2019

THE ROLE OF CD8 T CELL IMMUNODOMINANCE AND REGULATORY T CELLS IN NEONATAL IMMUNITY TO INFLUENZA VIRUS

Luke Heil
University of Kentucky, luheil123@gmail.com
Digital Object Identifier: https://doi.org/10.13023/etd.2019.403

STUDENT AGREEMENT:

I represent that my thesis or dissertation and abstract are my original work. Proper attribution has been given to all outside sources. I understand that I am solely responsible for obtaining any needed copyright permissions. I have obtained needed written permission statement(s) from the owner(s) of each third-party copyrighted matter to be included in my work, allowing electronic distribution (if such use is not permitted by the fair use doctrine) which will be submitted to UKnowledge as Additional File.

I hereby grant to The University of Kentucky and its agents the irrevocable, non-exclusive, and royalty-free license to archive and make accessible my work in whole or in part in all forms of media, now or hereafter known. I agree that the document mentioned above may be made available immediately for worldwide access unless an embargo applies.

I retain all other ownership rights to the copyright of my work. I also retain the right to use in future works (such as articles or books) all or part of my work. I understand that I am free to register the copyright to my work.

REVIEW, APPROVAL AND ACCEPTANCE

The document mentioned above has been reviewed and accepted by the student's advisor, on behalf of the advisory committee, and by the Director of Graduate Studies (DGS), on behalf of the program; we verify that this is the final, approved version of the student's thesis including all changes required by the advisory committee. The undersigned agree to abide by the statements above.

Luke Heil, Student

Dr. Beth A. Garvy, Major Professor

Dr. Carol Pickett, Director of Graduate Studies

THE ROLE OF CD8 T CELL IMMUNODOMINANCE AND REGULATORY T CELLS IN NEONATAL IMMUNITY TO INFLUENZA VIRUS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine at the University of Kentucky

By

Luke Harrison Heil

Lexington, Kentucky

Director: Dr. Beth A Garvy, Professor of Microbiology, Immunology, and Molecular Genetics
Lexington, Kentucky

2019

ABSTRACT OF DISSERTATION

THE ROLE OF CD8 T CELL IMMUNODOMINANCE AND REGULATORY T CELLS IN NEONATAL IMMUNITY TO INFLUENZA VIRUS

Neonates are more susceptible to influenza virus infection than adults, resulting in increased morbidity and mortality as well as delayed clearance of the virus. Efforts to improve influenza infection outcomes in neonates typically center on prevention, although current vaccines fall short of complete protection and can only be administered in humans after 6 months of life. We propose that as the neonatal immune system responds differently than the adult immune system, interventions that are efficacious or tolerable in adults cannot be guaranteed to perform the same in neonates. T cell vaccines that target conserved influenza virus epitopes have been proposed for conferring protection to multiple influenza virus strains, but if T cell vaccines will be used in infants and adults, neonates must be able to respond to the same T cell antigens as adults. Mouse pups responded to influenza virus peptide $PA_{224-233}$ but not $NP_{366-374}$ during influenza virus infection in contrast to the codominant adult response. Mice infected as pups also generated diminished T cell memory compared to mice infected as adults and displayed skewed immunodominance during secondary infection. Adult bone marrow derived dendritic cells (BMDCs) improved viral clearance when loaded with influenza virus and promoted $NP_{366-374}$-specific $CD8^+$ T cell responses in infected pups. BMDC peptide vaccination could stimulate $PA_{224-233}$-specific but not $NP_{366-374}$-specific $CD8^+$ T cell responses in pups, but, $PA_{224-233}$ vaccination offered no protection to pups during lethal infection. These data suggest that altered immunodominance must be considered when stimulating $CD8^+$ T cell responses in adults and neonates.

Immaturity and active suppression of immune responses are both factors in neonatal vulnerability to disease. Specifically, active suppression of neonatal immunity by regulatory T cells (Tregs) has been proposed as a driving factor in diminished neonatal immunity, but removing these cells can compromise viral defense or increase deleterious inflammation. Mice that lacked Tregs displayed compromised anti-influenza antibody responses and decreased lymph node responses during influenza virus infection. A high proportion of pup Tregs also expressed Gata3. Transgenic pups with a Treg specific Gata3 knockout displayed an increase in Tbet expression in both conventional and regulatory T cells and an increase in IFNγ producing $CD4^+$ T cells in the lungs during infection. These data suggest that Tregs are required for effective humoral responses to influenza virus and that Gata3 expression influences Treg suppressive function in neonates.

KEYWORDS: Neonates, Influenza virus, T cells, Lung, Immunodominance, vaccine.

Luke Heil

6/4/2019

THE ROLE OF CD8 T CELL IMMUNODOMINANCE AND REGULATORY T CELLS IN NEONATAL IMMUNITY TO INFLUENZA VIRUS

By
Luke Harrison Heil

Beth Garvy
Director of Dissertation

Carol Pickett
Director of Graduate Studies

6/4/2019
Date

To Kate and everyone else in my life who now know far more than they ever wanted to about immunology.

ACKNOWLEDGMENTS

I would like to thank my mentor, Beth Garvy, for her time, patience, and insight throughout my time in her lab. I want to thank Jerry Woodward, Becky Dutch, Sarah D'Orazio and outside examiner Thomas Chambers for challenging me, answering my questions and serving as my committee. I am also extremely grateful to Melissa Hollifield for providing endless technical assistance as well as being my friend. During this project, I continued the work of Janet Louise Lines and Samantha Jewell, and I am thankful for all the data, protocols and influenza virus they left me. I could not have done this project without their hard work. Finally, I want to thank the NIH tetramer core facility for providing MHC-peptide tetramer reagents.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1. OVERALL INTRODUCTION

1.1 Introduction

Influenza virus is a single stranded enveloped orthomyxovirus responsible for mild to severe recurrent, seasonal respiratory disease in humans worldwide (Cottey et al., 2001; Oshansky et al., 2012). Disease burden varies year to year, but the CDC estimates that influenza infections were responsible, in the United States, for 9.3- 49 million symptomatic illnesses, 140,000- 960,000 hospitalizations and 12,000- 79,000 deaths each year since 2010. This significant burden remains despite vaccination efforts (Antonova et al., 2012; Nelson et al., 2014; Reed et al., 2015). The elderly and the young are particularly impacted due to less robust vaccine responses and less effective anti-viral immune responses, and special effort must be dedicated to protecting these populations (Neuzil et al., 2002).

Both vaccines and natural infections fail to induce long-lasting protective immunity to influenza virus due to the virus's high mutation rate and host responses that preferentially target epitopes of the virus that are prone to mutation (Sridhar et al., 2015; Thommes et al., 2017). Seasonal influenza vaccines offer some protection by predicting the common Influenza A and B strains that will be prevalent in a specific year and generating antibody responses against those viruses (Grohskopf et al., 2018). Vaccine effectiveness hovers around roughly 40-60% effectiveness each year, but some seasons have much poorer results such as the 2014-15 season (19%) (Jackson et al., 2017) and the 2004-05 season (10%) (Belongia et al., 2009).

Multiple efforts are underway to address the limitations of current influenza vaccination strategies, such as using subunit T cell antigens or broadly neutralizing antibodies to generate memory that is not susceptible to influenza virus's usual rapid immune evasion (Estrada et al., 2019). Both of these strategies attempt to bypass the limitations of reactions generated by natural infections and conventional vaccination methods to protect against a wide range of influenza virus strains (Gottlieb et al., 2014; Lee et al., 2019).

Influenza virus is the leading cause of hospitalizations and deaths for vaccine preventable illnesses in children (Reed et al., 2015). Immune responses are different in children than in adults, resulting in both decreased protection from natural infections as well as diminished responses to vaccines (Basha et al., 2014). Children under six months of age cannot be vaccinated against influenza virus, and children under five years of age require two injections of the vaccines instead of the one adults and older children require (Grohskopf et al., 2018). Any novel vaccination strategy must be evaluated in the context of early life immune responses.

The purpose of this dissertation is to investigate neonatal responses to influenza virus in two ways. First, to test the hypothesis that neonates must be vaccinated differently than adults when considering T cell vaccines, and second, to test the hypothesis that Tregs perform a vital anti-viral role in influenza virus clearance in neonates.

1.2 Lung

Like all mucosal surfaces, the lung requires specialized immune functions to protect the host from the consequences of exposing a region of the body to external microorganisms without the barrier of skin to protect it. The respiratory tract is generally divided into the upper and lower respiratory tracts with the upper tract comprising the nose, nasal cavities, sinuses, pharynx, and the upper part of the larynx and the lower tract comprising the lower larynx, trachea, bronchi, bronchioles and most importantly the alveoli where gas exchange occurs (Hsia et al., 2016). Most contact with microorganisms occurs in the upper respiratory tract, but bacteria can also be found in the lower alveoli under normal conditions (Hilty et al., 2010).

The lungs contain colossal surface area and extensive vascularization to facilitate gas exchange, and perturbations to the steady state conditions can easily cause damage or blockages that could be devastating. Immune responses in the lungs must balance potency with tolerance to avoid pathology, particularly in neonates as infants are still undergoing lung development after birth and cannot afford to lose any of their already limited respiratory capacity (Harbeson, Ben-Othman, et al., 2018; Harbeson, Francis, et al., 2018; Lee et al., 2010). Additionally it is well recognized now that infections early in life can have lasting consequences for lung function, particularly in the development of asthma (Merkus, 2003). Early infections with Respiratory Syncytial Virus (RSV) (Feldman et al., 2015) and human rhinovirus (HRV) (Miller et al., 2009) have been closely linked to later development of asthma.

The immune system in the upper respiratory tract functions much like it does in other mucosal surfaces, such as the gut, with extensive immunosurveillance by dendritic cells and secretion of IgA. The conducting airways have an epithelial layer containing some dendritic cells that extend processes into the airway to sample airborne antigens (Geurts van Kessel et al., 2008; Neyt et al., 2013). The lamina propria contains other immune cells such as macrophages, T cells and plasma cells that secrete IgA and maintain surveillance for potential pathogens (Habibi et al., 2015; Lanka et al., 2016).

The alveoli have a few additional components due to the unique challenges and needs of the environment. Alveolar macrophages live within the airspace and function both as cleanup for debris that accumulates in alveolar spaces as well as sentinels for infection (Hussell et al., 2014). The parenchyma surrounding the alveoli contains dendritic cells, T cells and other immune cells that are poised to quickly intervene if a pathogen is detected. During an infection, many cells will move into the alveolar spaces including monocyte-derived macrophages, neutrophils and both $CD4^+$ and $CD8^+$ T cells (Strickland et al., 1996). This appears to be important to clearance of many pathogens, but an influx of cells and fluid often compromises local alveolar function until the inflammation is resolved (Das et al., 2016; Lines et al., 2010).

The neonatal lung in humans and mice is immature at birth with thick walled alveoli resembling buds from the ends of bronchioles. These proto-alveoli then expand and thin to form mature adult alveoli (Hsia et al., 2016). In humans, alveolar development occurs both before and after birth and is complete by around eight years of life (Langston, 1983). In mice, most of this development occurs in the first two weeks of life (Foster et al., 2006). This process is mediated by several growth factors including

constitutive expression of transforming growth factor beta (TGFβ). TGFβ is required for proper lung development, but it also may play a role in establishing the immunosuppressive environment of the neonatal lungs (Alejandre-Alcazar et al., 2008; Nakanishi et al., 2007).

Neonates consistently demonstrate reduced immune function across a wide variety of infections, although they can clear low doses of pathogens (Basha et al., 2014). This observed deficiency has been theorized to be necessary to accommodate the delicate demands of the neonatal lungs (Harbeson, Francis, et al., 2018; Medzhitov et al., 2012). Early in life, the neonatal immune system must learn to tolerate the vast array of novel, innocuous antigens that every human encounters in daily life without succumbing to pathogens. This balance plays out in the unique functions of neonatal immunity, although the cost is undeniable. Neonates are more susceptible to infections, and although efforts are being made to improve outcomes for infected infants, care must be taken to maintain the balance. Enhanced clearance does little good if extensive pathology is the byproduct (Delgado et al., 2009).

1.3 Influenza virus

Influenza viruses comprise four of the seven genera of *Orthomyxoviridae*, although most attention in human health is given to influenza A and B. Influenza A is the subject of this study and the focus of this section. Influenza A is an enveloped, single-stranded RNA virus with a segmented genome. Influenza A has eight RNA gene segments that encode ten to fourteen proteins depending on the strain (Nakatsu et al., 2016). The most common list of these proteins includes the surface proteins

hemagglutinin (HA) and neuraminidase (NA), RNA polymerase subunits PB1, PB2 and PA, the nucleoprotein NP, the matrix proteins M1 and M2, two non-structural proteins NS1 and NEP, and an apoptosis inducing protein PB1-F2 (Edinger et al., 2014).

Influenza viruses are classified by their HA and NA serotype. H1N1 and H3N2 are the influenza A types responsible for seasonal influenza infections (Grohskopf et al., 2018). H5N1 and less commonly H7N9 are avian influenza strains that cause severe but uncommon disease in humans (Brankston et al., 2007). Genetic drift occurs within these serotypes so that one H1N1 strain is often not identical to another, but these broad categorizations are helpful in delineating general groups of influenza viruses (Stellrecht et al., 2017) (Figure 1.1).

Hemagglutinin is responsible for viral attachment, entry and uncoating. It has a highly conserved stalk region called HA1 and a more globular head region called HA2 that binds to sialic acids on target cells (Krammer et al., 2019). This binding is highly specific with human influenza virus strains binding to α(2,6) sialic acids and avian viruses binding to α(2,3) sialic acids (Edinger et al., 2014). Pigs have both α(2,3) and α(2,6) sialic acids allowing human and avian viruses to co-infect pig cells and to mix and re-assort into dangerous new strains. α(2,6) sialic acids are primarily expressed on the surface of ciliated epithelial cells of the upper respiratory tract in humans, and as a result, almost all infected cells in humans are respiratory epithelial cells (Ibricevic et al., 2006). Once the virus is endocytosed, the pH within the endosome drops causing a conformational change in the HA protein that allows the viral membrane to fuse with the endosome. The viral contents are then released into the host cytoplasm (Banerjee et al., 2014; Edinger et al., 2014).

The viral gene segments each comprise a viral RNA surrounded by multiple NP proteins and a polymerase complex of PA, PB1 and PB2 (Lakdawala et al., 2016). These segments traffic to the host cell nucleus where viral replication begins (Oymans et al., 2018). NS1 inhibits host cell protein production to conserve resources for viral replication (Diebold et al., 2003). The ER processes HA, NA and M2 before they are glycosylated in the golgi apparatus and move to the apical side of the cell membrane (Edinger et al., 2014). There, new viruses are assembled and loaded with all eight gene segments (Takizawa et al., 2016). Evidence suggests that this process is not random and that the vast majority of assembled viruses contain the complete set of viral gene segments (Nakatsu et al., 2016; Samji, 2009).

As influenza is an enveloped virus, complete virions bud from infected cells. The HA on the virus's surface binds to sialic acids on the host cell, which could prevent spread of the virus to new cells, but NA cleaves the sialic acids and allows the virus to escape. (Kosik et al., 2019) (Figure 1.2). Influenza viruses with neutralized NA accumulate on the host cell membrane, preventing effective spread (Palese et al., 1974).

1.4 Influenza disease

Influenza is typically acquired through contact with infected materials from an infected person. Respiratory secretions of infected humans contain large quantities of the virus and this can be spread via airborne droplets or surfaces contaminated by droplets (Brankston et al., 2007). These droplets are typically created by sneezing or coughing, although there is evidence that simply breathing can spread them as well (Ortigoza et al.,

2018). The droplets must be breathed in to come in contact with the respiratory epithelial cells required for viral infection of host tissues (Ibricevic et al., 2006).

The incubation period for influenza virus in humans is one to four days at which point symptomatic disease manifests. Uncomplicated influenza begins with onset of fever, myalgia, malaise and headache that persists for several days (Nicholson, 1992). Viral shedding occurs before symptomatic disease and continues for an average of 6.4 days (Carrat et al., 2008; Lee et al., 2009). Degree of severity of symptoms correlates with magnitude of viral shedding (Lau et al., 2010; Peteranderl et al., 2016). After resolution of symptoms, some patients experience persistent fatigue and weakness (Nicholson, 1992).

