC and NK cell subsets in the lung and their functional response to severe acute respiratory infection such as H5N1 provides a promising avenue for understanding the early innate response to influenza infection.

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PREFACE

I would like to give my sincerest thanks to Dr. Simon Barratt-Boyes, for accepting me into his lab and offering his support throughout this project. I would also like to thank the members of the Barratt-Boyes lab, both current and former, for their help and friendship, with special thanks to Elizabeth Wonderlich for all of my training in the Regional Biocontainment Laboratory. I thank my committee members, Drs. Hartman and Wang, for their time and feedback. Finally, I would like to express my appreciation and gratitude to my academic advisor, Dr. Jeremy Martinson, for all of his advice and mentorship throughout my time here at Pitt. I could not have done it without each and every one of you.

1.0 INTRODUCTION

Influenza virus is a small, enveloped RNA virus belonging to the family Orthomyxoviridae that can be found in many different species. Phylogenetically, influenza viruses originate from wild waterfowl as their natural host, but have evolved to routinely infect domestic poultry and a wide variety of mammals[1]. Avian influenzas circulate worldwide in both wild and domestic birds. Human seasonal influenza strains are endemic in the population, causing between 3 to 5 million infections a year and resulting in approximately 250,000-500,000 deaths worldwide[2]. Influenza viruses are divided into three subtypes (A, B and C), of which influenza A viruses are primarily responsible for the burden of human disease. Influenza virus mutates rapidly, which results in a wide variety of strains whose epidemiology shifts from year to year. The virus has two main surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), which are used to subtype the virus based on reactivity to human antibodies. Influenza A subtypes are named after their species of origin (if other than human), site of isolation, number of isolate, year of isolation, and then by HA and NA subtype (e.g. A/goose/Guangdong/1/96 H5N1)[3]. Currently, the circulating human strains of influenza A are of the H1N1 and H3N2 subtypes[2].

In humans, influenza virus infection is transmitted by inhalation of airborne particles, droplets, and contact with contaminated surfaces via fomites. Infection causes acute respiratory disease, as well as symptoms such as fever and general malaise, although

complications such as secondary pneumonia can arise[4]. The case fatality rate of seasonal influenza disease is relatively low, although there are several populations who are at higher risk of mortality and complications due to influenza virus infection, including people over the age of 65, children under the age of 5, and pregnant women[2]. While mortality is typically low, due to its widespread prevalence in the human population and ability to mutate into new strains, influenza virus is widely considered to be one of the greatest threats for a human pandemic disease. Pandemics occur when a new strain is introduced to the human population, typically through a species crossover event, and spreads rapidly. In 1918 for example, a strain of H1N1 influenza virus known as the Spanish Flu caused the worst recorded influenza pandemic in human history that is estimated to have killed 50 million people worldwide. Influenza pandemics of some degree occur about every 40 years[5]. As such, influenza monitoring and surveillance is an ongoing public health priority.

1.1 INFLUENZA BIOLOGY AND EPIDEMIOLOGY

The influenza viral genome is single stranded, negative sense RNA that is divided into eight segments which encode a total of 10 proteins: 3 RNA polymerase components (PB1, PB2 and PA), the viral nucleoprotein, two matrix proteins (M1 and M2), two nonstructural proteins (NS1 and NS2), and the surface glycoproteins HA and NA[3].

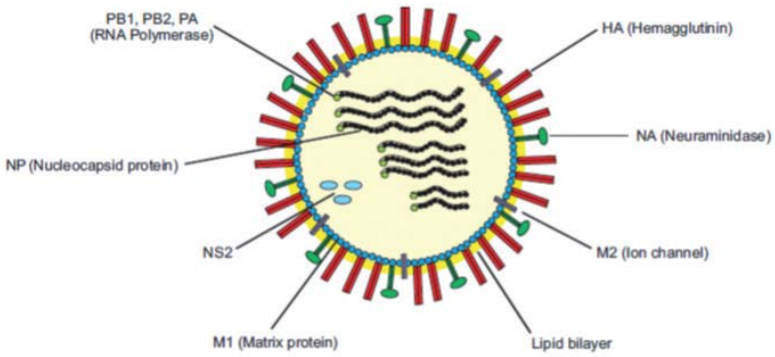

Figure 1. Influenza Virion

Schematic representation of influenza virion and structural proteins. Source: Szewczyk et al.[3]

The surface proteins, HA and NA play an integral role in virus entry and release, respectively. HA binds sialic acid-galactose receptors on the surface of host cells to facilitate entry. Variations in HA protein structure contribute to the species-specificity of virus, by preferentially binding different forms of glycosidic linkages. In human influenza viruses, the preference is for sialic acids covalently attached to galactose via an α2,6. Avian strains of the virus, however, primarily use α2,3 linkages to infect cells which line the intestinal tract of many species of waterfowl and domestic poultry. This explains the fact that strains have evolved some degree of species-specificity, but it is important to note that the binding of these linkages is preferential, not absolute[3]. Point mutations in the HA protein or infection with high inoculum doses of the virus can be enough to overcome this preference to cause cross-species infection. The NA protein is responsible for cleaving sialic acid from the surface of infected cells, facilitating viral release by preventing recapture of virions through HA re-binding to host cells.

Rapid evolution is a key aspect of influenza epidemiology, which is driven by two factors, an error-prone RNA polymerase, and the segmentation of the influenza genome. The RNA polymerase used to copy the influenza genome during viral replication possesses no error-checking activity, and as such is highly likely to introduce point mutations during genomic

replication. The accumulation of these mutations over time leads to what is referred to as antigenic drift, as gradual changes to the genome result in new viral variants that are not neutralized by the immune system. This applies to the surface proteins HA and NA in particular, as they are the primary target of neutralizing antibody activity and immunological memory, hence why influenza strains are classified according to antibody cross-reactivity of these proteins. Strain variants resulting from antigenic drift often contain some degree of overlap, and the immune system is able to at least partially recognize similar strains from year to year, resulting in varying degrees of immunity throughout the population to seasonal strains.

Pandemic strains, on the other hand, often arise from what is termed antigenic shift. Antigenic shift is the reassortment of genome segments between two different influenza viruses when more than one virus strain infects the same cell in a host. This can lead to rapid jumps in evolution that create entirely new genetic variants to which the host has no prior immunity, and these can transmit rapidly through a susceptible population. Cross-species antigenic shifts commonly take place in an intermediary host that can be infected with multiple strains from different species. Domestic swine are commonly considered to be a mixing vessel for reassortment between human and avian strains of influenza. The resulting virus is considered to have pandemic potential if it is easily transmissible from person-to-person. Furthermore, viruses that undergo antigenic shift and make a zoonotic jump between species could undergo further antigenic drift mutations of their HA protein to select for preferential binding of the new host's sialic acid receptors, resulting in a new HA variant subtype that is newly species-specific for that host.