In mice, the disease course is very similar although there are recognized differences in murine infections verses human infections (Bouvier et al., 2010; Thangavel et al., 2014). (See mouse model of influenza, page 9). After intranasal inoculation with A/Puerto Rico/8/1934 influenza virus (PR8), adult mice experience an infection that peaks around day seven of infection before the virus is rapidly cleared by day 10 of infection. Neonates experience a longer course with clearance around day 14 of infection (Lines et al., 2010). Neither C57Bl/6 adults nor pups transmit the virus although a few other mouse strains can transmit certain influenza viruses as pups (Ortigoza et al., 2018). Morbidity is assessed by weight loss in adults which can be profound. Pups typically will not lose any weight during infections, but they will stop gaining weight as an indication of morbidity (Lines et al., 2010).

1.5 Mouse model of influenza

Influenza virus infection in immunocompetent humans is an uncomplicated, predominantly upper respiratory infection that may be transmitted between hosts for 8 to 9 days after infection (Brankston et al., 2007). Several animal systems exist to model this infection including mice, ferrets, and non-human primates (Bouvier et al., 2010; Thangavel et al., 2014). Ferrets and non-human primates most closely model the disease course of humans as they primarily develop upper respiratory infections, may be infected with human isolates of influenza virus without adaptation, and can transmit the virus between hosts through coughing, but they are expensive to maintain and few reagents exist for these species (Smith et al., 1933; Thangavel et al., 2014). Mice are not a natural host for influenza, but they may be infected with mouse-adapted strains of influenza virus (Thangavel et al., 2014). They develop lower respiratory infections and do not typically transmit the virus to other mice (Ortigoza et al., 2018). For these reasons, mice are often viewed as a poor model of influenza virus infection in humans, but the advantages of mice as a model organism are widely recognized and may justify the use of mice in answering many questions regarding immunity to influenza virus. Mice are readily available, cheap, and easy to breed. Many reagents exist for use in the species and many genetically modified mouse strains already exist. The use of mouse models has already allowed for the discovery of much of the current knowledge of influenza virus infection: knowledge that would have been much harder to discover in more challenging animal models. I believe these considerations justify the use of mouse models in influenza research, although care must be taken to interpret results in light of the limitations of this artificial infection system.

The second consideration for the use of a mouse model in these studies is the suitability of mice for studying neonatal immunology and infection. The lungs of mouse and human infants differ in early life with humans undergoing significant alveolarization in the third trimester while mice alveolize predominantly shortly before birth and through the first two weeks of life (Alejandre-Alcazar et al., 2008; Langston, 1983). Additionally, there are lymphocyte trafficking differences, with mice populating their peripheral tissues with lymphocytes after birth, while humans typically do so during gestation. This means that immune cell populations in the lung may look significantly different between mice and humans until mice finish populating their lungs. Some studies have suggested that a 7 day old mouse immune system resembles the immune system of a human infant (Adkins, 2005).

Interestingly, mice may be a better model for neonatal influenza infection than for adult influenza virus infection. While humans typically develop upper respiratory infections when exposed to influenza virus, human infants can develop severe interstitial pneumonia and lower respiratory infections (Guarner et al., 2006). In this way, the interstitial pneumonia and lower respiratory infections displayed by neonatal mice match human illness fairly well. Of course, the underlying reality is that influenza studies in human infants are extremely challenging for ethical and practical reasons. Mice represent a system to glean answers about neonatal immunobiology that would be impossible to otherwise obtain. For this reason, mice are a reasonable model for studying influenza virus infection in neonates.

1.6 Typical viral immunity in adults

The response to viral pathogens follows a general pattern in humans and mice. Sterilizing viral immunity will be considered here, as influenza is not typically considered a chronic infection. First, the virus is detected, and early innate immune functions such as NK cells and type I interferons keep the virus from undergoing unchecked replication before the adaptive immune system can reach full functionality (Fensterl et al., 2015; Schoggins et al., 2011). Once adaptive immune cells are differentiated and expanded, the virus is sterilized and the expanded populations of cells contract until memory populations of T and B cells remain (Chen, Liu, et al., 2018). Vaccination efforts are mainly interested in the adaptive immune response, especially neutralizing antibody responses, but the entire process is worth considering when evaluating an adult or neonatal immune response to a specific pathogen.

There are several methods for a host to detect a viral infection. These include receptors such as Toll-like receptors (TLRs), nucleotide oligomerization and binding domain (NOD)–like receptors (NLRs), C–type lectin receptors (CLRs), intracellular retinoic acid inducible gene–I (RIG-I)-like receptors (RLRs), and the Pyrin and HIN domain (PYHIN) receptors (Braciale et al., 2013). These receptors recognize pathogen associated molecular patterns (PAMPs) such as viral nucleic acids or danger-associated molecular patterns (DAMPs) such as ATP or other components released from necrotic/apoptotic cells (Yoo et al., 2013). Once a PRR binds to a PAMP, signaling cascades are activated leading to the production of cytokines.

Type I interferons are critical mediators of innate immunity in the early stages of viral defense. IFN-α and IFN-β are the major members of this group, although there are other members (Schoggins et al., 2011). Type III interferons also play a role in viral defense and include IFN-λ. These are much more recently described than type I interferons and are structurally distinct from them, but they perform similar functions, namely inhibition of viral replication (Durbin et al., 2013; Mordstein et al., 2008). Animals that lack type I interferons are exquisitely sensitive to viral infections (Clay et al., 2014). In fact, C57BL/6 mice lack MX1, a gene responsible for some of the functional response to interferon signaling, and as a result, these mice, as well as may other lab mouse strains that lack MX1, are more susceptible to influenza virus infection than mice with an intact MX1 (Ciancanelli et al., 2016; Shin et al., 2015).

Dendritic cells are one of the bridges between innate and adaptive immunity, as they are potent activators of T cells and assist in the activation of B cells as well as detecting pathogens and producing cytokines (Neyt et al., 2013). Dendritic cells are not a homogeneous population, but in general, they express a range of PRRs and are able to phagocytose pathogens. This allows them to recognize pathogens and internalize them for processing and presentation on MHC class I and MHC class II to T cells (Dinter et al., 2014). After encountering a virus in the periphery, dendritic cells migrate to secondary lymphoid organs such as lymph nodes to encounter naïve T and B cells (Geurts van Kessel et al., 2008). They express high levels of co-stimulatory molecules like CD40 and CD80/86, making them the most potent antigen presenting cells (APC) for stimulating T cell responses to a viral infection (Larsson et al., 2000).

Naïve pathogen-specific $CD8^+$ and $CD4^+$ T cells encounter dendritic cells and other APCs in the secondary lymphoid tissue and become activated (Ho et al., 2011). Activated T cells then migrate to the site of infection or remain in the lymphoid tissue to help activate B cells and promote the production of high quality antibodies for viral neutralization (Inoue et al., 2018; Nolz, 2015). In the infected tissue, $CD4^+$ and $CD8^+$ T cell cytokine production helps recruit effector cells to the site of infection and $CD8^+$ T cell cytotoxicity kills virally infected cells, removing sites of viral replication (Chen, Liu, et al., 2018). Although innate mechanisms may be able to protect against some viruses or infectious doses, T cell activity is usually required for sterilizing immunity to a viral pathogen (Epstein et al., 1998; Graham et al., 1991). In fact, many viruses downregulate MHC class I in an attempt to evade $CD8^+$ T cell recognition. The absence of MHC class I helps activate natural killer cells which can then kill infected cells in a non-antigen dependent manner (Mahmoud et al., 2016).

B cells are activated by antigens transported to the secondary lymphoid tissue and begin to proliferate forming germinal centers with the help of T follicular helper cells (Tfh) (Xu, Ziani, et al., 2018). During this time they undergo selection and can switch antibody class in a process known as affinity maturation. The goal of this process is to generate plasma cells that can create vast quantities of high-quality antibodies for viral neutralization and clearance (Inoue et al., 2018).

After the pathogen is cleared, contraction of adaptive immune cell populations occurs with some T and B cells differentiating into a memory phenotype. Long-lived plasma cells may migrate to the bone marrow for maintenance. Some memory T cells remain in the tissue, while others become circulating memory cells (Nguyen et al., 1999).

1.6.1 General viral immunity in neonates

The neonatal immune response to viruses follows the same steps as the adult response, but differences in both the timing and magnitude of the various components of that response result in a general increase in susceptibility to infection, morbidity, and mortality in this population.

TLR expression is similar between neonates and adults, but neonates display much lower type I interferon production than adults after TLR stimulation (De Wit et al., 2004), hampering initial viral defense. Neonates have dendritic cells that can take up virus and present it to the adaptive immune system, but these cells are both less abundant in the periphery and less active than their adult counterparts resulting in delays throughout the reaction (Gold et al., 2007; Ruckwardt et al., 2014). Activation and proliferation of T and B cells in the secondary lymphoid tissues happens later in neonates resulting in delayed migration of adaptive immune cells to infected tissues (Lines et al., 2010). Once there, neonatal T and B cells display reduced functionality including lower antibody production, reduced cytotoxicity and reduced and skewed cytokine production (Adkins et al., 1998; Fernandez et al., 2008b; Holbrook et al., 2015).

These factors all contribute to the overall outcomes of neonatal infections. Low infectious doses of virus are not adequately controlled by low levels of type I interferon. This allows virus to expand, leading to a serious infection more easily than in adults. After this, the delayed T cell migration and diminished quality and quantity of anti-viral antibodies allows further expansion of the virus and delays clearance, extending disease course and contributing to morbidity and mortality (Basha et al., 2014).

Much less work has been done characterizing neonatal immunity compared to the extensive study of adult immunity. Studies with neonates are challenging to perform, and as a result, basic information about neonatal responses to many pathogens is currently lacking.

1.6.2 Respiratory syncytial virus and the study of viral immunity

RSV is a ubiquitous infection in human neonates. All humans are seropositive for RSV infections by 2 years of age (Miller et al., 2013). As such, RSV is frequently studied to understand neonatal viral immunity (Ruckwardt et al., 2014; Schmidt et al., 2018). These studies can give insights into certain other viral infections like influenza virus as RSV is also a negative-sense, single-stranded, enveloped RNA virus (Ascough et al., 2018).

1.7 $CD8^+$ T cells

$CD8^+$ T cells are required for sterilizing immunity to viral infections because they kill virally infected cells thus disrupting viral replication. Antigen naïve $CD8^+$ T cells express chemokine receptors CD62L and CCR7 which helps them home to secondary lymphoid tissues (Ge et al., 2012; Teijeira et al., 2017). Their T cell receptor (TCR) then can bind to a specific peptide (usually 9-10aa in length) in the context of MHC class I. T cells specific for viral peptides encounter dendritic cells presenting those peptides on

MHC class I alongside costimulatory molecules like CD80/86. T cell activation occurs after engagement of the TCR with MHC class I/ peptide and CD80/86 to CD28 (Veiga-Fernandes et al., 2000) (Figure 1.3). Cytokines such as type I IFN and IL-12 are made by antigen-presenting cells and provide a third signal for T cell activation (Galani et al., 2017).

Activated $CD8^+$ T cells downregulate CCR7 and CD62L to allow them to leave the lymph node and barring them from reentering secondary lymphoid tissue. They then upregulate CXCR3, CCR4, CCR9, CCR6, and CCR10: chemokine receptors that promote trafficking to sites of inflammation. They also upregulate CD44 giving activated and memory T cells a phenotype of $CD44^{hi}$ $CD62L^{lo}$ when analyzed by flow cytometry (Lines et al., 2010). Inflamed tissue expresses selectins, ICAMS and VCAMS, to allow cell adherence and homing and also produce chemokines that bind to $CD8^+$ T cells and upregulate expression of integrin on the T cell surface. These bind to selectins and arrest $CD8^+$ T cells at inflamed sites and allow them to invade the tissue (Teijeira et al., 2017).

The classical effector function of $CD8^+$ T cells is cytotoxicity, but they also produce copious amounts of IFNγ and TNFα. Activated $CD8^+$ T cells bind to MHC class I/ peptide complexes on infected cells to direct cytotoxicity. $CD8^+$ T cells induce apoptosis in infected target cells through several mechanisms. They can activate Fas/FasL mediated killing as well as expressing TRAIL (Doherty et al., 1997). TRAIL interacts with the receptors D4/D5 on target cells to induce killing (Shrestha et al., 2012). $CD8^+$ T cells produce perforin and granzymes for direct killing. Perforin makes pores in target cells to allow granzymes into the cytoplasm. Granzymes then induce apoptosis

(Zhao et al., 2018). Immunological synapses form between cytotoxic T cells and target cells ensuring that delivery of granzymes and other toxins is highly specific. This synapse is formed from rings of receptors and cytoskeletal elements (Murugesan et al., 2016).

Even with the highly specific nature of $CD8^+$ killing, these cells are linked to immunopathology in infections. Mice deficient in IFNγ or mice that receive IFNγ deficient $CD8^+$ memory cells lose less weight during sublethal infections, suggesting that these cells play a central role in infection-associated morbidity (Ostler et al., 2002; Ruckwardt et al., 2010). Ideally, $CD8^+$ T cell activity will be just enough to enable viral clearance without causing unacceptable damage, but different infections and treatments can skew this balance. For example, increasing the magnitude of $CD8^+$ T cell responses to a virus can improve viral clearance while also inducing lethal immunopathology. $CD8^+$ T cells are powerful effectors and care must be taken when manipulating their function.

Formation and reactivation of $CD8^+$ memory is essential to the long term health of the host, and may provide an avenue of attack for vaccinating against pathogens that can evade antibody mediated immunity (Xiang et al., 2015). After an acute reaction, a subset of $CD8^+$ T cells will differentiate into memory cells. Some of these will remain in the tissue as tissue-resident memory cells (Trm) and others will return to circulation as central memory cells (Tcm) (Ballesteros-Tato et al., 2014; Connors et al., 2018). The timing of expansion and contraction of CD8+ T cell populations can vary greatly between infections, but for murine influenzavirus infections, the peak of the $CD8^+$ T cell response occurs around day 7 of infection in adults and day 10 of infection in pups (Lines et al.,

2010). Although much attention is given to antibody mediated protection from influenza infections, memory $CD8^+$ T cells provide substantial protection against subsequent infections (Wu et al., 2014). Some studies have suggested that this protection can be heterosubtypic as well, which is usually not the case for antibody mediated protection (Nguyen et al., 1999). Heterosubtypic viruses share some components, such as internal proteins, while differing on other ways that usually evade antibody-mediated immunity, such as the exterior proteins HA and NA. Experiments in mouse models have mostly used heterosubtypic influenza virus infections rather than $CD8^+$ T cell targeted vaccines to show that $CD8^+$ memory can reduce disease severity without B cell protection (Liang et al., 1994). Vaccines targeting $CD8^+$ T cells directly have been shown to protect against influenza virus as well as other viral pathogens (Knuschke et al., 2014).

Unlike naïve $CD8^+$ T cells, memory $CD8^+$ T cells do not need to traffic to secondary lymphoid tissues for activation, as they can be activated directly by inflammatory conditions and infected cells. This allows secondary responses to be much faster than primary responses (Crowe et al., 2003). During influenza virus infection, Trm cells rapidly kill infected respiratory epithelial cells, potentially preventing symptomatic disease altogether (Epstein et al., 1998; Nguyen et al., 1999). In mice, the protection of Trm is sufficient as blocking Tcm infiltration of the lungs does not compromise protection (Wu et al., 2014). In humans, $CD8^+$ memory has been shown to promote less serious disease during influenza infection with heterosubtypic memory providing milder disease course to patients with the 2009 H1N1 (Xing et al., 2009). Patients infected with 2009 H1N1 also displayed reduced viral shedding during subsequent H3N2 seasonal

infections (Sridhar et al., 2013). $CD8^+$ memory populations can wane over time (Rai et al., 2014), but many memory cells persist for at least a decade (Akondy et al., 2017).

1.7.1 $CD8^+$ T cells in neonates

Neonatal $CD8^+$ T cell responses have diminished magnitude and function in part due to lower expression of CD28 mediated costimulation from neonatal DCs (Ruckwardt et al., 2014). Studies have shown that with proper stimulation, neonatal $CD8^+$ T cells are capable of responding in an adult manner to many antigens, suggesting that it is lack of stimulation rather than T cell intrinsic characteristics that underlie the altered function of $CD8^+$ T cells in neonates (Fadel et al., 2006).

Regardless of the cause, $CD8^+$ T cells in neonates are hypoactive and contribute to the vulnerability to infection that young mice and humans experience. There are fewer $CD8^+$ T cells in the lungs of influenza virus infected pups than adults, and the peak response is later than in adults (Lines et al., 2010). They produce less IFNγ, perforin and granzyme, and kill less effectively than adult cells *in vivo* (Fernandez et al., 2008b). They also do not move into the airways during infection, resulting in interstitial pneumonia. Adult airways contain many $CD8^+$ T cells, and some evidence suggests that this is important for efficient viral clearance (Lines et al., 2010). One study showed that neonatal $CD8^+$ T cells in infection sites are less functional than circulating $CD8^+$ T cells in the same animal, suggesting that neonatal $CD8^+$ T cells may also be actively suppressed in some way during infection (Fike et al., 2018).

Neonates develop memory T cells like adults, but evidence suggests that this process may differ from adults. Neonatal infections produce fewer tissue-resident

memory cells with diminished heterosubtypic protection during subsequent infections (Connors et al., 2018). Additionally, one study showed that neonatal infection with herpes simplex virus 1 (HSV-1) can "lock in" sub optimal neonatal responses that interfere with later adult challenges (Rudd et al., 2013). There is evidence that this memory defect is at least partly due to lower TCR avidity of neonatal memory $CD8^+$ T cells compared to adult memory $CD8^+$ T cells (Ruckwardt et al., 2011; Rudd et al., 2013). This suggests that the defect in neonatal T cell memory responses may be T cell intrinsic, contrasting with the extrinsic activation constraints on neonatal primary responses.

Improving neonatal infection outcomes is partially reliant on safely improving $CD8^+$ T cell functionality in neonatal responses. While several mechanisms have been suggested to explain diminished neonatal $CD8^+$ T cell function, the relative importance of these factors has not been established. Additionally, safe and effective strategies for circumventing these limitations have not been discovered, although work is being done to address this issue.

1.8 Dendritic cells

There is some disagreement as to how to definitively distinguish dendritic cells (DC) from other cell types, but in the lung, we will consider three main subsets. These are $CD103^+$ or $CD11b^+$ conventional dendritic cells, otherwise called cDC1 and cDC2 respectively, and $CD11c^{dim}$plasmacytoid dendritic cells (Neyt et al., 2013). Of these, the conventional DCs also express CD11c as a consistent but not definitive marker and are most relevant to antigen presentation during viral immunity (Geurts van Kessel et al., 2008). CD11c is also expressed on alveolar macrophages rendering it insufficient as a

sole marker of DCs in the lung (Hussell et al., 2014). Plasmacytoid dendritic cells are often identified by SiglecH expression and produce high levels of type I interferons making them important during viral infection as well (Liu et al., 2009).

Dendritic cells normally reside in the parenchymal spaces of the lung and monitor both their immediate surroundings as well as nearby airways through the use of cellular extensions that extend into the airways. During an infection, dendritic cells must detect and take up pathogens, mature to an inflammatory DC phenotype and migrate to secondary lymphoid organs to activate T and B cells (Geurts van Kessel et al., 2008; Ho et al., 2011). Dendritic cells express Toll-like receptors that allow them to detect (PAMPs) (Baharom et al., 2015). Viruses such as influenza virus can attempt to subvert this direct activation through NS1 mediated inhibition of TLR responses, but PAMP detection is only one of the methods of inducing DC maturation (Bender et al., 1998; Ioannidis et al., 2012).

The inflammatory environment of a viral infection includes multiple cytokines and cell types that can activate dendritic cells. Lung epithelial cells can produce IL-1 and GM-CSF (Willart et al., 2012). GM-CSF is an important growth factor for dendritic cells and may be essential for their survival (Zhan et al., 2011). IL-1 is a potent activator of dendritic cells and is necessary for inducing anti-influenza viral $CD8^+$ T cell responses (Pang et al., 2013). Type I interferons are produced by a variety of cells and are probably the best at inducing DC maturation (Rudd et al., 2007). NK cells have been shown to promote DC migration to lymph nodes. (Ge et al., 2012) They also kill virally infected cells creating infected cellular debris for DCs to phagocytose and process for antigen presentation (Ge et al., 2012). Finally, complement also plays a role in DC migration,

particularly C3 which is necessary for migration to the lymph node in animal studies (Kandasamy et al., 2013).

Dendritic cell migration occurs rapidly after infection through upregulation of CCR7 receptor, although this change is tempered after 48hrs in influenza virus infection by upregulation of PGD2 which suppresses CCR7 (Grayson et al., 2007). It is not clear if this is to prevent further induction of inflammation and avoid excessive pathology or if this inhibition of migration is caused by the virus.

DCs acquire antigen either through direct infection, phagocytosing pathogens, phagocytosing infected cellular debris, or direct presentation of peptides processed by other cells (Bachmann et al., 1996; Chen et al., 2000; Mintern et al., 2015) (Figure 1.4). Peptides from other cells can simply occupy vacant MHC class I, displace peptides from MHC class I on the cell surface, or be processed and presented on MHC class I through a process called cross presentation (Cruz et al., 2017). In the case of influenza virus infection, DCs are infected directly but they also seem to take up apoptotic virally infected epithelial cells for cross-presentation on MHC class I (Bachmann et al., 1996; Hemann et al., 2016).

Monocytes differentiate into dendritic cells in inflammatory conditions and play a role in continuing $CD8^+$ T cell proliferation and activation in the lungs. The inflammation generated by these induced DCs contributes significantly to the pathology experienced by the host (Baharom et al., 2015; Cook et al., 2016). These monocyte derived dendritic cells are also important for mediating allergic responses in the lungs (Plantinga et al., 2013).

1.8.1 Neonatal DCs

Neonatal dendritic cells theoretically perform the same role in the immune responses of infant humans and mice as they do in adults, but differences can be observed across multiple aspects of dendritic cell function and phenotype that cause these cells to behave differently in neonates and adults. On a broad level, neonatal dendritic cells seem to be less stimulatory than adult DCs at steady state and during infections, expressing lower levels of costimulatory molecules CD40, CD80/86 and MHC class II in cord blood and mouse pups (Papaioannou et al., 2019; Qureshi et al., 2005). They are also less prevalent in neonatal lungs than in adult lungs (Ruckwardt et al., 2014). Neonates have both $CD11b^+$ and $CD103^+$ dendritic cells, although the $CD11b^+$ population is severely diminished and $CD103^+$ dendritic cells have diminished function in neonates (Malloy et al., 2017; Ruckwardt et al., 2018). Plasmacytoid dendritic cells represent a significant fraction of lung DCs early in life, but this proportion diminishes with age (Dakic et al., 2004; Sun et al., 2003). Like the other DC subsets, neonatal plasmacytoid dendritic cells are less active than their adult counterparts (De Wit et al., 2004).

Expression of TLRs is similar on adult and neonatal cells at steady state, but the response to TLR stimulation is not equivalent. Neonatal dendritic cells produce IL-10, IL-6 and IL-23 after TLR stimulation rather than IL-12 that is important in Th1 T cell responses in adults. This shifts towards a more adult-like response of IL-1 and TNF-α after about a year of life in humans (Malloy et al., 2017). TNF-α production, in particular, is much lower in neonatal dendritic cells, although adult-like function can be induced in these cells by pretreatment with IFNγ (van Haren et al., 2016).

Overall, neonatal dendritic cells are much less proficient at activating T cells and are strongly biased towards production of more Th2 cytokines (de Kleer et al., 2016). This is possibly a significant contributing factor to the neonatal susceptibility to viruses as Th2 cytokines are useful mainly for eukaryotic parasites and suppress the expression of Th1 cytokines which are important for immunity to intracellular pathogens like viruses (Graham et al., 1994). Neonatal DCs are also less proficient at processing and presenting antigens as has been demonstrated in the context of RSV infection (Ruckwardt et al., 2014). This may have important implications for which antigens neonates can respond to in a given infection.

Although the general insufficiency of neonatal dendritic cells is well recognized, it is not clear how important this deficiency is in promoting neonatal susceptibility to influenza disease. It is also not clear how neonatal DCs effect the use of T cell vaccines in neonates.

1.8.2 Bone marrow derived dendritic cells

Generation of bone marrow-derived dendritic cells (BMDCs) is a common method of obtaining large numbers of mouse dendritic cells for study. The general procedure to create these cells is to isolate bone marrow from mice and then culture it in the presence of GM-CSF to differentiate hematopoietic precursors into dendritic cells (Ebrahimi-Nik et al., 2018; Inaba et al., 1992). Many studies have been done with these convenient cells to examine dendritic cell function and other immune functions. It has long been recognized that BMDC culture gives rise to a somewhat heterogeneous population of cells, but the consequences of this as it pertains to assessing dendritic cell physiology with BMDCs is becoming a cause for concern (Jones et al., 2017).

Cells obtained from BMDC culture typically express CD11c and MHC class II, but further examination shows that some of these cells are macrophages and some are dendritic cells. The DCs in the culture mature more upon stimulation with LPS than the macrophages, while the macrophages are more sensitive to interferon stimulation (Helft et al., 2015). Cytokine production can also vary between these subsets (Helft et al., 2015). These factors make it difficult to use BMDCs as a direct proxy for native dendritic cells, and studies hoping to examine the *in vitro* behavior of conventional dendritic cells should consider using Flt3L to stimulate DC differentiation (Jones et al., 2017).

However, studies have demonstrated that GM-CSF BMDCs potently stimulate T cells and can reasonably be used to study T cell activation (Bhattacharya et al., 2011; Ebrahimi-Nik et al., 2018; Zhan et al., 2011). In regards to the study of neonatal immunology, BMDCs are adult-like both in stimulatory capacity and phenotype (Qureshi et al., 2005). Additionally, generating DCs from neonatal bone marrow or cord blood produces cells that resemble natural adult DCs rather than neonatal DCs, suggesting that *in vitro* work characterizing neonatal dendritic cells must be done with isolated DCs *ex vivo* (Qureshi et al., 2005).

1.9 Immunodominance

During B and T cell development, naïve cells are generated with a broad range of antigen specificities. This means that theoretically, during an infection cells exist that could recognize many different epitopes on a given pathogen. In practice, however, most

of the adaptive response to a pathogen is directed towards just a few epitopes. This principle is known as immunodominance. The epitopes with the largest responses are called immunodominant, and the epitopes with smaller responses are called subdominant (Belz et al., 2000; Cukalac et al., 2014; Kim et al., 2015). The immunodominance hierarchy for a particular infection in a particular individual is thought to be the result of several variables including MHC haplotype, antigen processing and presentation, naïve antigen specific cell precursor frequency, TCR affinity, and suppression of subdominant clones by dominant clones (Chen et al., 2000).

MHC haplotype is the easiest factor to understand, although it can be difficult to study. Simply put, a particular MHC can only present peptides that fit in its groove. As such, the immunodominance hierarchy of an individual will only include epitopes that can be presented by that individual's MHC proteins (Day et al., 2011; Kim et al., 2015).

Antigen availability and processing can be complex and likely plays a key role in establishing immunodominance hierarchies. There can be much more of one antigen expressed by a pathogen than another, leading to unequal quantities of various antigens for presentation to immune cells. For example, there are many NP proteins in an influenza virus for every PA protein, leading to potentially different levels of these antigens in infected cells. Additionally, different antigens can be processed differently by APCs and infected cells. For example, dendritic cells of C57Bl/6 mice infected with the A/Puerto Rico/8/1934 strain of influenza virus (PR8) can present both immunodominant peptides $NP_{366\text{-}374}$ and $PA_{224\text{-}233}$, but infected epithelial cells only present $NP_{366\text{-}374}$ (Crowe et al., 2003).

Precursor frequency refers to the numbers of naïve cells specific for a particular epitope. This can vary somewhat between specificities for reasons that are not currently clear, but there are multiple studies that show that differences in precursor frequency can significantly shape the immunodominance hierarchy. High precursor frequencies for a specific epitope can lead to rapid initial expansion, but other factors can overcome this head start to establish a different hierarchy as the infection progresses (Cukalac et al., 2014; La Gruta et al., 2010). B and T cell receptors vary widely in their affinity for binding to an antigen. Higher affinity cells recive higher intensity of TCR stimulation during a response and will likely proliferate more, becoming dominant in the hierarchy (Chen et al., 2000; Cukalac et al., 2014). This can override precursor frequency.

Finally, suppression of subdominant clones by dominant clones is likely related to competition for stimulation (Willis et al., 2006). This happens in lymph nodes as B cells undergo affinity maturation and among T cells as cells compete for access to dendritic cells (Schone et al., 2017).

T cell immunodominance is much less predictable outside of inbred mouse strains and controlled viral infections, but some influenza virus components are often consistently more immunogenic than others. For example, the NP protein of influenza virus commonly contains a dominant epitopes for $CD8^+$ T cells in both mice and humans (Belz et al., 2000; Lee et al., 2019). HA is another common target for $CD8^+$ T cells across species (Chen et al., 2000). In C57Bl/6 mice infected with PR8, $NP_{366-374}$ and $PA_{224-233}$ are the dominant $CD8^+$ epitopes (Chen et al., 2004a). These will be used extensively in this study.

1.10 Flu immunodominance in mice

Most of what is known about immunodominance during influenza virus infection is from work done in adult C57Bl/6 mice infected with PR8, and of this, most is known about $CD8^+$ T cell immunodominance hierarchy. B cell immunodominance to influenza is almost always directed against HA. The mechanics of this process are of great importance to B cell vaccination efforts and the development of broadly neutralizing antibodies (Angeletti et al., 2018). $CD4^+$ T cell hierarchy is much less well understood than B or $CD8^+$ T cell immunodominance due to the difficulty of making MHC class II tetramers, but this limitation is less of a problem now thanks to technological improvements. One study suggests that the C57Bl/6 $CD4^+$ T cell hierarchy for PR8 infection contains a broader range of relevant epitopes than the $CD8^+$ hierarchy with responses to NP, PA, HA, and PB1 (Crowe et al., 2006).

The $CD8^+$ T cell immunodominance hierarchy for C57Bl/6 mice infected with PR8 is well established in adults. $NP_{366-374}$ and $PA_{224-233}$ co-dominate and $PB1_{10-34}$ and other less common epitopes following as subdominant specificities (Luciani et al., 2013). The precise proportions of these populations are not consistent, but the magnitudes of the response to each epitope in an individual animal will follow this established hierarchy. This hierarchy is set by several factors. $PB1_{10-34}$-specific cells exist at the highest frequencies prior to infection and dominate early in the infection (La Gruta et al., 2010). $PA_{224-233}$-specific cells are the second most common precursor and these also proliferate early in the infection (Cukalac et al., 2014). $NP_{366-374}$-specific cells are less common prior to infection and proliferate later in the infection, but by the peak of the response at day 7 of infection, $NP_{366-374}$ cells are fully co-dominant with $PA_{224-233}$-specific cells

(Cukalac et al., 2014). Although they are fairly prevalent early in infection, $PB1_{10-34}$-specific cells are of low affinity and do not proliferate to the same levels as the dominant $NP_{366-374}$ and $PA_{224-233}$ (Uddback et al., 2016).

Between $NP_{366-374}$ and $PA_{224-233}$-specific cells, $PA_{224-233}$-specific cells in C57BL/6 mice have higher precursor frequency and TCR affinity than $NP_{366-374}$-specific cells. One study showed that at high antigen doses, this allows them to dominate over $NP_{366-374}$ specific cells. At lower antigen doses, the relative effectiveness of presentation of $NP_{366-374}$ and $PA_{224-233}$ mattered more with $NP_{366-374}$ dominating over $PA_{224-233}$ (Luciani et al., 2013). This is important in memory responses because in secondary infections, $NP_{366-374}$ becomes strongly dominant over $PA_{224-233}$. While $NP_{366-374}$ and $PA_{224-233}$ are both presented by dendritic cells, generating $PA_{224-233}$ and $NP_{366-374}$ specific cells in the lymph nodes, only $NP_{366-374}$ is reliably presented by influenza virus infected respiratory epithelial cells (Chen et al., 2004a; Crowe et al., 2003). As a result of this, $PA_{224-233}$ specific cells are not effective for viral clearance and proliferate less in secondary infections where memory cells may be re-activated directly by infected epithelial cells (Crowe et al., 2005).