1.1.1 H5N1 High Pathogenicity Avian Influenza

As previously mentioned, the natural host of avian influenza viruses are wild waterfowl, and transmission among wild birds is common. Often infection within these species is asymptomatic. Influenza viruses also can circulate between wild birds and domestic poultry. Avian influenza typically refers to a group of viruses which normally infects only birds, and there are several different avian influenzas currently circulating throughout the world, including H5N1, H5N8, H5N6, H7N9 and H9N2 subtypes[6]. In birds, there are two classifications of avian influenza, low or high pathogenicity. Low pathogenicity avian influenza (LPAI) only causes mild symptoms, whereas high pathogenicity avian influenza (HPAI) is characterized by severe systemic disease and fatality rates sometimes nearing 100% in domestic poultry[6]. The pathogenicity of the strain can vary greatly between species, however, strains that cause disease in domestic poultry are of particular concern both due to their potential economic impact as well as the proximity to humans and potential for zoonotic transmission. There have been several instances of avian influenza viruses infecting humans, usually with cases of human-to-human transmission limited to close primary contacts such as immediate family and caregivers, but these spillover events tend to cause high morbidity and mortality[4]. H5 viruses in particular have been a cause for concern because there have been multiple cases of human H5 infection the past several decades[6].

The first known case of H5 influenza transmission to humans occurred in Hong Kong in 1997, as a result of the A/goose/Guangdong/1/96 virus. Viruses belonging to this lineage have since spread throughout the world and mutated to form a diverse repertoire of strains. Since 1997, human cases of H5 viruses have been reported in more than 60 countries[6]. There have been 856 human cases reported as of January 2017 across five continents, with a case fatality

rate exceeding 50%[6]. Human infection with H5N1 results in severe disease that is characterized by rapid onset of acute respiratory distress syndrome (ARDS), high viral load, and intense inflammation, which leads to rapid death[7]. Despite the severity of disease seen in humans, there have been no recorded instances of sustained human-to-human transmission. However, as H5N1 viruses continue to circulate in bird populations throughout the world, it continues to be monitored as a potential pandemic threat, and monitored for potential mutations that could facilitate human-to-human transmission.

1.1.2 Host response to influenza infection

The host response to influenza virus comprises of both innate and adaptive immune responses. After infection through aerosolized droplets or contact with fomites, infection initiates in the respiratory tract where the virus can infect both immune and non-immune cells. Recognition of the virus by innate immune sensing mechanisms stimulates the production of type 1 interferons (IFNs) which lead to induction of an antiviral state. The production of pro-inflammatory cytokines leads to many of the generalized symptoms of influenza, such as fever, and malaise, but also serves to begin the recruitment of innate cell types, including neutrophils, monocytes and natural killer cells.[8] Adaptive immune responses to influenza infection are quite complex, and have been reviewed many times in the context of immune response to vaccination[9]. Briefly, influenza is able to induce both an antibody response, largely against the two main surface glycoproteins, HA and NA, as well as a robust CD8+ and CD4+ T cell response. This leads to the generation of immunological memory, but the rapid mutation of the virus means that the strain-specificity of the adaptive immunity does not protect against new and emergent strains after a single infection.[9] Another important feature of the host response to influenza is the

potential for immunopathology caused by intense immune responses. Due to widespread viral replication, recruitment and activation of immune cells occurs rapidly. While viral mediated cell death and tissue damage causes much of the pathology of influenza, there is evidence of CD8+ T cell mediated tissue damage as well, and excess production of cytokines causes exacerbation of inflammation in severe disease[10].

In this study, we specifically focus on the innate response to influenza virus as mediated by innate lymphoid cell subsets.

1.2 INNATE LYMPHOID CELLS

ILCs are a broad, heterogeneous population of cells that are responsible for several crucial functions of the early innate immune response, including promoting homeostasis, tissue repair, and regulation of inflammation[11-13]. They are distributed throughout the body in various lymphoid and non-lymphoid tissues, but have been shown to be enriched at mucosal surfaces[13]. All ILCs develop from a single common lymphoid progenitor[12], which then differentiates into a common natural killer (NK) cell progenitor and a common helper ILC progenitor. The common helper ILC progenitor further differentiates into three subtypes[14] (ILC1, 2 and 3) of non-lytic ILCs that are defined based on their required transcription factors and cytokine expression.

There is limited universal nomenclature and definition of various subtypes of ILCs, and some research suggests that expression of surface markers can vary depending on the tissue of residence and cytokine environment, as well as the fact that there is some level of plasticity between the ILC types[13, 14]. All the non-cytotoxic ILCs react indirectly to pathogens by sensing

cytokines derived from myeloid or epithelial cells. In turn, they are believed to drive and amplify both innate and adaptive immune responses by producing cytokines associated with particular T cell types and feedback loops[13]. This makes them potent activators of the immune response, and they play a critical role in the regulation of immunity and inflammation.

1.2.1 Type 1 ILCs and classical NK cells

ILC1s include classical natural killer (NK) cells, as well as non-cytotoxic ILC1s. All ILC1s including NK cells express the transcription factor Tbet, while non-toxic ILC1s express both Tbet and Eomes[12]. Classical NK cells express the functional proteins perforin and granzyme b, and non-toxic ILC1s produce IFN-γ in response to interleukin (IL)-12[15]. There are also several emerging subsets of ILC1 that are as of yet poorly characterized[16].

While NK cells are traditionally classified in with ILC1s due to their expression of the characteristic transcription factor Tbet, they are distinct from other ILC subtypes in several ways. For one, NK cells are effector cells, involved in direct recognition and killing of infected host cells, as opposed to the rest of the ILCs which take on a non-cytotoxic helper role by producing cytokines that marshal the immune response[11]. Additionally, NK cells differentiate from the common innate lymphoid progenitor cell earlier in their development than the rest of the non-cytotoxic ILC subtypes[14]. NK cells are the body's first line of defense in response to intracellular pathogens. They are primarily located in secondary lymphoid tissues, and are recruited to the site of infection via chemokine signaling, again in contrast to other ILCs, which are tissue-resident[13]. They play a pivotal role in the innate response against viruses, and have been implicated in control of both acute and chronic viral infection[17]. NK cells express several

activating receptors, NKp46, NKp44 and NKp30 (alternatively referred to as NCR1, NCR2 and NCR3), as well as the NKG2 family of activating and inhibitory receptors[18].