Immunodominance hierarchies influence the outcome of infections. In PR8 infections of C57BL/6 mice, $PA_{224-233}$ vaccination promotes a strongly $PA_{224-233}$-dominant $CD8^+$ T cell response, but these mice experience increased morbidity compared even to naïve mice. This was linked to a lack of $PA_{224-233}$ presentation on infected airway epithelial cells (Crowe et al., 2005). PB1 vaccination results in similarly poor protection (Uddback et al., 2016). If vaccination can set hierarches that are ineffective for promoting protection, then care must be taken with what antigens are selected for subunit vaccines.

1.10.1 Immunodominance in Neonates

Influenza virus infection immunodominance studies have almost exclusively been done in adult mice. Studies have suggested that neonatal mice do not always respond with the same immunodominance hierarchy as adults. This was first shown with RSV infection of CB6F1 mice as pups responded codominantly to $K^dM2_{82\text{-}90}$ and $D^bM_{187\text{-}195}$ while adults displayed a strongly $K^dM2_{82\text{-}90}$-dominant response (Ruckwardt et al., 2011). Ruckwardt et al (2011) proposed that this difference was due to an intrinsic characteristic of neonatal T cells as adoptively transferred neonatal T cells in adult mice displayed the same hierarchy. Later, dendritic cells were implicated in establishing the altered immunodominance hierarchy in RSV infected pups, and that stimulating neonatal dendritic cells with TLR4 or TLR9 agonists could promote a more adult-like $CD8^+$ T cell hierarchy (Malloy et al., 2017; Ruckwardt et al., 2014).

Work has also been done investigating the immunodominance hierarchy of neonates during influenza virus infection. Studies have shown that pups do not respond to $NP_{366\text{-}374}$ to the same magnitude as adults (Carey et al., 2016). This was linked to T cell intrinsic factors as transgenic OVA- specific T cells expanded in adult and neonatal hosts, but nontransgenic cells from neonates did not expand in adults (Carey et al., 2016). Additionally, Vβ sequencing of pup and adult TCR repertoires demonstrated clear differences between the age groups with young pups displaying lower diversity of Vβ usage than adults. Older pups Vβ usage began to resemble adult patterns by day 7 of life (Carey et al., 2016).

There is variation on how deficient the neonatal response to $NP_{366-374}$ is reported to be in different studies. Studies with 3 day-old pups show very low $NP_{366-374}$-specific populations in pups and studies with 2 week-old pups show levels similar to adults (Carey et al., 2016; Fike et al., 2018; Zens et al., 2017). From these data, it is clear that neonatal immunodominance can be different than adult hierarchies, but many questions remain. It is not clear if neonates can be made to respond to adult antigens if given proper stimulation. It is also unknown whether the neonatal immune environment favors a different hierarchy for a functional reason. In the case of influenza virus infection, there is not a clear picture of what actually comprises the neonatal immunodominance hierarchy other than the lack of $NP_{366-374}$-specific cells. This study hopes to address these questions.

1.11 B cells and antibody response

Naïve B cells are generated in the bone marrow and then migrate to secondary lymphoid organs for activation (Hardy et al., 2001). During influenza infection, virus is carried to the lymph nodes by dendritic cells where it comes into contact with B cells (Legge et al., 2003). Activation can also occur in ectopic sites in the infected tissue as cell aggregates such as inducible bronchus associated lymphoid tissue (iBALT) (Moyron-Quiroz et al., 2004). Influenza-specific B cells bind virus with membrane-bound antibody otherwise known as the B cell receptor (BCR) (Hinton et al., 2008). Some B cells, particularly the innate-like B1 type, can undergo activation without T cell help, but

many antigens, including influenza virus antigens, stimulate B2 cells and require T cell help (Kaji et al., 2012).

Activated B cells may directly differentiate into short-lived plasma cells or migrate to the germinal centers of the secondary lymphoid tissue (Abbott et al., 2018; Tarlinton et al., 2013). In the germinal center, activated B cells proliferate and interact with T follicular helper cells through CD40/CD40L (Chevalier, 2015). They also receive stimulation from IL-4 and IL-21 (Mastelic et al., 2012). As the B cells proliferate, somatic hypermutation and class switching occurs. These are the processes where B cells mutate the BCR gene locus to hone antibody affinity and swap antibody isotypes from IgM to other types such as IgA or IgG (Abbott et al., 2018). At the end of this process, B cells producing high quality, virus-neutralizing antibodies will hopefully have been generated. Some of these cells become memory B cells while others become plasma cells for antibody generation (Figure 1.5). Some plasma cells migrate to the bone marrow where they are maintained potentially for the entire lifetime of the organism (Sallusto et al., 2010). Survivors of the 1918 “Spanish” influenza pandemic were found to have circulating antibodies against the virus in 2005 (Yu et al., 2008).

Antibodies play arguably the most important role in influenza virus defense. Antibody against the virus has been shown to provide protection against subsequent infections provided that the virus has not drifted enough to evade the response (Sallusto et al., 2010). These antibodies are typically directed against epitopes on HA and NA. HA-specific antibodies prevent viral entry and are usually dominant, however, NA-specific antibodies also reduce disease severity by helping to trap influenza viruses on the sialic acids of the host cells (Kosik et al., 2019; Skehel et al., 2000).

1.11.1 B cells in neonates

Neonates mount antibody responses to pathogens including influenza virus, but there are several crucial differences from adult responses (Basha et al., 2014). Neonates do not respond well to T cell independent B cell stimulation and as such have trouble responding to antigens like polysaccharide antigens. Vaccines that require responses to such antigens use polysaccharides conjugated to protein toxins to promote T cell help and adequate antibody generation (D'Angio et al., 1995). This is not thought to be a problem for responses to influenza viral proteins because HA is not a polysaccharide antigen.

Neonates have less organized germinal centers and produce less antibody than adults (Mastelic et al., 2012). These antibodies typically display little affinity maturation or somatic hypermutation and can be of lower quality than adult antibodies (Debock et al., 2013; Montecino-Rodriguez et al., 2011). Maternal antibody helps make up for these deficiencies until B cell immunity is able to mature. Influenza virus naïve mothers, such as the mice used in these studies, however, cannot protect their pups with maternal antibody allowing direct analysis of the pup's response (Waaijenborg et al., 2013).

1.12 $CD4^+$ T cells

$CD4^+$ T cells have diverse functions but during influenza virus infection, they are primarily important for cytokine production and T cell help to B cells for antibody production (Doherty et al., 1997). Like $CD8^+$ T cells, naïve $CD4^+$ T cells are activated in the lymph nodes where they can remain to help B cells or migrate back to the site of

infection to produce cytokines (Mozdzanowska et al., 1997). $CD4^+$ T cells are vital to clearance of many infections as is demonstrated by the consequences of their loss during Human Immunodeficiency Virus (HIV) infection and subsequent development of AIDS (Utay et al., 2016). Susceptibility to and severity of many infections including influenza virus infection increases dramatically as $CD4^+$ T cell count falls, pointing to their essential role in combating these diseases (Sheth et al., 2011).

1.12.1 T cell transcription factors and Th subsets

$CD4^+$ T cells are also called helper T cells and are often divided into subsets based on function. These subsets have characteristic transcription factors that help set their transcriptional programs (Figure 1.6). These are Tbet for Th1, Gata3 for Th2, RORγ for Th17 Bcl6 for Tfh and Foxp3 for Tregs (Figure 1.4).

Tbet drives Th1 function in $CD4^+$ T cells, but it is expressed in other cells as well. For example, Tbet expression is important for $CD8^+$ T cell, NK cell and Regulatory T cell (Treg) function (Szabo et al., 2000). Low expression of Tbet has been linked to decreased $CD8^+$ function in CD31+ $CD8^+$ T cells in neonates (Fike et al., 2018). In T helper cells, STAT1 and STAT4 are activated by IFNɣ and IL-12 and lead to Tbet expression. Tbet expression activates Th1 effector function, especially IFNγ production (Afkarian et al., 2002). It also blocks differentiation into other Th subsets, especially Th2 by suppressing Gata3 expression (Kanhere et al., 2012). Th1 function, especially expression of IFNγ, is important for activating macrophages and driving immunity

against intracellular pathogens (Bettelli et al., 2004). Th1 immunity is considered more inflammatory than Th2 in regard to classic immunopathology (Graham et al., 1994).

Gata3 drives Th2 function in $CD4^+$ T cells, but it is expressed in many other cells and may drive a wide range of critical functions (Wan, 2014). In T cells, IL-4 drives STAT6 to activate Gata3. This in turn leads to Th2 effector functions such as production of IL-4, IL-5, and IL-13. It also blocks differentiation into other Th subsets, especially Th1 by suppressing Tbet expression (Kanhere et al., 2012). Th2 cytokines stimulate eosinophils, basophils, mast cells and B cells and are associated with allergic and anti-parasite reactions (Chen, Hou, et al., 2018).

IL-6 and IL-23 act through STAT 3 and drive expression of RORγ which activates Th17 function (Yang et al., 2007). RORγ is thought to be opposed to Treg function and Foxp3 expression, although Foxp3/RORγ double positive cells have been found, particularly in the case of pathogenic inflammation (Ren et al., 2017). RORγ drives production of the characteristic Th17 cytokine IL-17. IL-17 is a proinflammatory cytokine that helps to attract innate immune cells, particularly neutrophils, to the site of inflammation (Veldhoen, 2017). Th17 cells are important in reactions to extracellular pathogens and fungi. They are also central players in many forms of autoimmunity (van der Waart et al., 2014).

Bcl6 was discovered in T follicular helper cells (Tfh) where it acts as a transcriptional repressor and opposes the function of Blimp-1 (Chevrier et al., 2017). Bcl6 is essential for Tfh differentiation and function and actively inhibits differentiation into other Th subsets by suppressing expression of transcription factors like Tbet, Gata3 and RORγ (Chevrier et al., 2017). Tfh cells express CXCR5 and PD1 as

well as high levels of CD40 and IL-21 for B cell activation (Chevalier, 2015). Tfh reside in the T cell zone of germinal centers in secondary lymphoid tissues and provide help for B cell activation, maturation, and class switching (Debock et al., 2013).

Foxp3 is closely associated with Treg differentiation and function. It drives Treg effector functions and is necessary for the maintenance of these cells (Zhou et al., 2009). In humans, Foxp3 is expressed transiently in activated T cells making it unreliable as a sole marker of Tregs, but in mice, it is thought to be exclusive to Tregs (Devaud et al., 2014; Sakaguchi et al., 2010).

1.12.2 Neonatal $CD4^+$

Neonatal $CD4^+$ T cells have been studied extensively and this work clearly shows a strong Th2 bias in neonatal T cell function with production of IL-4, IL-5 and IL-13 production predominating (Adkins et al., 1998). Some evidence suggests that IFNγ is epigenetically repressed in children under 6 months of age, although strong stimulation can overcome this restriction (Verhoeven, 2019; Verhoeven et al., 2016). Similarly, IL-4 is epigenetically favored in neonatal cells creating an environment where IL-4 strongly dominates the Th1/Th2 balance (Adkins et al., 1992). The Th2 bias in neonates is likely part of a toleregenic environment that helps prevent immunopathogenic IFNγ responses, but it may also be related to the development of asthma and other atopic conditions after childhood infections (Miller et al., 2011; Tang et al., 1994).

1.13 Regulatory T cells

Regulatory T cells (Tregs) are a subset of $CD4^+$ T cells that play an important role in immunosuppression and tolerance (Kim et al., 2007). In mice, the transcription factor Foxp3 is sufficient to identify $CD4^+$ T cells that are Tregs. In humans, Foxp3 can be expressed by other cells, requiring cells to also be $CD25^{hi}$ and $CD127^{lo}$ before they may be considered Tregs (Devaud et al., 2014). Ultimately, demonstration of suppressive capability is the final gold standard of whether a T cell is a Treg, but in mice, Foxp3 expression is widely accepted as a good marker.

Unlike conventional T cells, Tregs perform an immunosuppressive role in the immune system through a variety of mechanisms. Tregs are generated in the thymus from a selection of autoreactive cells that would otherwise be negatively selected during T cell development (Dhamne et al., 2013; Sakaguchi et al., 2010). Tregs can also be generated in the periphery with non-self TCRs. These are thought to help control activated immune responses (Bedoya et al., 2013).

Tregs utilize several mechanisms to suppress immune responses including IL-2 sequestration, APC deactivation, direct killing of effectors, and production of anti-inflammatory cytokines (Vignali et al., 2008). Tregs express high levels of CD25, the high affinity IL-2 receptor subunit, and this allows them to compete with effector T cells for available IL-2 (Leon et al., 2014). Tregs have been shown to decrease APC function by simply occupying autoreactive MHC- peptide complexes or downregulating APC expression of costimulatory molecules (Rueda et al., 2015). Surprisingly, Tregs have also been shown to directly kill effector cells using a granzyme system similar to $CD8^+$ T

cells (Gondek et al., 2005). The most well recognized mechanism of Treg suppression is production of anti-inflammatory cytokines, particularly TGFβ and IL-10 (Yu et al., 2017). IL-10 defecient mice spontaneously generate ulcerative colitis (Kuhn et al., 1993). Overall, mice that lack Tregs and humans with Treg defects manifest rampant multi-organ autoimmunity placing Tregs as a central mediator of tolerance (Bacchetta et al., 2016).

While Tregs express Foxp3 as a central regulator of their function, they have also been shown to express conventional T cell transcription factors like Tbet, Gata3, and RORγ (Kluger et al., 2016; Yu et al., 2015). There are several suggested explanations and consequences of this expression. Tregs expressing RORγ are thought to be cells that are losing suppressive function and transitioning to Th17 cells in cases of pathogenic inflammation (Ren et al., 2017). Similar theories have been proposed for Tbet and Gata3 expression (Koch et al., 2012; O'Hagan et al., 2017), but other studies have shown that Tbet and Gata3 are essential for Treg function as knocking out both these transcription factors in $Foxp3^+$ T cells results in rampant autoimmunity (Wohlfert et al., 2011; Yu et al., 2015). Specifics of what these functions are is unclear, particularly for neonates.

1.13.1 Tregs in Neonates

Almost all Tregs in neonates are thymus-derived which is consistent with the overall lack of antigen experience in the young immune system (Gervassi et al., 2014). Neonatal Tregs are less potent at suppressing T cells and dendritic cells than adult Tregs (Rueda et al., 2015), but they are still required to prevent catastrophic autoimmunity in newborn humans and mice (Kim et al., 2007). Scurfy mutant mice lack Foxp3 and

therefore lack Tregs (Godfrey et al., 1991). Hemizygous scurfy mice die by three weeks of age from multi-organ autoimmunity.

Tregs are essential for a fetus to prevent rejection by the maternal immune system (La Rocca et al., 2014), and even after birth, neonatal T cells may be predisposed to becoming Tregs after TCR stimulation (Wang et al., 2010). Perhaps because of these factors, Tregs are more numerous in many tissues including the neonatal lung than in adult lungs (Oliphant et al., 2015).

1.13.2 Depletion of Tregs and infection

Given the powerful role of Tregs in preventing autoimmunity and controlling inflammatory responses, considerable effort has been devoted to understanding whether Tregs constrain host responses to pathogens in ways that are detrimental to clearance. The simplest way to study this is to deplete Tregs from mice using anti-CD25 antibodies or use Foxp3 DTR mice that express the diphtheria toxin receptor under the control of the Foxp3 promoter. Administering diphtheria toxin to these mice specifically depletes Tregs (Betts et al., 2011; Wang et al., 2016).

The effect of Treg depletion on infections varies with the pathogen used. Treg depleted mice clear *Listeria monocytogenes* faster than undepleted mice (Heit et al., 2008). Additionally, herpes simplex virus 1 infected mice had lower viral titers after Treg depletion (Fernandez et al., 2008a). Other infections show the opposite. RSV infected mice have higher viral burdens after Treg depletion (Lee et al., 2010), and we have shown that pups lacking Tregs were not able to clear influenza virus (Oliphant et al., 2015).