While NK cell activity has been shown to play a critical role in influenza immunity, there are also several demonstrated mechanisms of evasion of NK cell defenses[18]. The NK cell natural cytotoxicity receptor NKp46 contains sialic acid residues, and as such are able to recognize the viral HA protein[19]. This interaction makes influenza virus a ready target for NK cell-mediated viral clearance, but also means that NK cells are susceptible to infection. Additionally, it is hypothesized that rapid mutation of HA among influenza strains contributes to influenza's effective evasion of NK cell defenses. Additionally, it has been shown that both seasonal and avian influenza strains can directly infect NK cells[18, 20] and have been shown to induce NK cell death as well as inhibition of activation[20]. Therefore, studying NK cell responses in an in vivo model could provide insights into their reaction to fatal influenza infection.

1.2.2 Type 2 ILCs

Type 2 ILCs are characterized by the expression of IL-13 and IL-5 in response to stimulation by IL-33 and IL-25, a cytokine expression profile that is analogous to type 2 helper T cells (Th2)[12, 21-23]. They rely on the transcription factor GATA-3 for their development, and express several characteristic surface markers including IL7-Rα, CD25, Sca-1, ST2, CD161 and CRTH2[12, 21, 23] in mice and humans. CRTH2 is a marker that is associated with Th2 immune responses[23] that prevents apoptosis and mediates chemotaxis to sites of allergic inflammation.

The cytokines produced by ILC2s, especially IL-13 have been shown to induce allergic inflammation, and ILC2s have been studied in various allergic diseases, asthma and also response to helminth infection[14]. Various innate immune cell subtypes produce IL-33 as a

danger signal and activate a feedback loop[12] that results in the recruitment of ILC2s. ILC2s also produce IL-5 which induces eosinophils and related effector responses, further resulting in the priming of Th2 response through recruitment or facilitating antigen presentation[12]. ILC2s have been extensively studied in the lung as a mediator of airway inflammation and cellular repair mechanisms, which is particularly important in the context of influenza infection.

1.2.3 Type 3 ILCs

Type 3 ILCs produce IL-22 and IL-17 in response to IL-23[12, 23], which is analogous to the functional profile of Th17 cells. They rely on the transcription factor RORγ for their development and function[12]. There are two main groups of ILCs, referred to as natural cytotoxicity receptor (NCR) expressing or NCR- subsets. NCR+ ILC3s express the NK cell receptors NKp46 and NKp44, as well as CD117[24, 25]. ILC3s have been implicated in a wide variety of roles in disease progression. They interact with the microbiome and have been implicated in the control of various bacterial and fungal infections, as well as the formation of various secondary lymphoid tissues[16]. As with ILC2s, ILC3s are important in driving the inflammatory response due to their early production of IL-17 in response to proinflammatory signaling by monocytes and other innate cells. The interaction between various ILC and helper T cell derived cytokines is important in the regulation of overall immune response.

1.2.4 ILCs in the Respiratory Tract

To date, there are no studies that we know of examining ILC responses in the lung of macaques or any other non-human primate model. ILCs have, however, been studied in a variety of human

lung diseases, including chronic obstructive pulmonary disease[24], pulmonary fibrosis[26], lung cancer[27], helminth infection[14], asthma[28] and allergic airway inflammation[29]. In these studies, ILC populations have been found which correspond to populations previously identified in mice, and express the same phenotype and cytokine profiles as ILCs identified in the sinuses, gut, lymph node and other secondary mucosal tissues such as gut-associated lymphoid tissue and splenic fat-associated lymphoid clusters[14, 21]. ILCs have been studied in the gut of rhesus macaques in relation to SIV infection[25, 30], but these studies have been restricted to examining IL-17 producing ILC3s. However, based on these similarities, we expect that comparably analogous populations exist in macaque lung mucosa.

ILC2s are the primary type of ILC that has been studied in the lung. The cytokines they produce, as well as the cellular product amphiregulin, have been shown to promote mucus production and tissue repair in lung[14], and the ILC2 product IL-13 has been shown to promote epithelial proliferation[21]. In influenza infection, these functions have been shown to be critical to maintaining the integrity of the alveolar epithelial barrier[22], and in fatal infection with H5N1, breakdown of this barrier integrity is a driving factor in progression of disease. In mouse models, ILC2s were shown to accumulate in the lung in response to H1N1 influenza infection, and provided a protective effect[22]. ILC2s have also been shown to enhance and regulate T cell responses in IL-13 dependent manner in lung[14]. ILC3s have also been identified in the human respiratory tract[24, 27]. There is evidence of interplay between ILC2s and ILC3s which further regulates inflammation[29]. IL-22 dependent ILC3s promote epithelial cell function and suppressing inflammatory response[14, 29]. In chronic inflammatory diseases, there has been a demonstrated shift in ILC populations away from ILC2s to ILC3s, indicating that ILC3 play a

role in mediation of inflammation as well as ILC2[24], and it has been demonstrated that the distribution of ILC populations altered in human lung disease[14, 24]

It is important to note, however, that not all ILC responses that have been seen in the lung are protective. There are several instances where detrimental ILC2 response were seen in atopic and allergic diseases[13], including allergic airway hyperactivity[13, 31]. This ILC mediated airway hyperreactivity has also been demonstrated in mouse models of influenza after infection with H3N1viruses[32], and the associated inflammation contributed to influenza pathology. Therefore, while ILCs promote barrier integrity and cell repair in acute respiratory infection, they may also play a role in the pathology of cytokine storms and inflammatory damage.

Overall, ILCs have been shown to be a relatively minor population in the lungs of both mice and humans, making up a total of around 0.02-0.08% of all CD45+ cells in a healthy adult lung[23], but they play an important role in the progression of many diseases. Identifying these cells in a macaque model could prove useful in understanding their role in the control of respiratory diseases.

2.0 SPECIFIC AIMS

To elucidate the mechanisms of the host immune response to H5N1 infection, the aims of this project are to develop a strategy to identify ILCs and NK cells in cynomolgous macaque tissues via flow cytometry. Specifically, we want to identify whether infection with H5N1 alters the populations of these cells in the respiratory tract.

In order to understand the dynamics that NK cells and ILCs play in H5N1 infection, we plan on first characterizing the cells in lung tissue, and quantifying them in order to compare between infected macaques and uninfected control animals. Previous data generated by our lab suggests that there is a depletion of NK cells in the peripheral blood of infected animals. We hypothesize that there will be a similar viral-mediated depletion of NK cells in the respiratory tract. Based on previous studies, there are indications that ILCs will be increased in the site of infection, so we hypothesize that we will be able to identify a population of lung-resident ILCs that proliferating. Because both NK cells and ILCs have been indicated in mediating protective immune responses against influenza, characterizing them in relation to severe disease will be helpful in understanding the immune responses to H5N1 infection, and if there are any potential links between these innate immune cells and correlates of protection against severe disease or mortality. This information can be used to help aid the treatment of severe influenza infections or develop novel therapeutic strategies.