Treg depletion undoubtedly increases inflammation in infected animals, and this increases immunopathology across nearly all infections tested (Suvas et al., 2004). This inflammation may not always be productive, however, as studies have reported disruptions in adaptive immune responses after Treg depletion. Tfh function is compromised by Treg depletion in influenza virus infections leading to lower titers of virus-specific antibody (Leon et al., 2014). This study was done in adults and did not test viral clearance, but other studies have suggested that Treg depletion does not alter influenza disease course in adults (Betts et al., 2011). In adult RSV infection, the increased viral burden observed in Treg depleted animals was associated with a delayed $CD8^+$ T cell response (Ruckwardt et al., 2009).

Treg clearly influences the response to influenza in both pups and adult mice, but the reasons for this are unclear in pups. The observation that Treg depletion did not alter the course of influenza virus infection in adult mice while compromising viral defense in pups suggests either that different mechanisms are at work in young animals or that the defects in antibody and germinal center formation in adults are more serious in the already low-function neonatal immune response. This is a technically difficult question to answer given the fragility of infected pups made even sicker by Treg depletion, but this study attempts to shed some light on the issue.

1.14 Overall hypothesis

The overall hypothesis of this dissertation is that neonatal immunity can be manipulated to improve outcomes during influenza infection. Chapter 3 will characterize neonatal immunodominance to influenza virus infection address whether neonatal

immunodomicance can be manipulated. Chapter 4 will explore whether $PA_{224-233}$-specific $CD8^+$ T cells can protect pups against influenza virus infection. Chapter 5 will examine whether regulatory T cells (Tregs) are required for anti-influenza virus antibody production, and characterize the role of Gata3 in Treg function during influenza virus infection.

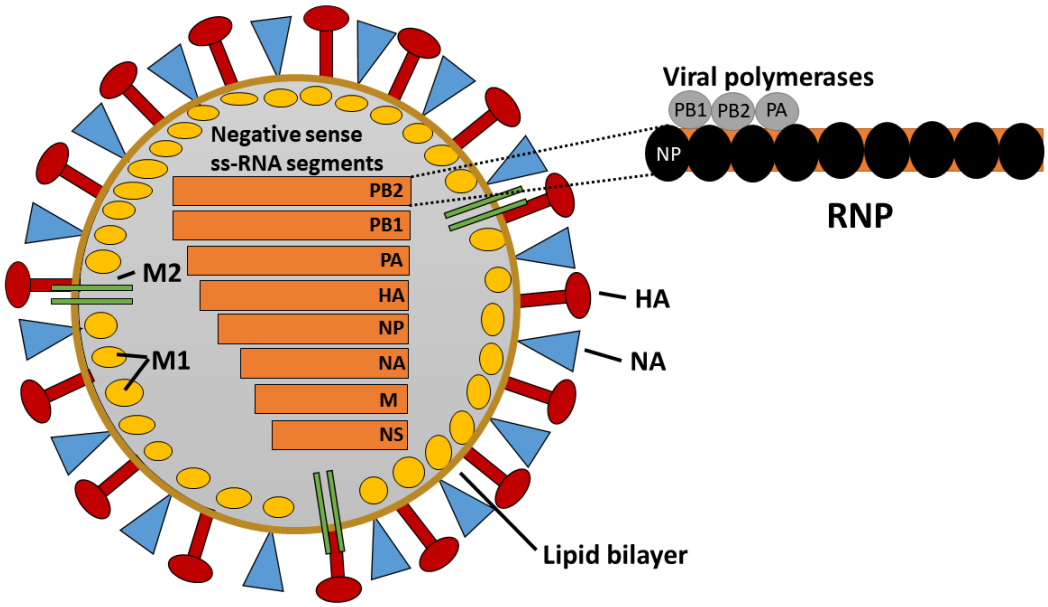

Figure 1.1 Influenza A virus structure.

Influenza A virus is enveloped by a lipid bilayer that contains the surface proteins hemagglutinin (HA), neuraminidase (NA), and the proton channel matrix protein 2 (M2). Matrix protein 1 (M1) forms the capsid. Inside the virion are eight RNA genome segments coated with nucleocapsid protein (NP) called ribonucleoprotein complexes (RNP). The RNPs are associated with the three components of the viral RNA dependent RNA polymerase, polymerase basic protein 1 (PB1) polymerase basic protein 2 (PB2) and polymerase acidic protein (PA).

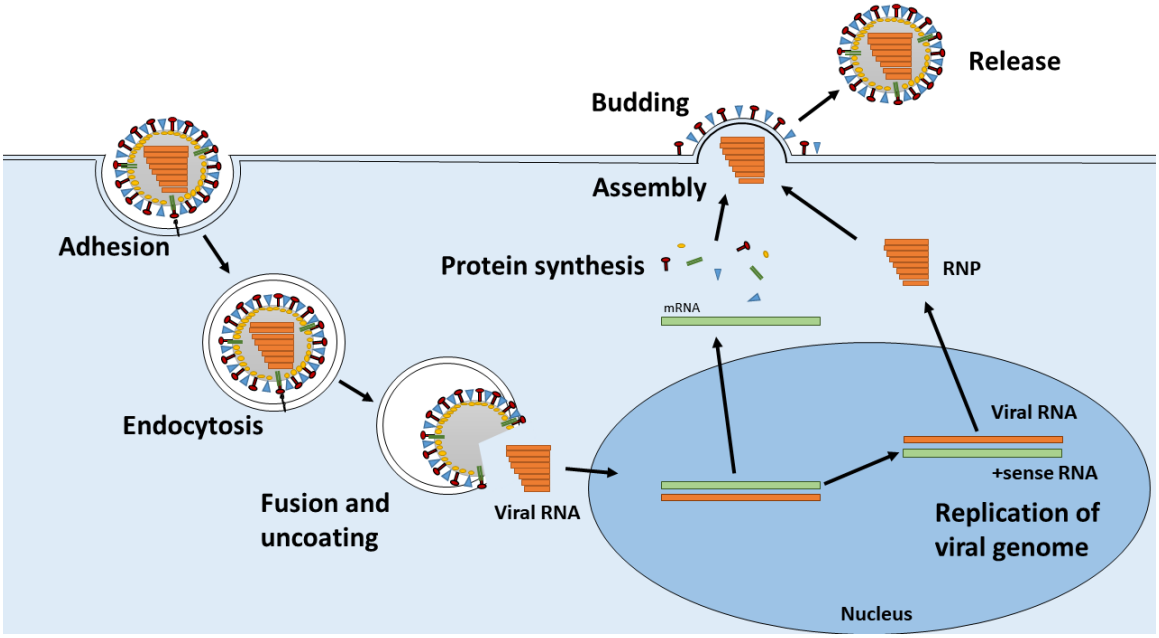

Figure 1.2 Influenza A virus infection cycle.

Hemagglutinin (HA) binds to sialic acids on host cells before being endocytosed. Acidification of the endosome induces a conformational change in HA that allows fusion with the endosome membrane and uncoating of the viral genome. Viral gene segments are transferred to the nucleus and undergo replication into mRNA and + sense template for viral genome replication. Viral proteins are synthesizes in the cytoplasm and new ribonucleoprotein complexes (RNPs) are packaged. Virions are assembled at the host cell membrane and bud from the surface after RNPs are packaged. NA cleaves host sialic acids to allow release of the virus.

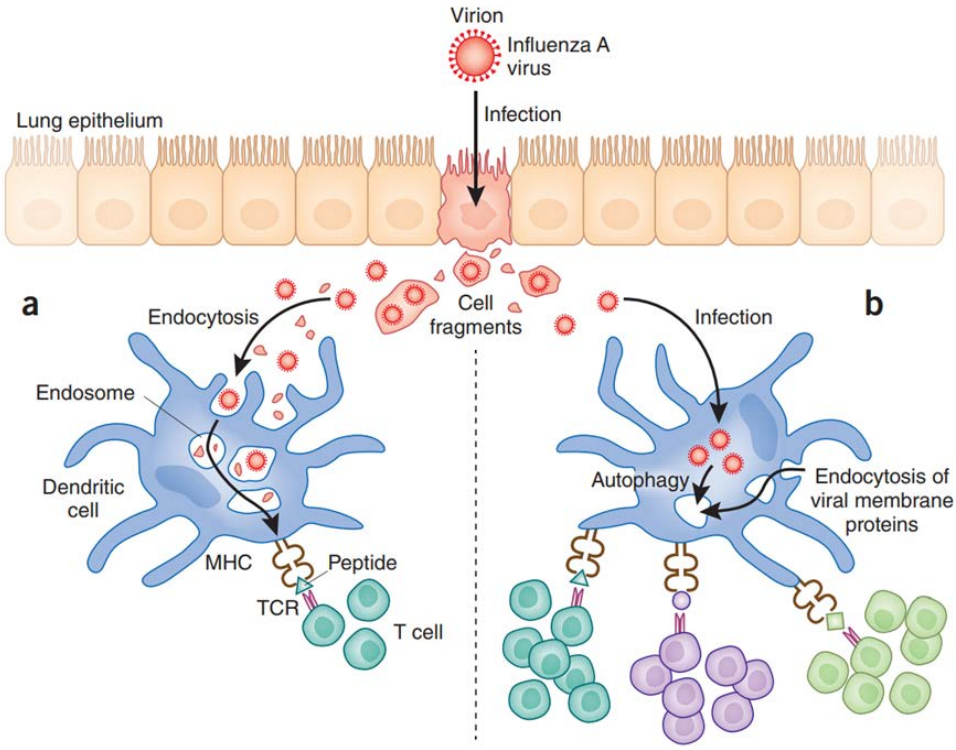

Figure 1.3 Dendritic cell antigen acquisition.

Adapted with permission from (Mintern et al., 2015)

During influenza virus infection, respiratory epithelial cells become infected and lyse releasing cell fragments and infectious viruses. Dendritic cells may endocytose cellular debris (a) or become infected (b) to acquire influenza antigens. Endocytosed antigens are processed and presented on MHC class II or cross presented on MHC class I for activation of T cells. Infected DCs process viral proteins through autophagy and directly present viral peptides on MHC class I to $CD8^+$ T cells.

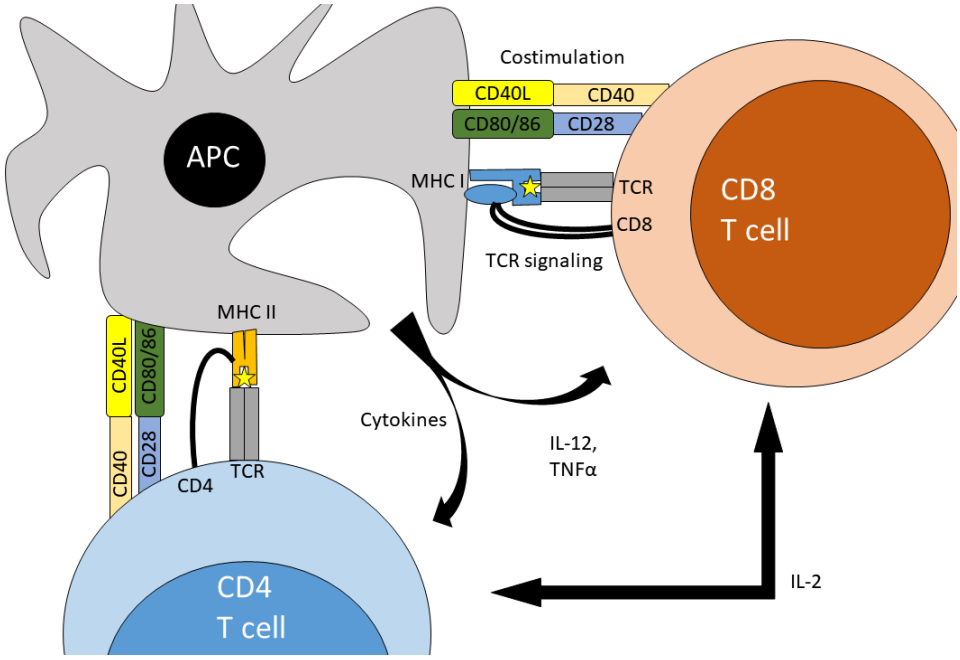

Figure 1.4 T cell activation.

During infection, dendritic cells are activated by pathogen-associated molecular patterns (PAMPs) and acquire antigens in the inflamed tissue. They then migrate to lymphoid tissue to interact with T cells. Activated dendritic cells present peptides bound to MHC class I and MHC class II and express costimulatory molecules like CD40L and CD80/86. Naïve $CD4^+$ and $CD8^+$ T cells interact with MHC class II and MHC class I respectively with the aid of the $CD4^+$ and $CD8^+$ proteins. CD28 interacts with CD80 or CD86 to provide a second signal for activation. CD40/CD40L interaction provides costimulation as well. Activated dendritic cells produce cytokines like IL-12 and type I interferons that impact T cell activation and polarization into various T helper subsets. T cells produce IL-2 to promote T cell survival and proliferation.

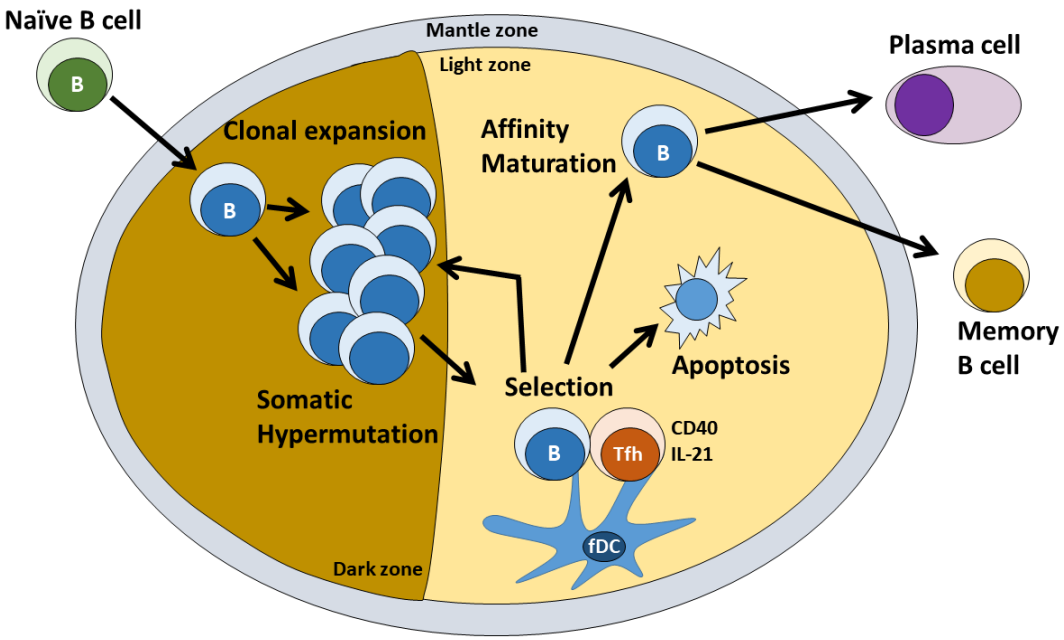

Figure 1.5 Germinal center reaction.

Naïve B cells encounter antigen in the periphery and become activated. They migrate to lymph nodes and proliferate to form germinal centers (GC). Clones expand in the dark zone of the GC and undergo somatic hypermutation of the B cell receptor gene. B cells migrate to the light zone for interaction with follicular T helper cells and GC dendritic cells to select for mutations that improved antibody affinity and destroy mutants that reduced antibody affinity. After affinity maturation, B cells with T cell help can undergo class switching and differentiate into plasma cells or memory B cells.

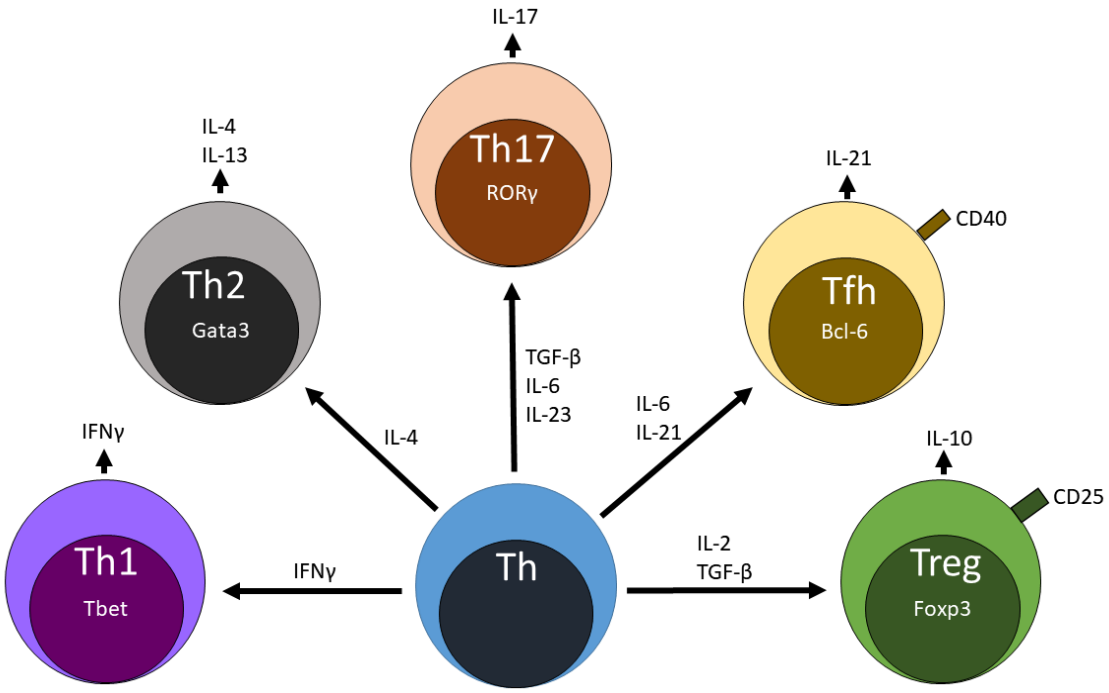

Figure 1.6 T helper subsets.

Naïve $CD4^+$ T cells differentiate into Th subsets upon activation. Th1 cells are polarized by IFNγ, express the transcription factor Tbet, and produce IFNγ. Th2 cells are polarized by IL-4, express the transcription factor Gata3 and produce IL-4 and IL-13. Th17 cells are polarized by TGF-β, IL-6 and IL-23, express the transcription factor RORγ and produce IL-17. T follicular helper (Tfh) cells are polarized by IL-6 and IL-21, express the transcription factor Bcl-6, and produce IL-21 while expressing CD40. Regulatory T cells (Tregs) are polarized by IL-2 and TGF-β, express the transcription factor Foxp3, and produce IL-10 and TGF-β while expressing CD25.

CHAPTER 2. MATERIALS AND METHODS

2.1 Mice

C57BL/6J (Stock #000664), Gata3$^{tm1.1Mbu}$/J (Stock #028103), B6.129(Cg)-*Foxp3*$^{tm4(YFP/icre)Ayr}$/J (Stock #016959), and B6.129(Cg)-*Foxp3*$^{tm3(DTR/GFP)Ayr}$/J (Stock #016958) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B.Cg-*Foxp3*^{sf} (Stock #004088) (scurfy) heterozygous female mice were also purchased from The Jackson Laboratory and bred with wild-type C57BL/6 males under specific pathogen free conditions. Scurfy mutation was determined by flow cytometry for male pups and Foxp3 cre/ Gata3 flox status was determined by PCR after breeding as described below. Timed pregnancies were generated by co-housing female mice for two weeks to synchronize estrus before co-housing with one male to two females for breeding. All mice were maintained at the University of Kentucky Division of Laboratory Animal Research facilities with the approval of the University of Kentucky Institutional Animal Care and Use Committees (IACUC) and Institutional Biosafety Committees. Male and female pups were used in all experiments.

2.2 Phenotyping and genotyping of scurfy and Gata3-Foxp3+ mice

Presence of scurfy null mutation of Foxp3 on the X chromosome was confirmed by flow cytometry of TBLN CD4$^+$ T cells. Scurfy mice were identified by the complete lack of Foxp3 intracellular staining in CD4$^+$ cells.

Foxp3$^{tm4(YFP/icre)Ayr}$/J genotype was determined by PCR on tail snip DNA with the primers CGGGTCAGAAAGAATGGTGT and CAGTTTCAGTCCCCATCCTC according to the protocol provided by The Jackson Laboratory.

Gata3$^{tm1.1Mbu}$/J genotype was determined by PCR on tail snip DNA with the primers TGTCAGGGCACTAAGGGTTG and CACAGTGGGGTAGAGGTTGC according to the protocol provided by The Jackson Laboratory.

B6.129(Cg)-*Foxp3*$^{tm3(DTR/GFP)Ayr}$/J were maintained as hemizygous transgenic animals and required no genotyping.

2.3 Viral stocks and influenza virus peptides

Influenza viruses, A/Puerto Rico/8/34 (H1N1) (PR8) and A/X-31(H3N2) (X-31) (originally provided by Linda Cauley, University of Connecticut Health Center) were grown in 10-day-old specific-pathogen-free embryonated chicken eggs (Charles River) as has been described before (Cottey et al., 2001). Both viral stocks were verified to contain only IAV by RADIL (University of Missouri). Viral titers were measured in egg infectious doses (EID_{50}) by titrated infectious doses in new eggs as described before (Cottey et al., 2001). Viral aliquots were stored at -80°C.

$NP_{366-374}$, $PA_{224-233}$, PB1 (10-34) and PB1-F2 (62-70) were purchased from GenScript as custom peptides (Table 2.1).

2.4 Infections

Mice were given LD_{10} doses of PR8 or HKx31 intranasally (i.n.) under isoflurane anesthesia. The virus was thawed and diluted in HBSS for delivery. Volume was 10µL for pups and 50µL for adults. For PR8, this was equivalent to 2.5 $(EID)_{50}$/g of body weight for adults and 0.25$(EID)_{50}$/g of body weight in pups. For HKx31 this was equivalent to 10 $(EID)_{50}$/g of body weight in adults and 1 $(EID)_{50}$/g of body weight in pups. Following infections, all mice were monitored daily for body weight and euthanized by CO_2 or isoflurane asphyxiation in accordance with the University of Kentucky IACUC's criteria.

For heterosubtypic infections, primary infections were performed with PR8 or HKx31 as described above. For secondary infections, an LD_{100} dose was delivered intranasally under isoflurane anesthesia. This corresponded to 25 $(EID)_{50}$/g of body weight for PR8 and 100 $(EID)_{50}$/g of body weight for HKx31. Lethal doses were determined by dose dependent survival studies in pups and adults for both HKx31 and PR8.

2.5 Cell isolation

Lungs were lavaged five times with cold HBSS/3mM EDTA to extract alveolar cells as described before (Sun et al., 2017). Lungs were excised and minced before digestion at 37°C with 50U/mL DNAse (Sigma) and 1mg/mL collagenase (Sigma) in RPMI (ThermoFisher) with 3% FCS (Atlas Biologicals, Fort Collins, CO). Lungs were

collected in RPMI with 3% FCS and TBLN were collected in cold HBSS. Single-cell suspensions of lungs and TBLN were generated separately by pressing tissues though 70μm cell strainers (BD Biosciences, San Jose, CA). Red blood cells were lysed using ammonium chloride/potassium (ACK) lysis buffer. Cells were then washed and resuspended in HBSS (ThermoFisher).

2.6 Flow Cytometry

Surface staining was performed on $5x10^5$ to 10^6 cells with fluorochrome conjugated antibodies or MHC-I tetramers in PBA buffer (phosphate-buffered saline containing 0.1% BSA and 0.02% NaN3) for 30min at room temperature (Table 2.2). Cells were fixed with 10% formalin at room temperature for 20min before wash with HBSS and a spin at 300xg for 7 min.

For intracellular cytokine staining, cells were stimulated for 4hrs at 37C with 50ng/mL PMA (Enzo Life Sciences) and 1 **μ**g/mL of ionomycin (Sigma). Brefeldin A (10 **μ**g/mL final concentration) was added 2hrs after simulation to trap cytokines in the golgi apparatus. Cells were surface stained at room temperature as described above with the addition of 10**μ**g/mL Brefeldin A (Sigma) to the PBA. Cells were fixed 5% neutral buffered formalin (Sigma), permeabilized with saponin (5mg/mL) and stained for 30min with antibodies specific for IFN_γ. Analysis was performed by flow cytometry with an LSRII cytoflurometer (BD Biosciences, Mountain view, CA). 50,000 events were

collected for each sample and analysis was performed with FlowJo software (Tree Star, Inc., Ashland, OR).

For intracellular transcription factor staining, cells were surface stained as described above before being fixed and permeabilized with the eBioscience Foxp3/ transcription factor staining buffer set (ThermoFisher cat # 00-5523-00) according to the manufacturer's instructions. Cells were stained with anti-Foxp3, anti-Tbet, anti-Gata3 or anti-RORɣt for 30min at room temperature. Analysis was performed by flow cytometry with an LSRII cytoflurometer (BD Biosciences, Mountain View, CA). 50,000 events were collected for each sample and analysis was performed with FlowJo software (Tree Star, Inc., Ashland, OR).

2.7 Antigen presentation assays

Bone marrow-derived dendritic cells were generated by flushing the femurs of adult, female C57BL/6 mice with cold RPMI to isolate bone marrow. Red blood cells were lysed with ACK as described above, and bone marrow was plated with 10 mL RPMI containing 10%FCS, 0.1% 2-Mercaptoethanol and 1ng/mL GM-CSF (Sigma). After 10 days, BMDCs were washed with RPMI and incubated for 1hr at 37°C and 5% CO_2 in 14mL polypropylene culture tubes in RPMI containing 10%FCS with either PR8 at a 0.1 multiplicity of infection, 0.1 mM peptide, or left untreated. Treated BMDCs were cultured 1:5 with lymphocytes taken from lymph nodes of mice 10 days post-infection and prepared as a single cell suspension as described above. After 3 days, plates were centerfuged at 300xg for 7 min and supernatants were collected and frozen at

-80°C. Supernatant was later analyzed for IFN_γ by ELISA per the manufacturer's instructions (eBiosciences).

For lung tissue presentation assays, lungs were taken from naïve 2-day-old pups and adults and digested as specified before. Spleens were taken from PR8 infected adults at day 7 post infection and $CD8^+$ T cells were isolated by $CD8^+$ T cell enrichment column (Cat# MCD8C-1000, R&D). 10^4 $CD8^+$ T cells were co-cultured with 10^5 naive lung digest with and without peptide or co-cultured with peptide loaded BMDCs. After 3 days, supernatants were frozen at -80°C and later analyzed for IFN_γ by ELISA (Cat# BMS606) per the manufacturer's instructions (ThermoFisher)

2.8 CpG vaccination

CpG vaccination was prepared with 1mg/mL $NP_{366-374}$, 1mg/mL $PA_{224-233}$, and 0.4mg/mL CpG (OD2395, Invitrogen) mixed 1:1 with Montinide (Seppic, Henrico County VA) to form an emulsion. Vaccine was injected i.p. to mice on day 0 and day 7 of vaccination.

2.9 Adoptive transfer of PR8 infected or peptide pulsed BMDCs

BMDCs were generated as described above. After 9 days, BMDCs were stimulated with 50ng/mL LPS (Sigma) overnight at 37°C and 5%CO_2. Then BMDCs were washed once with RPMI before resuspension in RPMI with 10% FBS and incubated with 10^7 EID PR8 or HBSS per 10^6 BMDC for 1hr at 37C, 5%CO_2. BMDCs were then washed once with RPMI and incubated for an additional 3hrs at 37C. BMDCs were

washed once with RPMI before resuspension in RPMI with 10% FBS and adoptive transfer. Peptide-pulsed DCs were incubated for 1hr with 1mM fina concentration of peptide and then washed once with RPMI before adoptive transfer. 10^4 BMDCs in 50μL of RPMI were injected I.P. to pups on day 0 of infection or vaccination. $5x10^4$ BMDCs were transferred to adults on day 0 of vaccination.

2.10 Hemagglutination inhibition assay.

Hemagglutinin inhibition assays were performed as described before (Cottey et al., 2001). Briefly hemagglutination capacity for viral stocks was determined by titrating virus with 1% chicken red blood cell (CRBC) solution (Colorado Serum Company). Stock virus was diluted to 4 hemagglutination units (HAU) and mixed with serial dilutions of serum from infected mice. 1% CRBC was added after 30min at room temperature. Agglutination was observed after a 40min incubation at room temperature.

2.11 Statistics

Data represent the mean ±SD or SEM for 3-10 mice per group and at least 2 separate trials were performed for each experiment. Statistical analysis was performed with SigmaPlot software (San Jose, CA). Student's t-tests or two-way analysis of variance (ANOVA) were performed to test for differences followed by Holm-Sidak *post-hoc* test for pairwise comparisons. Mann-Whitney rank sum tests or Kruskal-Wallis one-way ANOVA on ranks were performed at each time point followed by Dunn's pairwise

post-hoc test if normality or variance tests failed. $p<0.05$ was considered statistically significant.

Table 2.1 Influenza peptides

Peptide	Sequence
$NP_{366-374}$	ASNENMETM
$PA_{224-233}$	SSLENFRAYV
$PB1_{10-34}$	SSYRRPVGI
$PB1\text{-}F2_{62-70}$	LSLRNPILV

Table 2.2 Antibodies used for flow cytometry

Antibody/ tetramer	Flurocrome or flurophore	Vendor/Cat. No./Clone
Anti-mouse CD4	PerCP-eFluor710	Thermo/ e 46-0041 / GK1.5
Anti-mouse CD8	APC	Thermo/ e 17-0081/ 53-6.7
Anti-mouse CD8	FITC	Thermo/ e 11-0081/ 53-6.7
Anti-mouse CD19	PE	Thermo/ e 12-0193/ 1D3
H-2D(b) Influenza A NP 366-374 ASNENMETM tetramer	PE	NIH Tetramer Core Facility
H-2D(b) Influenza A PA 224-233 SSLENFRAYV tetramer	Alexa 488	NIH Tetramer Core Facility
Anti-mouse CD44	FITC	Thermo/ e 11-0441/ IM7
Anti-mouse CD62L	APC	Thermo/ e 17-0621/ MEL-14
Anti-mouse IFNγ	eFluor450	Thermo/ 48-7311-82/ XMG1.2
Anti-mouse Foxp3	APC	Thermo/ e 17-5773/ FJK-16s
Anti-mouse Tbet	PE	Thermo/ 12-5825-82/ 4B10
Anti-mouse Gata3	PE	Thermo/ 12-9966-42/ TWAJ
Anti-mouse CXCR5	FITC	Thermo/ 11-9185-42/ MU5UBEE
Anti-mouse PD-1	BV510	BioLegend/ 135241/ 29F.1A12
Rat IgG1 k isotype	PE	Thermo/ 21-4301-83/ eBRG1
Rat IgG2a k isotype	PE	Thermo/ 12-4321-83/ eBRG2a
Rat IgG1 k isotype	APC	Thermo/ 17-4301-83/ eBRG1
Rat IgG1 k isotype	eFluor450	Thermo/ 48-4301-83/ eBRG1

CHAPTER 3. THE CD8$^+$ T CELL IMMUNODOMINANCE HIERARCHY IS ALTERED IN NEONATES DURING INFLUENZA VIRUS INFECTION

3.1 Introduction

Neonates and children are vulnerable to influenza virus infection, suffering significant morbidity and even mortality as a result of this common infection (Antonova et al., 2012; Nelson et al., 2014; Reed et al., 2015). This remains the case despite yearly vaccination efforts (Neuzil et al., 2002; Thommes et al., 2017). Children under 6 months of age cannot be vaccinated for influenza, and even after that period, two injections are required to confer protection instead of the one adults require (Sridhar et al., 2015). As a result, influenza causes more hospitalizations and deaths in American children than any other vaccine-preventable disease (Reed et al., 2015).