3.0 METHODS

3.1 VIRAL INOCULATION AND GENERATION OF ANIMAL SAMPLES

Samples for my work were previously generated during a study of macaques infected with H5N1 virus as described previously by Wonderlich et al.[33] Briefly, seven healthy adult female cynomolgus macaques were inoculated via the aerosol route with highly pathogenic avian influenza A/Vietnam/1203/2004 (H5N1) virus. Virus was provided by Dr. Daniel Perez at the Department of Population Health, University of Georgia. Macaques were exposed to small-particle aerosols of virus for a mean duration of 24.3 minutes, and dose was calculated based on each individual macaque's respiratory volume and nebulizer viral concentration. The mean dose of inoculation was $6.72\log_{10}$ PFU (Table 1). Macaques all either succumbed to respiratory failure or were humanely sacrificed due to symptom progression by six days post-infection, with a mean time-to-death of 3.2 days. For this analysis, cryopreserved single cell suspensions of various tissues were thawed and stained for flow cytometric analysis. Tissue samples were prepared by Elizabeth Wonderlich and Simon Barratt-Boyes as described[33]. All work was carried out in the University of Pittsburgh's Regional Biocontainment Laboratory biosafety level 3 facility.

Table 1. Characteristics of Animals' Aerosol Exposure

Animal ID	Viral Titer (log_{10} PFU)	Time to death (days)
132	7.26	3
133	7.17	2*
134	7.07	6*
135	6.16	3
136	6.46	2*
137	6.37	2*
138	6.57	4
Mean	6.72	3.2

*- animal humanely sacrificed

3.2 FLOW CYTOMETRY

Classical NK cells have been relatively well studied, and NK cell activation panels in macaques have previously been established[34]. The definition of NK cells used in this study was CD45+ lymphocytes, lacking expression of lineage markers for T cells, B cells and monocytes. In macaques, NK cells also express CD8α. They can further be divided into subsets that express either CD16 or CD56. There are several NK cell specific surface markers that can be used as additional positive inclusion gating, but for this panel we chose to use NKG2A, which has been shown to be widely expressed on macaque NK cells.

ILC subsets were more difficult to define via extracellular surface markers alone, since they are less well-studied, and there is not a universally agreed-upon definition of pan-ILC markers. Therefore, development of the ILC panel is described in detail below.

3.2.1 ILC Surface Marker Identification

Since universal nomenclature based on surface markers have not been established, the first step we undertook was to establish a literature review to decide how to define and identify ILCs in tissues. Because we had previously determined that in this study, we were primarily interested in the behavior of classical NK cells, and there was very little information that suggested non-lytic ILC1 would be of particular import in acute respiratory infection, we decided to look exclusively at Type 2 and 3 ILCs. Different studies have identified different gating strategies for various tissues, species, and disease states, so we needed to synthesize the results of previous studies to create a profile of surface markers that we would identify as ILCs. Table 2 summarizes the distribution of surface markers that have been used to identify Type 2 and 3 ILCs in previous studies, and which tissue/species they have been identified in.

Table 2. ILC Surface Markers

Surface Marker	ILC2 in Human [22-24]	ILC3 in Rhesus Macaque[25]
CD16	-	-
CD25	+	-
CD56	-	-
CD90	+	+
CD117	+/-	+
CD127	+	+
CD161	+	-
CRTH2	+	-
NKp30	-	+
NKp44	-	+
T1-ST2	+	-

It is believed that in humans, all ILC subtypes require IL-7 to drive their development, and therefore express CD127, which is the IL-7 receptor subunit α. Additionally, another surface marker which has been demonstrated on all ILC subtypes is CD90, which is a costimulatory molecule for activation and proliferation signaling. It has been shown that anti-CD90 antibodies leads to pan-ILC depletion[22], however, while ILCs express CD90 at increased levels, it is not ubiquitously expressed by all cells. Another point of interest to note is that while CD117 has been indicated as a ILC3-specific surface marker in both macaques and humans[24, 30], other studies suggest that some fraction of CRTH2 expressing ILCs also express CD117. Whether this holds true in the respiratory tract of nonhuman primates is unknown.

3.2.2 Panel Development

Having decided on the definition to use for both ILCs and NK cells, two full flow cytometry panels were developed. While I had hoped to be able to identify both NK cells and ILCs out of a single panel to identify both subsets of ILCs at the same time, to better facilitate quantitative

analysis of changes in populations, ultimately due to constraints in available fluorochrome conjugations, the panels were separated into one identifying NK cells, and one identifying ILCs, keeping lineage markers consistent between the two.

Table 3. Flow Cytometry Panels for NK Cell and ILC Identification

Laser	BP Filter	Fluorochrome	NK Cells	ILC
UV 335nm	450/50	DAPI	Live/Dead	Live/Dead
Violet 405nm	450/50	Pac Blue	CD3/CD20	CD3/CD20
	525/50	V500		CD90
Blue 488nm	530/30	FITC	CD45	CD45
	575/26	PE	CD56	CD127
	710/50	PerCP- Cy5.5	CD163	CD163
	780/60	PE-Cy7	CD8	
Red 633nm	660/20	APC	NKG2A	CRTH2
	780/60	APC-Cy7	CD16	CD117

3.2.3 Staining and Analysis

Cryopreserved single-cell tissue suspensions were stained with extracellular labeling antibodies. Prior to staining, all cells were counted using trypan blue exclusion assay, and 1 X 10^6 live cells per test were stained with antibodies for 35-45 minutes as per protocol. Non-viable cells were excluded by staining with Live/Dead Fixable UV stain kit from ThermoFisher. Single or multiple antibody leave-outs were used as fluorescence-minus-one controls to determine negative gating. All samples were run on BD LSRII cytometer using BD FACSDiva software, and analyzed using FlowJo software version 10.x. Statistical analysis was performed using All analyses were performed using GraphPad Prism v7. A p value of <0.05 was considered

significant, using two-tailed nonparametric Mann-Whitney test, comparing means between naïve control and infected animal samples.

4.0 RESULTS

4.1 IDENTIFICATION OF ILC SUBSETS

4.1.1 ILCs reside in hilar lymph node and lung tissue of cynomolgous macaques

The ILC panel was run in two tissues of interest to see if there were ILCs which could be identified using the ILC panel in different tissue. I looked at hilar lymph node, and lung tissue to try and identify both type 2 and type 3 ILCs. ILCs were defined as CD45+ lymphocytes lacking expression of lineage markers identifying T cells, B cells and monocytes using CD3, CD20 and either CD163 or CD14, respectively. CD163 was used in lung tissue while CD14 was used as a monocyte marker in lymph node. These populations then were examined for their differential expression of CD127, CD117 and CRTH2.