The neonatal immune response exhibits a variety of differences from the adult response that may underlie the observed vulnerability of infants to influenza virus infection (Adkins et al., 2002; Adkins et al., 1998; Basha et al., 2014; Ruckwardt et al., 2014). We have shown that CD4$^+$ and CD8$^+$ T cell migration into the lungs is delayed in mouse pups during influenza virus infection (Lines et al., 2010). Additionally, these T cells accumulate in the interstitium of murine lungs instead of moving into the alveolar spaces as is seen in adults (Lines et al., 2010). This is similar to the interstitial pneumonia observed in severe human infant influenza virus and RSV infections (Welliver et al., 2007). These diminished T cell populations correlate with changes in function as well, with neonatal T cells displaying reduced cytotoxicity and cytokine production compared to adult cells in a variety of viral illnesses (Adkins et al., 2002; Ruckwardt et al., 2014; You et al., 2008). Intriguingly, neonatal T cells can respond

robustly like adult cells given adult APCs (Fadel et al., 2006; Smith et al., 2014). We have seen in our murine model of influenza infection that with this diminished immune function, pups struggle to clear even minute quantities of virus (Lines et al., 2010). Understanding the functional aspects of the neonatal influenza virus response is necessary for effectively designing specific treatments that improve infection outcomes while avoiding excess inflammation in this fragile population.

During T cell receptor (TCR) generation, a diverse repertoire of T cell specificities is created (Carey et al., 2017). During an immune response, however, most of the expanded T cell population derives from clones specific for a few of the many potential epitopes in a given antigen: a principle known as immunodominance (Chen et al., 2000; Crowe et al., 2003; La Gruta et al., 2010). In C57Bl/6 mice, the peptides $NP_{366-374}$ and $PA_{224-233}$ are known to be co-dominant during adult A/PR/8/34 (PR8) influenza virus infections, but it has been suggested that immunodominance can be shifted in neonates (Carey et al., 2016; Ruckwardt et al., 2014). Neonates respond co-dominantly to epitopes from the M2 and M proteins of respiratory syncytial virus compared to the strong M2 restricted response in adult infections (Ruckwardt et al., 2011). Similarly, in influenza virus infection, published evidence as well as our preliminary data suggested that pups do not respond to the $NP_{366-374}$ epitope (Carey et al., 2016). $CD8^+$ T cells are typically considered essential to effectively clear primary influenza virus infection (Wu et al., 2014), but pups are so exquisitely sensitive to the virus that they may simply not produce an effective $CD8^+$ T cell response during this infection. Nonetheless, we were interested in investigating what, if anything, neonatal $CD8^+$ T cells were responding to instead of $NP_{366-374}$.

In this study, we identify what may be the immunodominant peptide in neonatal C57Bl/6 murine infections using an acute model of influenza virus infection. Responses were compared between adult mice and pups to evaluate $CD8^+$ specificity in primary and secondary responses. We found the primary neonatal response to be strongly biased towards $PA_{224-233}$ rather than the adult codominant response to $NP_{366-374}$ and $PA_{224-233}$. $CD8^+$ T cell specificity is less predictable in the secondary infections of mice first infected as neonates compared to mice first infected as adults. Some mirror the strong dominance of $NP_{366-374}$-specific $CD8^+$ T cells, while others exhibit $NP_{366-374}$ and $PA_{224-233}$ codominance or even $PA_{224-233}$ dominance alone. Regardless of hierarchy, $CD8^+$ memory was less effective in mice first infected as pups. Additionally, we found that transfer of PR8-loaded bone marrow-derived dendritic cells improved viral clearance in pups as well as promoting $NP_{366-374}$-specific $CD8^+$ T cells. We believe these data support previous findings about neonatal T cell specificity and demonstrate that the immunodominance hierarchy can be manipulated to some degree. These data also show the importance of examining the neonatal period separately from adult responses when establishing an immunodominance hierarchy. This notion has important implications for the development of subunit vaccines for patients of different ages, as well as furthering our general knowledge of viral defense in early life.

3.2 Results

3.2.1 Neonates do not produce $CD8^+$ T cells specific for $NP_{366-374}$ during primary influenza virus infection and instead respond to $PA_{224-233}$

The $CD8^+$ immunodominance hierarchy for adult C57Bl/6 mice has been clearly demonstrated during PR8 infection (Belz et al., 2000; Crowe et al., 2003), but much less is known about neonatal $CD8^+$ T cell specificity. We wanted to test whether the dominant adult response to $NP_{366-374}$ could be recapitulated in pups. We infected 2-day-old or 8-week-old mice with an LD_{10} dose of PR8 influenza virus. $CD8^+$ T cells were isolated from the lymph nodes of pups and adults at day 10 of infection and stimulated with BMDCs loaded with $NP_{366-374}$, $PA_{224-233}$, $PB1_{10-34}$ subunit of the viral polymerase (PB1) or $PB1\text{-}F2_{62-70}$ protein encoded by the +1 alternative open reading frame in the PB1 gene (PB1-F2). IFNγ production was measured in supernatants at day 3 of culture by ELISA. Neonatal cells produced more IFNγ in response to $PA_{224-233}$ and $PB1_{10-34}$ than to $NP_{366-374}$ (Figure 3.1 A). Conversely, the adults displayed similar responses to $NP_{366-374}$, $PA_{224-233}$ and $PB1_{10-34}$ (Figure 3.1 A). There was some variability in the $PB1_{10-34}$ response between trials with another experiment showing much lower response in both pups and adults (data not shown).

To further test the shift in the neonatal $CD8^+$ T cell response, we infected 2-day-old or 8-week-old mice with an LD_{10} dose of PR8 influenza virus and analyzed whole-lung digest and bronchial alveolar lavage (BAL) $CD8^+$ T cells for antigen specificity by flow cytometry using tetramers for $NP_{366-374}$ and $PA_{224-233}$. At 10 days post-infection, pups displayed an essentially absent $NP_{366-374}$- specific $CD8^+$ T cell response compared to adults in both lung digest (Figure 3.1 B, D) and BAL (Figure 3.1 C, E). This is consistent

with previous findings suggesting that pups do not respond to $NP_{366-374}$ during PR8 infection. (Carey et al., 2016).

Adults also displayed $PA_{224-233}$-specific populations in addition to the $NP_{366-374}$-specific cells. Although pups did not respond to $NP_{366-374}$, they did display robust $PA_{224-233}$-specific cell populations in the lung digest (Figure 3.1 F, G). There was some variation amongst the pups as to what percentage of the $CD8^+$ T cells in the lung and BAL were $PA_{224-233}$-specific, but none of the $PA_{224-233}$-dominant mice showed any appreciable $NP_{366-374}$ response (Figure 3.1 F, G). As expected, neonates displayed fewer overall $CD8^+$ T cells in the alveolar spaces during infection, which translates to lower overall numbers of antigen-specific T cells in this location as well (Figure 3.1 I). This is consistent with our previous work suggesting that neonatal $CD8^+$ T cells are unable to efficiently enter the airways during infection (Lines et al., 2010). These data indicate that pups display a $PA_{224-233}$ response instead of the adult co-dominant $NP_{366-374}$ and $PA_{224-233}$-specific response.

3.2.2 Neonatal influenza virus infection generates weak T cell memory and results in altered $CD8^+$ specificity during secondary infection

A significantly altered immunodominance hierarchy during a primary infection likely impacts the specificity of the memory response generated by that infection (Chen et al., 2004a; Crowe et al., 2003). We used heterosubtypic influenza virus infections to examine memory responses in mice first infected at 2 days or 8 weeks of age. HKx31 is an artificial reassortant influenza virus strain that shares all internal components including $NP_{366-374}$ and $PA_{224-233}$ with PR8 while differing with respect to exterior components like HA and NA. HKx31 is H3N2 and PR8 is H1N1. Therefore, only T cell

memory to the internal components of either virus will be protective when mice are challenged with the other virus. Mice were infected with a LD_{10} dose of HKx31 or PR8 and then rechallenged 2 months later with a lethal dose of PR8 or HKx31, respectively. Mice first infected as pups demonstrated more weight loss than mice first infected as adults, although both groups were better protected than naïve animals. All naïve mice were euthanized by day 7 of infection when they had lost 20% of starting body weight (Figure 3.2 A). Mice first infected as pups had significantly fewer IFNγ positive $CD4^+$ and $CD8^+$ T cells than mice first infected as adults at day 9 of infection (Figure 3.2 B). Mice first infected as pups showed similar numbers of $CD8^+$ T cells in the lung digest to the numbers displayed by mice first infected as adults (Figure 3.2 C), but they had fewer $CD8^+$ T cells in the BAL at day 5 of infection (Figure 3.2 D).

The secondary response immunodominance hierarchy in adult C57BL/6 mice has been shown to strongly favor $NP_{366-374}$ over $PA_{224-233}$ (Crowe et al., 2003), and our data correlates well with this. Mice first infected as adults demonstrated high levels of $NP_{366-374}$-specific $CD8^+$ T cells in whole-lung digest that arose around day 5 of infection and persisted through day 10 of infection (Figure 3.3 A). In contrast, levels of $PA_{224-233}$-specific cells remained easily detectable throughout the infection but never rose significantly above starting levels (Figure 3.3 B). Mice first infected as pups also demonstrated clear populations of $NP_{366-374}$-specific $CD8^+$ T cells, but they arose around day 7 of infection instead of day 5 (Figure 3.3 A). Unlike the mice first infected as adults, mice first infected as pups developed appreciable populations of $PA_{224-233}$-specific $CD8^+$ T cells (Figure 3.3 B). These cells also arose around day 7 of infection. These data suggest that primary influenza virus infection as a neonate alters the specificity and

kinetics of secondary influenza virus challenges in comparison to primary and secondary influenza virus infections in adults.

3.2.3 Adoptive transfer of bone marrow-derived dendritic cells alters $CD8^+$ T cell immunodominance during influenza virus infection and improves influenza virus clearance in pups

Poor activation by neonatal dendritic cells has been implicated in many of the altered T cell responses observed in neonates (Malloy et al., 2017; Ruckwardt et al., 2014; Ruckwardt et al., 2018). We transferred BMDCs stimulated with LPS and incubated with influenza virus or HBSS into 2-day-old pups before infecting them with an LD_{10} dose of influenza virus. At day 3, 5 and 7 post-infection, similar numbers of $CD4^+$ T cells (Figure 3.4 A) as well as activated (CD44hi CD62L lo) $CD4^+$ T cells (Figure 3.4 B) were found in the lung digests of mice that received either treatment. Similar results were found for $CD8^+$ T cell populations (Figure 3.4 C, D). Both treatments were associated with similar proportions of IFNγ-producing $CD8^+$ T cells in the lung digest at days 7 and 10 post-infection (Figure 3.4 E).

Despite these similarities in the overall magnitude of the response, transfer of PR8-infected BMDCs alters the appearance of antigen-specific $CD8^+$ T cell populations in the lungs. Significantly more $NP_{366-374}$ and $PA_{224-233}$-specific $CD8^+$ T cells were detected at day 7 post-infection in pups that received PR8 infected BMDCs than pups that received control BMDCs (Figure 3.5 A). By day 10 post-infection, $NP_{366-374}$ and $PA_{224-233}$-specific $CD8^+$ T cells were easily detectable in both treatment groups (Figure 3.5 A). Finally and most intriguingly, pups that received PR8 infected BMDCs cleared the virus by day 10 of infection while those that received control BMDCs did not (Figure 3.5 B). These data suggest that providing adult-like dendritic cell activation to pups allows them to respond

to $NP_{366-374}$ and that influenza virus-loaded dendritic cells improve viral clearance in pups.

3.3 Discussion

$CD8^+$ immunodominance during PR8 infections in adult C57Bl/6 mice is stable and predictable with $NP_{366-374}$ and $PA_{224-233}$ as clearly dominant epitopes during primary infections (Belz et al., 2000). Recent work, however, suggests that established immunodominance hierarchies may not hold in neonatal infections (Carey et al., 2016; Ruckwardt et al., 2011). If neonates cannot be guaranteed to mirror adult hierarchies, then the patterns of neonatal immunodominance and the means to manipulate neonatal immunodominance must be understood on their own terms. Subunit and peptide vaccines require predictable immunodominance hierarchies, and if infants do not respond to the same subunits as adults, then subunit T cell vaccines could be ineffective for this vulnerable population.

In our study, we found that neonatal C57BL/6 mice did not respond to the adult dominant $CD8^+$ T cell epitope of $NP_{366-374}$ during primary PR8 infections. Instead, pups responded to the $PA_{224-233}$ epitope (Figure 3.1). Heterosubtypic challenges in mice infected as pups or as adults displayed altered $CD8^+$ T cell response timing and immunodominance (Figure 3.3) that correlated with increased morbidity in mice first infected as pups (Figure 3.2). Transfer of adult BMDCs partially shifted the neonatal $CD8^+$ response to a more adult-like immunodominance hierarchy, with PR8 infected

BMDCs providing an earlier expansion of PR8-specific $CD8^+$ T cells and improved viral clearance when compared with mice that received unloaded BMDCs (Figure 3.5).

Effective clearance of viral pathogens, including influenza virus, requires antigen-specific $CD8^+$ T cells (Graham et al., 1994). We have previously shown that neonates display reduced homing of T cells to the lungs and airways during influenza virus infection (Lines et al., 2010). Neonates are able to clear low levels of influenza virus, although they do so much less effectively than adults (Lines et al., 2010). While $CD8^+$ T cells certainly play a major role in adult infections, it is unclear how significant their contribution is to the limited neonatal anti-viral response. C57BL/6 pups do not recognize the immunodominant adult $CD8^+$ T cell epitope $NP_{366-374}$. This deficit is clear in both the lung parenchyma and the airspaces (Figure 3.1 B-D). A recent study suggests that MHC-peptide tetramers can underestimate true antigen-specific cell populations, especially for low-affinity T cells such as tumor infiltrating lymphocytes or autoreactive T cells (Rius et al., 2018). It is possible that the lack of tetramer staining in neonatal lung digest and BAL is due to low-affinity anti-$NP_{366-374}$ cells instead of an absence of those cells.

Neonatal T cells are hyporesponsive *in vivo,* with poor IFNɣ production and proliferation (Basha et al., 2014; Graham et al., 1994; You et al., 2008). With proper stimulation, however, they are able to respond more robustly (Adkins et al., 1994). When a previous student cultured $CD8^+$ T cells from pups and adults with bone marrow derived dendritic cells (BMDCs) we saw that PR8 was able to provoke a strong IFNɣ response comparable to that produced by adult $CD8^+$ T cells (Lines, 2010). Conversely, $NP_{366-374}$ loaded BMDC stimulation was not enough to promote an adult level response to NP_{366-}

$_{374}$, supporting the idea that the $CD8^+$ population in pups simply lacks adult levels of $NP_{366-374}$-specific cells (Figure 3.1 A).

There is some controversy as to whether pups lack $NP_{366-374}$-specific $CD8^+$ T cells in primary infections with one study suggesting a diminished response in pups infected on day 3 or 7 of life (Carey et al., 2016), and another suggesting that pups respond to $NP_{366-374}$ at similar levels as adults (Zens et al., 2017). The chief difference between these studies was the age of the pups with 2-week-old pups responding to $NP_{366-374}$, 7-day-old pups, responding minimally, and the 3-day-old pups that, like the 2-day-old pups in this study, generally lacked $NP_{366-374}$-specific $CD8^+$ T cells altogether. This paints a picture of a rapidly evolving $CD8^+$ response with different immunodominance hierarchies at different ages. Two-week-old pups are still immunologically immature in other ways, but perhaps the altered immunodominance we observed is a feature only of the early neonatal period.

While $NP_{366-374}$ is the dominant $CD8^+$ epitope in adult infections, other epitopes such as $PA_{224-233}$ also generate detectable responses in adult primary infections (Cukalac et al., 2014). We looked to these subdominant epitopes to find potential targets for neonatal T cells. We found that pups display robust $PA_{224-233}$-specific populations visible through tetramer analysis of pup whole-lung digest and BAL (Figure 3.1 B-E). In pups, $PA_{224-233}$-specific cells make up a similar proportion of total $CD8^+$ T cells in pup and adult lungs, suggesting that this is a major population in neonatal anti-influenza virus responses (Figure 3.1 F). Neonates typically display few lymphocytes in the alveolar spaces during influenza virus infection, as we have previously shown (Lines et al., 2010),

but a large percentage of what $CD8^+$ T cells are there are $PA_{224-233}$-specific (Figure 3.1 G, I).