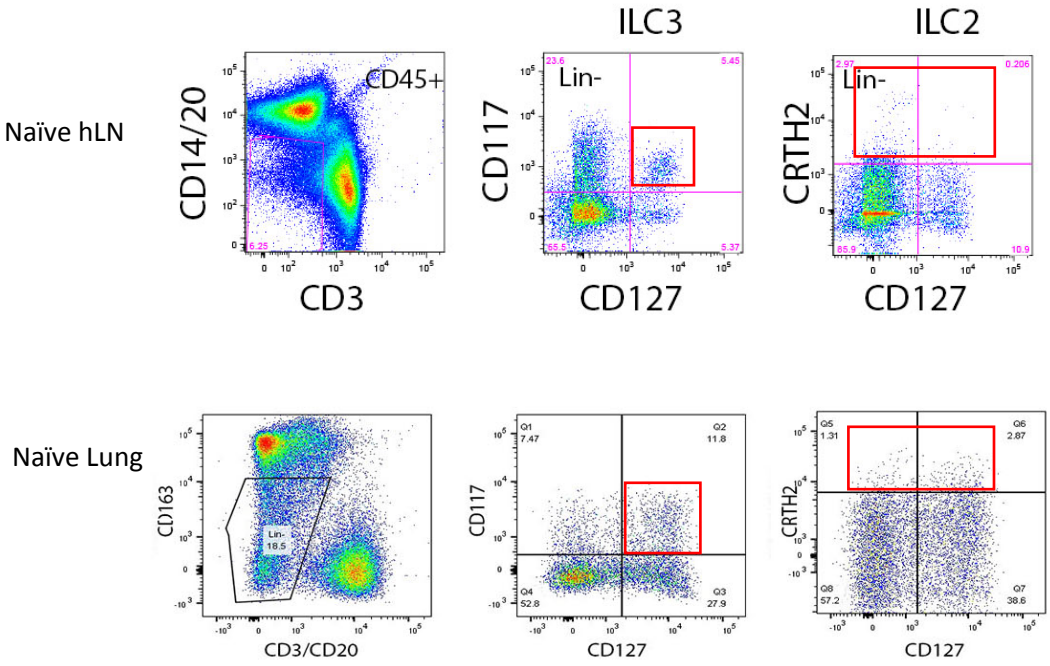

Figure 2. ILC2 and ILC3 Populations in Naive Hilar Lymph Node and Naive Lung Tissue

Populations highlighted in red are ILC2 and ILC3 subtypes expressing characteristic markers.

Both hilar lymph node and lung tissues had distinct populations of CD127/CD117 double positive ILC3s. While ILC3s have been previously demonstrated to be resident in various mucosal tissues, mesenteric and peripheral lymph nodes as well as PBMCs in macaques prior to this study, they have not been shown to be resident in lung tissue before. In hilar lymph node as well as lung there is a very limited population that expresses the characteristic ILC2 marker CRTH2. However, it is assumed that CRTH2+ cells that are potentially ILC2s.

4.2 COMPARISON OF ILC SUBSETS BETWEEN INFECTED AN NAÏVE ANIMAL SAMPLES

4.2.1 Changes in distribution of lymphocyte populations

There were several overarching themes that were seen when examining the differences between infected lung tissue compared to healthy controls. The proportion of live cells that were CD45+ lymphocytes was higher in infected animals (Figure 2). The remaining live cell population that did not express CD45 was presumably largely composed of epithelial cells. This corroborates results seen in our previous work[33] that virus-mediated epithelial cell death contributes to the breakdown of the alveolar barrier integrity. Another important change seen in infected lungs was that the proportion of T cells decreased, while the proportion of cells that were negative for all lineage markers (a population which includes both NK cells and ILCs) more than doubled in comparison to control animals. This is in respect to the entire proportion of live cells in the sample, not just CD45+ lymphocytes. These changes could be indicative of an overall infiltration of lymphocytes into the lung, in conjunction with epithelial cell death. Also of note is a relative increase in monocytes and a decrease in the population of T and B cells.

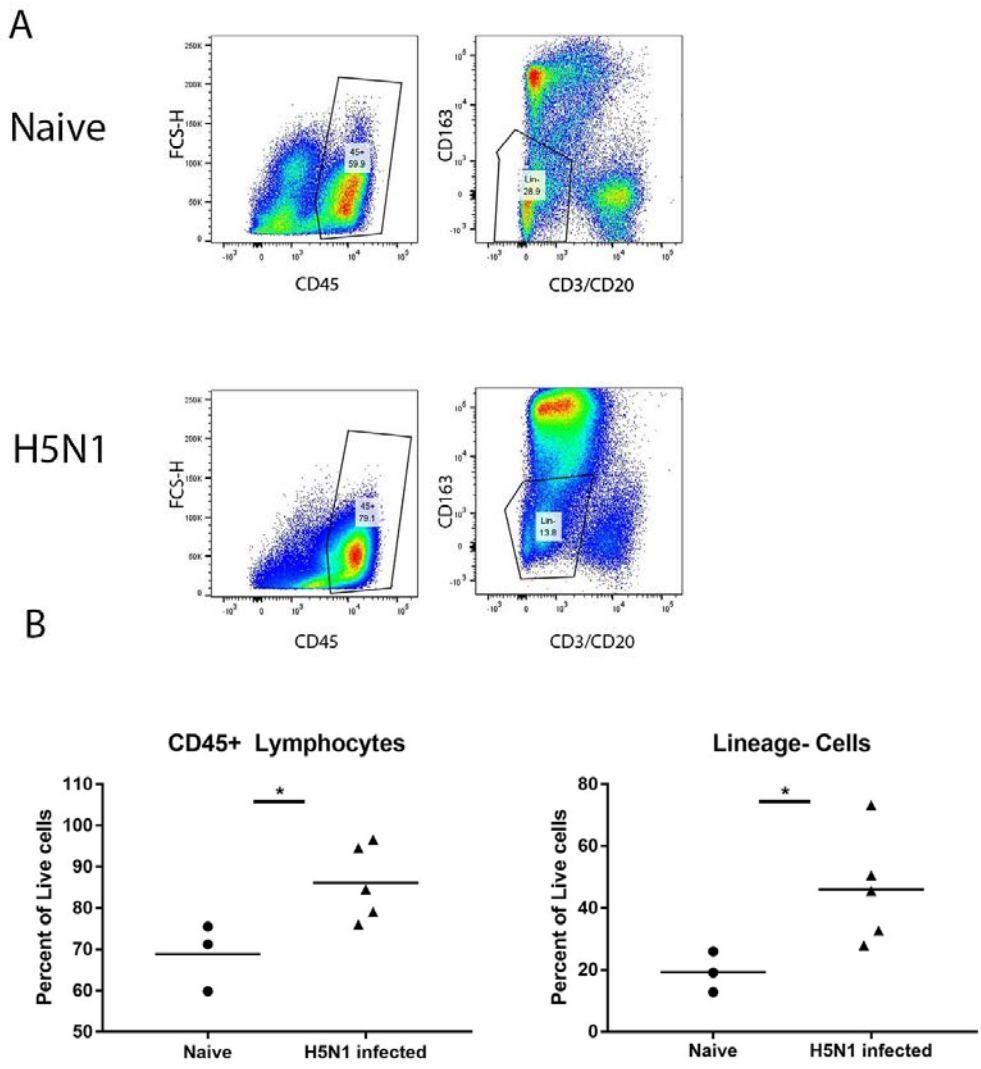

Figure 3. Change in Cell Populations in Lung Tissue

A) Representative images of gating for CD45 and lineage markers in naïve vs infected lung tissue. B) The percent of total live cells in each sample that are CD45+ lymphocytes as opposed to CD45- epithelial cells are significantly increased in the lung tissue of infected macaques. The percent of all live cells that lack lineage markers for T cells, B cells and macrophages are also significantly increased in the infected lung.

4.2.2 NK cells decrease in infected animals

NK cells were identified in lung suspensions of infected compared to naïve control animals. NK cells were defined as CD45+Lineage-CD8+ cells that were either CD16+ or CD56+. In the lungs of cynomolgous macaques, the NK cell population that was found was overwhelmingly CD16+,

with hardly any expression of CD56+ NK cells seen (Figure 4). While CD56 is considered an NK-specific cell marker in humans, in macaques CD56+ NK cells are a relatively minor population[34], however, there are typically a small percentage (<5%) that can be found in lymphoid or other tissues that we did not observe in lung tissue.

Overall, the proportion of Lineage- cells that were identified as CD16+ NK cells were significantly decreased ($p=0.036$) in infected animals compared to control animals. This is consistent with previous studies in vitro which suggest influenza evades NK cell activity by directly infecting them and inducing cell death. Furthermore, the inhibitory molecule NKG2A had significantly decreased expression on the population of CD16+ NK cells that were found in infected animals compared to uninfected controls. NKG2A is typically expressed ubiquitously on macaque NK cells, so this is clearly a departure from normal cellular function, but whether this represents increased activation of NK cells or a functional perturbation is unclear.

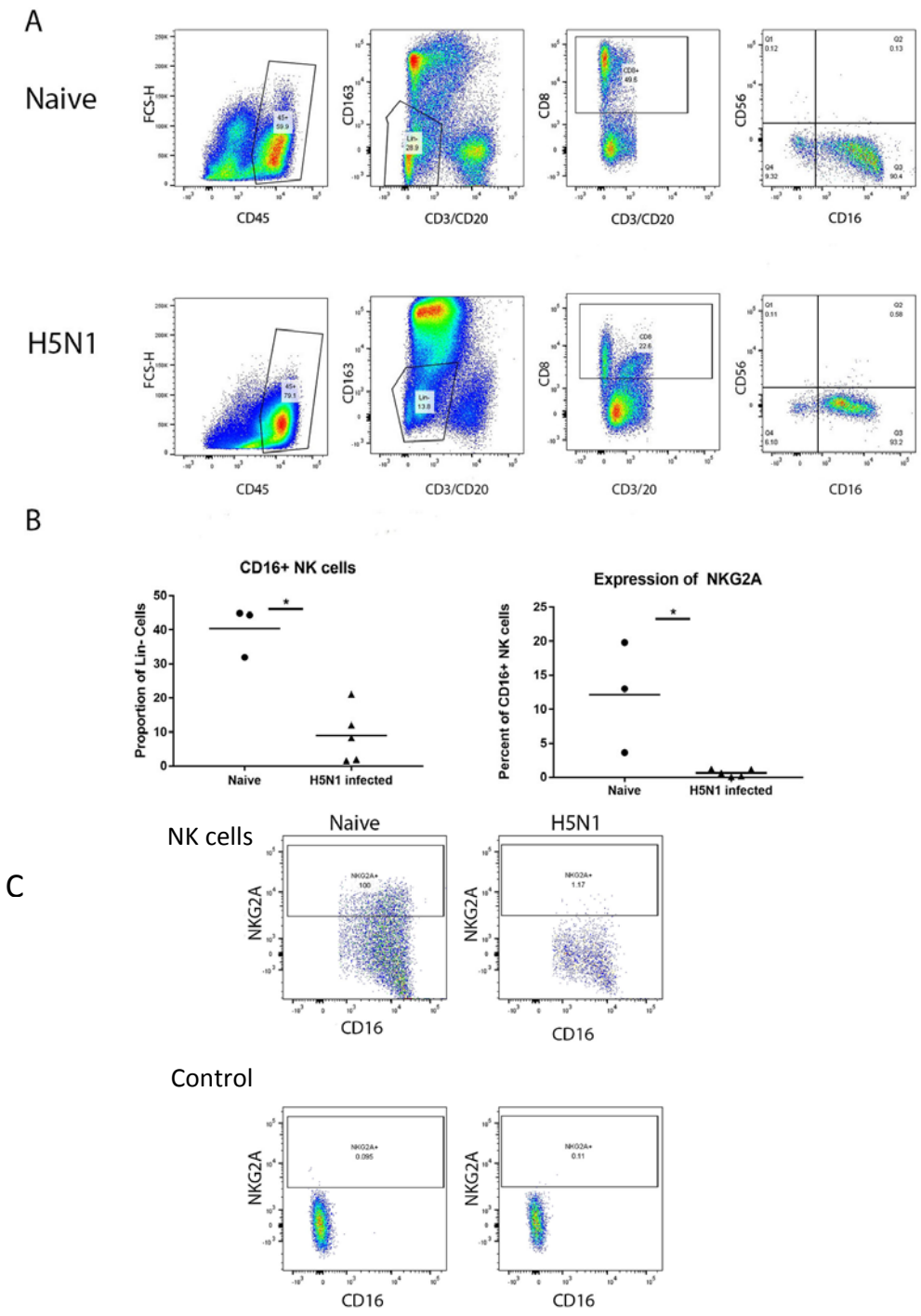

Figure 4. NK Cells Decrease in the Lungs of Infected Animals

A) Representative gating of naïve control animals compared to H5N1 infected lung tissue staining for NK cells. NK cells are defined as CD45+ Lineage-CD8+ cells that are either single or double positive for CD16 or CD56. Nearly all CD45+ Lineage-CD8+ cells in the lung are CD16 single positive NK cells in both infected and uninfected animals. B) The population of 16+ NK cells is significantly decreased in infected macaques as a proportion of all Lineage- cells. Of the remaining CD16+ NK cells in the lung, significantly fewer express the surface receptor NKG2A. C) Top: Representative gating of increase in NKG2A expression on naïve vs H5N1 infected lung NK cells Bottom: Double leave out negative fluorescence control.

4.2.3 Changes in ILC distribution during infection

ILCs were defined in this study as being CD45+, Lineage negative cells (CD3, CD20, and CD163) that expressed CD127 (IL-7Rα). We further explored the expression of CRTH2 and CD117, which are specific markers for Types 2 and 3 ILCs, respectively, as well as the expression of CD90, which is a surface receptor that provides costimulation for proliferation and activation of lymphocytes. Representative gating for various ILC populations is shown in figure 5.

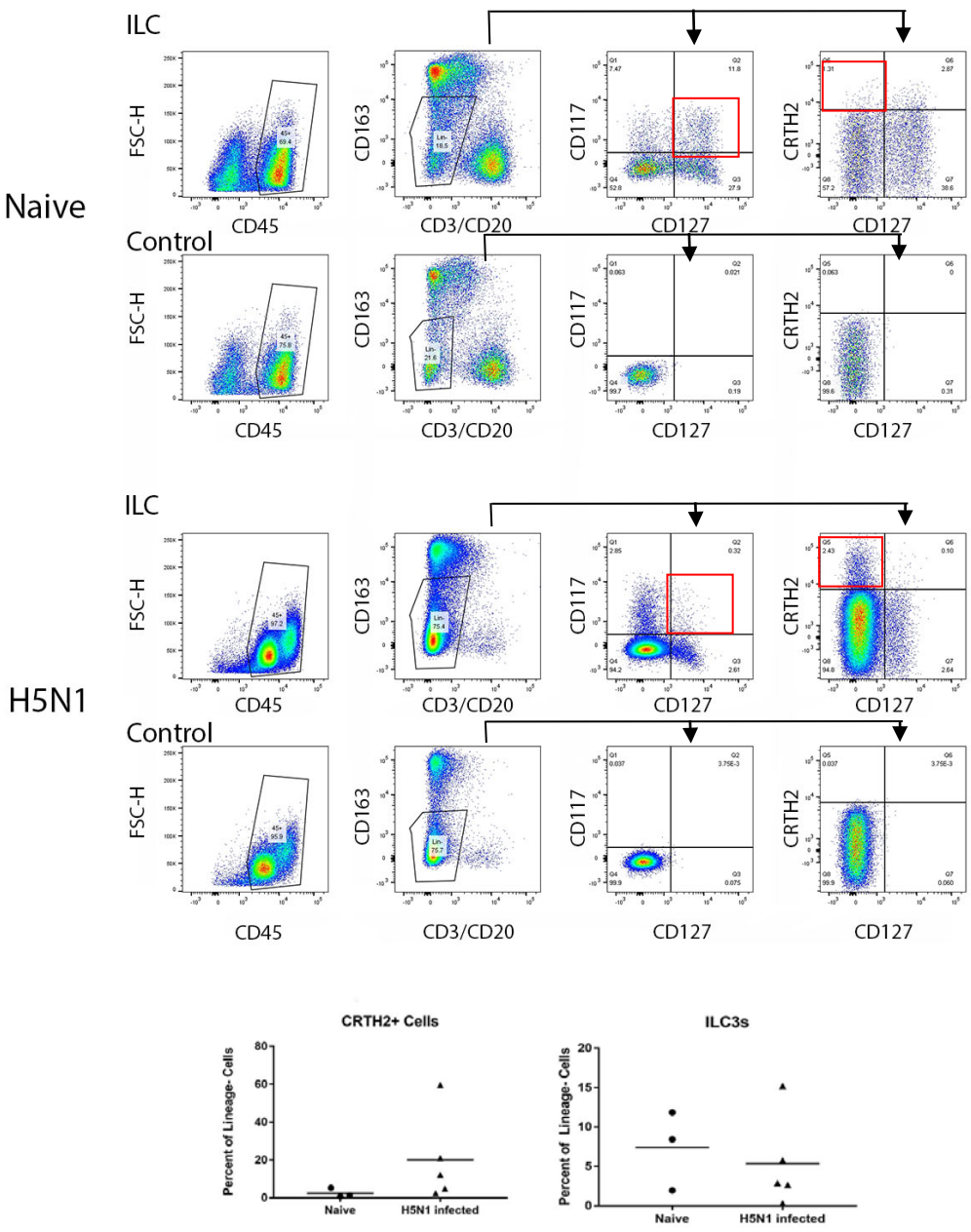

Figure 5. ILC Expression in Infected Compared to Control Lung Tissue

A) Representative gating of CD45+ Lineage- lymphocytes. Top: Populations highlighted in red represent CD117/CD127 double positive ILC3s, and CRTH2+ cells in panels 3 and 4, respectively. Bottom: Double leave out negative fluorescence control B) The mean percentage of lineage negative cells expressing CRTH2+ between naïve and infected animals increases non-significantly. Also shown is the mean percentages of CD117/CD127 double positive ILC3s, which had no significant change.

ILC expression was highly variable even among control animals. Type 3 ILCs were defined as CD127/117 double positive populations, but there was no statistically significant difference between control and infected animal lung. Likewise, there was no population seen that was positive for both CD127 and CRTH2, which would be identified as ILC2.

However, there was a large CRTH2 positive population seen that did not express CD127, and this population did show a trend to increase in infected lungs, but this was not significant between naïve and infected animals ($p=0.14$). While it has been demonstrated that all ILC types express CD127 in human and mouse lung populations, to our knowledge ILC2s have not been extensively studied in a macaque model, and further characterization of the functional capabilities of this CRTH2+ cell population could elucidate whether they are or are not truly ILC2s. A percentage of this single-positive population also expressed the costimulatory surface receptor CD90 (with a mean of 46% of cells, range 3%-90%) (data not shown). CD90 expression is commonly seen on ILC populations, and has been used to define ILC subsets, although there can be varying degrees of expression[22].

The fact that a portion of the CRTH2+ population expresses CD90 could indicate that they are ILCs, but further analysis of function, proving expression of the characteristic transcription factor GATA-3 or IL-13 would indicate that they share additional aspects of the ILC2 phenotype. CRTH2 can be expressed by other cell types, including T cells, however, it is unlikely that this population includes T cells due to the lack of CD3 expression. Another observation when classifying these subsets of ILCs is that a significant proportion of CRTH2+ cells also expressed CD117. While it has been shown in human respiratory tract ILCs that CRTH2 and CD117 define mutually exclusive populations of ILCs[24]

In all, ILCs represented less than 5% of all live cells in the lung in all animals. The mean percentage of CRTH2+ of all live cells in naïve animals was 0.57%, and a mean of 6.15% of all live cells in infected animals. CD117/CD127 double positive ILC3s represented an average of 1.4% of all live cells in naïve animals and 1.8% of in infected animals. Despite the apparent infiltration of CRTH2+ cells in infected animals, there were no significant changes in ILC populations between infected and uninfected animals.

5.0 DISCUSSION AND CONCLUSIONS

As hypothesized, the depletion seen in NK cell populations are consistent with influenza virus infection studies performed in vitro[18, 20]. The depletion of NK cells in the lung during severe respiratory disease provides insight into the pathology of influenza infection. Specifically, it is unknown what the functional mechanism of NK cells is in H5N1 influenza virus infection in humans, and whether they have a significant protective effect as has been demonstrated with other viral infections, or whether their depletion is in fact due to virally mediated cell death, and is a viral evasion mechanism. One hypothesis from previous studies is that the depletion of NK cells during H5N1 infection could be causally linked to the high pathogenicity and case fatality of the disease compared to milder seasonal influenzas[19], although much more work is needed in this arena to demonstrate this experimentally. Future work to identify whether NK cells in H5N1 infected lungs in our animal model are directly infected would provide further evidence that the NK cell depletion shown here is in fact mediated by direct viral infection of NK cells, or whether there are other additional immune evasion mechanisms at play would be useful in understanding the acute response to viral infection.

The identification of ILCs in macaques is a novel finding that has implications for the study of a variety of human diseases. Additional experimentation is necessary to determine whether the populations of cells that I identified as potential CRTH2+ ILC2s express cytokines and transcription factors consistent with ILC2s identified in humans and mice. However, if these

ILC2 and ILC3 subsets that were identified prove to have consistent functional profiles in addition to these suggestive phenotypes, it would be beneficial to the field of ILC research in several ways. For one, ILC2 have not been extensively studied in a macaque model, and so identifying them opens up whole new classifications of diseases in which ILC2s have been shown to be important, including respiratory disease but also diseases such as helminth infection and atopic allergic diseases, to study in a non-human primate model. However, the identification of this subset raises some additional questions about the phenotype of macaque ILC2s, such as why these CRTH2+ cells do not express CD127. Since CD127 is the receptor for IL-7, and IL-7 is thought to be required for ILC development[12], it was previously used as an identifier for all ILC subsets. If further experimentation bears out the hypothesis that these CRTH2+ cells have additional ILC2 characteristics, it would be pertinent to understand why they lack CD127, if this is an altered phenotype seen only due to infection, whether there is something fundamentally different about the development of ILCs in the macaque immune system, and whether this finding changes our approach to studying these cells in non-human primates.

While additional experimentation is needed to prove that the CRTH2 subsets identified phenotypically share other hallmarks of ILC function such as characteristic cytokine production or transcription factors, there is a strong trend suggesting that they are increasing during acute influenza infection. Since infiltration of ILC2s into influenza infected lungs was hypothesized, it seems plausible that the CRTH2+ expressing subset of lineage negative cells are likely to be ILC2s even if they do not express the characteristic surface marker CD127. Despite the fact that these cell types remain minor populations in both healthy and infected lung, they play a critical, as of yet under-studied role in shaping the early innate immune response to infection, and are worth studying in more depth in models of severe disease.

Further work characterizing the function of ILCs in the lung during HPAI H5N1 influenza infection would also be useful to understand the mechanisms underlying protective immunity to influenza. It is apparent that influenza pathology in severe disease is at least partially mediated by the immune response and cytokine storm caused by upregulation of inflammatory cytokines. As such, understanding the innate mechanisms that promote or control cytokine production and regulate early inflammation is critical in understanding the mechanisms of influenza pathology. Understanding which mechanisms of immunity lead to protective responses compared to those which increase the severity of disease could lead to novel treatments. Since breakdown of epithelial barrier integrity is a hallmark characteristic of ARDS, ILC products such as amphiregulin that support tissue repair and epithelial cell proliferation could prove to be an important mechanism for protecting against severe disease, and further research into these mechanisms could provide new avenues to explore towards therapeutic strategies for severe respiratory distress. If the role of ILCs were better understood in severe disease, this could lead to the development of new strategies to combat avian influenza outbreaks in humans.

The development of novel therapeutic strategies is particularly important when discussing the public health impact of avian influenza. Because H5N1 causes rapid onset disease with severe morbidity and mortality, treatment options are currently limited. If a crossover event or viral reassortment ever did lead to a strain of H5N1 virus that was easily transmissible between humans, there would be an incredible need for additional treatments. Currently, influenza preparedness centers largely around prevention of infection through vaccination[35]. While this strategy works well at preventing and containing infection, the potential for the sudden emergence of new strains for which vaccines are not yet developed (or are developed but we

have inadequate quantities to vaccinate large populations at once), leaves major gaps in global influenza pandemic preparedness. While there are several licensed H5 vaccines for poultry, and candidate vaccines exist for human use, there are still several drawbacks to this strategy of preparedness. For one, these vaccines are largely untested, and while their immunogenicity and efficacy can be tested in small-scale clinical trials, without an outbreak of cases it is impossible to completely predict their actual effectiveness at preventing disease or mortality. Antiviral drugs such as oseltamivir are another strategy to prepare against the potential of an influenza pandemic[35]. While modeling suggests that these drugs could be effective at preventing the large-scale spread of novel influenza viruses, there are also concerns about antiviral resistance and questions about the efficacy of antivirals as post-exposure prophylactics[36]. As such, the development of therapeutics that are particularly effective against avian influenza could be beneficial in terms of public health preparedness and pandemic response. Even more broadly, immunotherapeutics against severe respiratory inflammation could be beneficial for a number of human diseases, including a variety of acute viral infections that cause ARDS.

5.1.1 Future aims

Having a non-human primate model in which to study symptomatic H5N1 avian influenza is an incredibly useful tool to understanding the correlates of disease and immunity that cannot be studied in humans in the absence of a large outbreak. Additionally, having identified a potential population of cells that resemble human ILCs in the lung, the next step would be to examine their cytokine production when stimulated, and see if this also corresponds to human ILC populations. Further work with ILCs in the respiratory tract could lead to better understanding of not only the response to acute viral respiratory infections, but to a wide variety of inflammatory

and chronic lung diseases in which ILCs may play a vital part. Likewise, an in vivo NK cell model could contribute to more basic research about innate response to influenza viral infection, and understanding the mechanism of innate response is a key aspect of being able to control acute viral infections. Being able to study these cells in vivo in nonhuman primates could prove an important tool for understanding their role in the immune system.

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