Altered immunodominance in primary infections has important implications for vaccination. There is currently a significant effort in developing a universal influenza virus vaccine which is likely to rely on a subunit or peptide antigen (Estrada et al., 2019). We were concerned that altered immunodominance in neonates could cause problems when using these kinds of vaccines in young populations. Studies on herpes simplex virus 1 (HSV-1) have suggested that neonatal infections can "lock in" suboptimal immunodominance hierarchies for subsequent adult infections (Rudd et al., 2013). Additionally, it has been demonstrated in influenza virus infections that T cell memory from neonatal infections is less robust than memory from adult infections (Connors et al., 2018; Zens et al., 2017). In our model, the memory from a pup primary infection is clearly protective, as the animals survived an infection that was lethal to naïve animals (Figure 3.2 A), but differences in the responses still exist and may explain the relatively more severe disease course experienced by mice first infected as pups.

Mice first infected as adults show the previously described (Chen et al., 2004a; Crowe et al., 2003) strongly $NP_{366-374}$ dominant secondary response. Many of the mice first infected as pups also have a strong $NP_{366-374}$ response, however, the pattern was less predictable than in mice first infected as adults (Figure 3.3 A). A few of the animals first infected as pups were even $PA_{224-233}$ dominant in the secondary response, maintaining the neonatal immunodominance pattern (Figure 3.3 B). It has been suggested that a predominately $PA_{224-233}$-specific response might be detrimental in secondary infections

(Crowe et al., 2003), but we did not have enough $PA_{224-233}$ dominant animals to observe this effect.

Intriguingly, although both groups displayed similar levels of total $NP_{366-374}$ and $PA_{224-233}$-specific $CD8^+$ T cells by day 7 of infection, there was an earlier onset of $NP_{366-374}$-specific expansion in the mice first infected as adults (Figure 3.3 A).

Given that neonates seemed capable of responding to $NP_{366-374}$ in a few individual animals, we wanted to find a way to force a more consistent $NP_{366-374}$-specific response in pups. For this we turned to dendritic cells. Neonatal dendritic cells have been shown to be much less stimulatory than adult dendritic cells *in vivo* due to a number of factors including lower expression of costimulatory molecules and MHC class I and II (Ruckwardt et al., 2014). Studies of respiratory syncytial virus (RSV) infection in pups suggest that there are several populations of dendritic cells in the lungs of pups early in life. They found that one of these phenotypes, a $CD103^+$ population, was partially responsible for the altered neonatal hierarchy observed in RSV infected pups (Malloy et al., 2017; Ruckwardt et al., 2018). We reasoned that in influenza virus infections, adult bone marrow-derived dendritic cells (BMDCs) might make pups respond more like adults, immunodominance hierarchy included.

Pups that received BMDCs developed an $NP_{366-374}$-specific population, whether or not the dendritic cells were loaded with PR8, supporting the idea that neonatal dendritic cells may be responsible for the pups' altered hierarchy (Malloy et al., 2017; Ruckwardt et al., 2018). It is not surprising that both unloaded and PR8 loaded BMDCs could promote $NP_{366-374}$-specific $CD8^+$ T cell expansion, as unloaded BMDCs will also likely take up PR8 during an infection. However, while both BMDC treatments promoted

$NP_{366-374}$-specific $CD8^+$ populations at the peak of infection, PR8 loaded BMDCs did improve the time needed to clear the virus (Figure 3.5 B). This correlates with an increase in total $NP_{366-374}$ and $PA_{224-233}$-specific $CD8^+$ T cells at day 7 post-infection (Figure 3.5 A). Given that there were not differences in total $CD4^+$ or $CD8^+$ T cells, activation status of those cells or IFNɣ production, we suspect that this altered onset of antigen-specific $CD8^+$ T cell expansion may be an important factor in explaining the improved viral clearance PR8 loaded BMDCs provide (Figure 3.4). If $NP_{366-374}$-specific cells are more effective for enacting influenza viral clearance in pups as they are in adults (Crowe et al., 2005), then it is possible that it is the early onset of $NP_{366-374}$-specific cells rather than total $NP_{366-374}$ and $PA_{224-233}$-specific cells that improves viral clearance in these animals. Chapter 4 will address this question.

These data could contrast with the idea that neonates do not respond to $NP_{366-374}$ due to the neonates' immature TCR repertoires (Zens et al., 2017), but perhaps both concepts could be important factors in establishing the altered neonatal immunodominance hierarchy. Adult-like antigen presentation from BMDCs stimulated $NP_{366-374}$-specific $CD8^+$ T cells in the pups, but not to the clearly $NP_{366-374}$-dominant levels observed in adult infections. It is possible that this remaining difference is due to the immature TCR repertoire.

In conclusion, these data show a consistent shift in neonatal C57BL/6 $CD8^+$ T cell immunodominance during PR8 infection that influences but does not guarantee the hierarchy of secondary infections. Additionally, this altered hierarchy can be manipulated by altering the quality of dendritic cell activation available to neonatal $CD8^+$ T cells, such as provided by BMDCs. These data suggest that age should be considered

when designing subunit vaccines for infants, but that with proper stimulation, it may be possible to guarantee a response to the same targets in neonates and adults. Manipulating neonatal immune responses requires a delicate touch. Adult magnitude responses are effective for clearing many pathogens, but neonates frequently cannot tolerate the immunopathology that accompanies such robust responses (Harbeson, Ben-Othman, et al., 2018; Harbeson, Francis, et al., 2018). Improving infection outcomes in neonates will necessitate highly specific interventions such as expanding virus-specific T cell populations while avoiding more global T cell activation. We believe that neonatal dendritic cells are a promising target for accomplishing this goal.

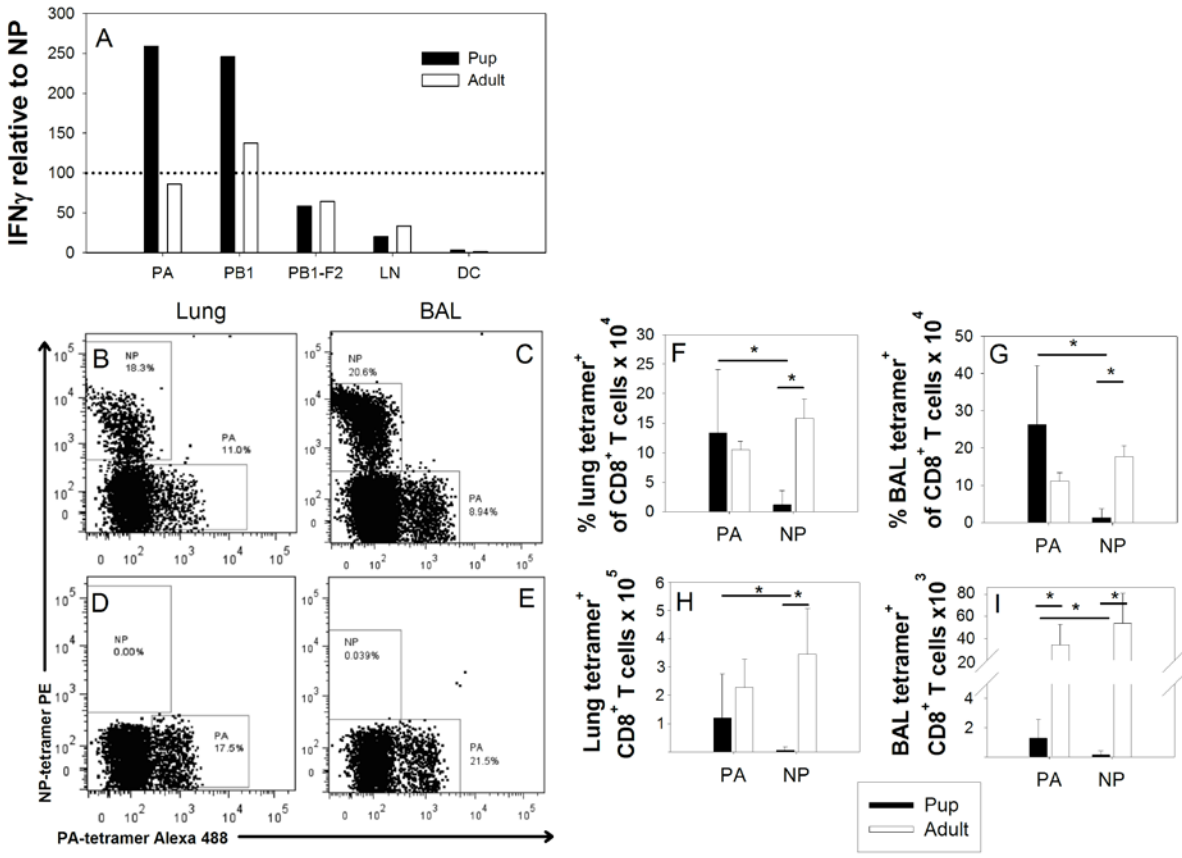

Figure 3.1 Pups display $PA_{224-233}$ dominance rather than the adult $NP_{366-374}$ and $PA_{224-233}$ codominance.

Adult or 2-day-old mice were infected i.n. with an LD_{10} dose of the PR8 strain of influenza virus. At day 10 post-infection, TBLNs were obtained from infected mice and cells were co-cultured with peptides and BMDCs. At day 3, supernatants were taken and IFNγ concentration was measured by ELISA (A). Cells from whole-lung digest, as well as bronchial alveolar lavage (BAL), were also obtained at day 10 post-infection and stained with anti-$CD8^+$ antibody as well as H-2D(b) $NP_{366-374}$ and H-2D(b) $PA_{224-233}$ tetramers. Representative scatter plots gated on $CD8^+$ cells are shown for cells from

adult lung (B) and BAL (C) as well as pup lung digest (D) and BAL (E). Data is summarized as the percent of lung $CD8^+$ T cells that are also tetramer positive in the lung digest (F) and BAL (G). Total numbers of tetramer-positive $CD8^+$ T cells are also shown for lung digest (H) and BAL (I). Data show mean ± SD for two separate experiments with at least 8 mice per group. * $P < 0.05$ by student's T test.

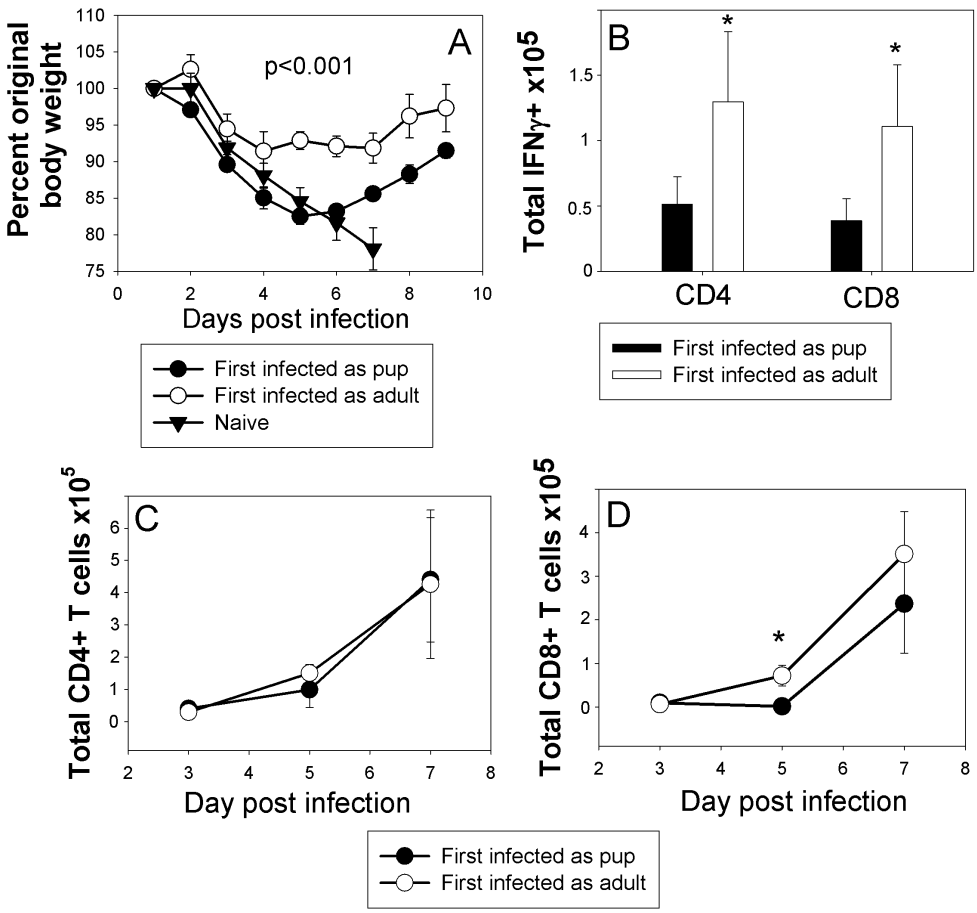

Figure 3.2 Mice infected as pups lose more weight and display altered T cell responses compared to mice infected as adults.

Adult or 2-day-old C57BL/6 mice were infected i.n. with an LD_{10} dose of the X-31 strain of influenza virus before being infected again with an LD_{100} dose of the PR8 strain of influenza virus 8 weeks later. Average percent of starting body weight is shown for the secondary infection (A). Total numbers of $CD4^+$ and $CD8^+$ lymphocytes that were also IFN_γ^+ are shown for the BAL at day 7 post-infection (B). Total $CD4^+$ (C) and $CD8^+$ (D) lymphocytes in the lung digest were assessed by flow cytometry. Data show mean ± SD for two separate experiments with at least 5 mice per group. * $p < 0.05$ compared to mice first infected as pups at the same time point by one way or Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's pairwise *post-hoc* test.

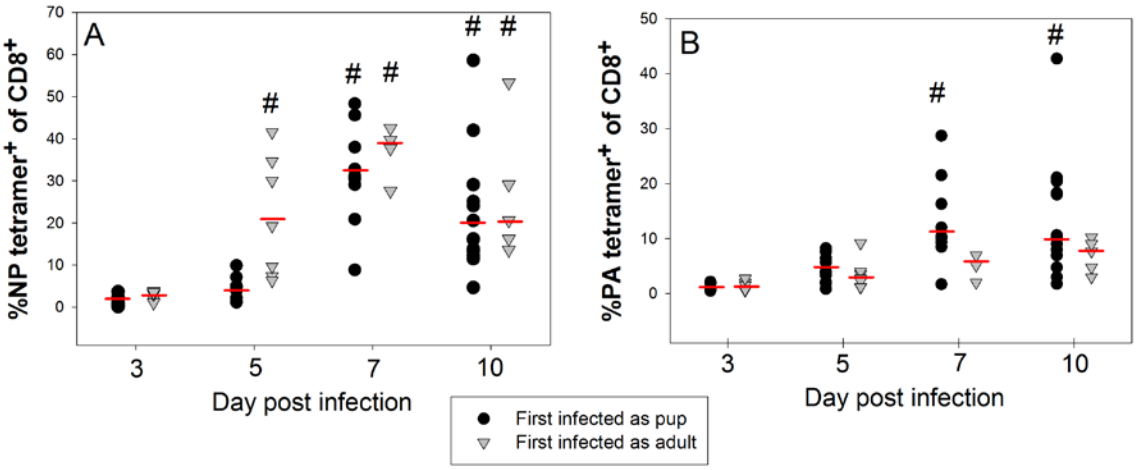

Figure 3.3 Mice infected as pups display altered $CD8^+$ T cell responses compared to mice infected as adults during secondary infections.

Adult or 2-day-old C57BL/6 mice were infected i.n. with an LD_{10} dose of the X-31 strain of influenza virus before being infected again with an LD_{100} dose of the PR8 strain of influenza virus 8 weeks later. Lung digests were acquired at day 3, 5, 7 and 10 of infection and stained with anti-$CD8^+$ antibody as well as H-2D(b) $NP_{366\text{-}374}$ and H-2D(b) $PA_{224\text{-}233}$ tetramers. Percent NP $tetramer^+$ (A) or PA $tetramer^+$ (B) of $CD8^+$ T cells in the lung digest was determined by flow cytometry. Data show individual mice and mean for pooled data from two separate experiments with at least 4 mice per group. # $p<0.05$ compared to mice in the same group at day 3 of infection by one way ANOVA followed by Holm-Sidak *post-hoc* test for pairwise comparisons.

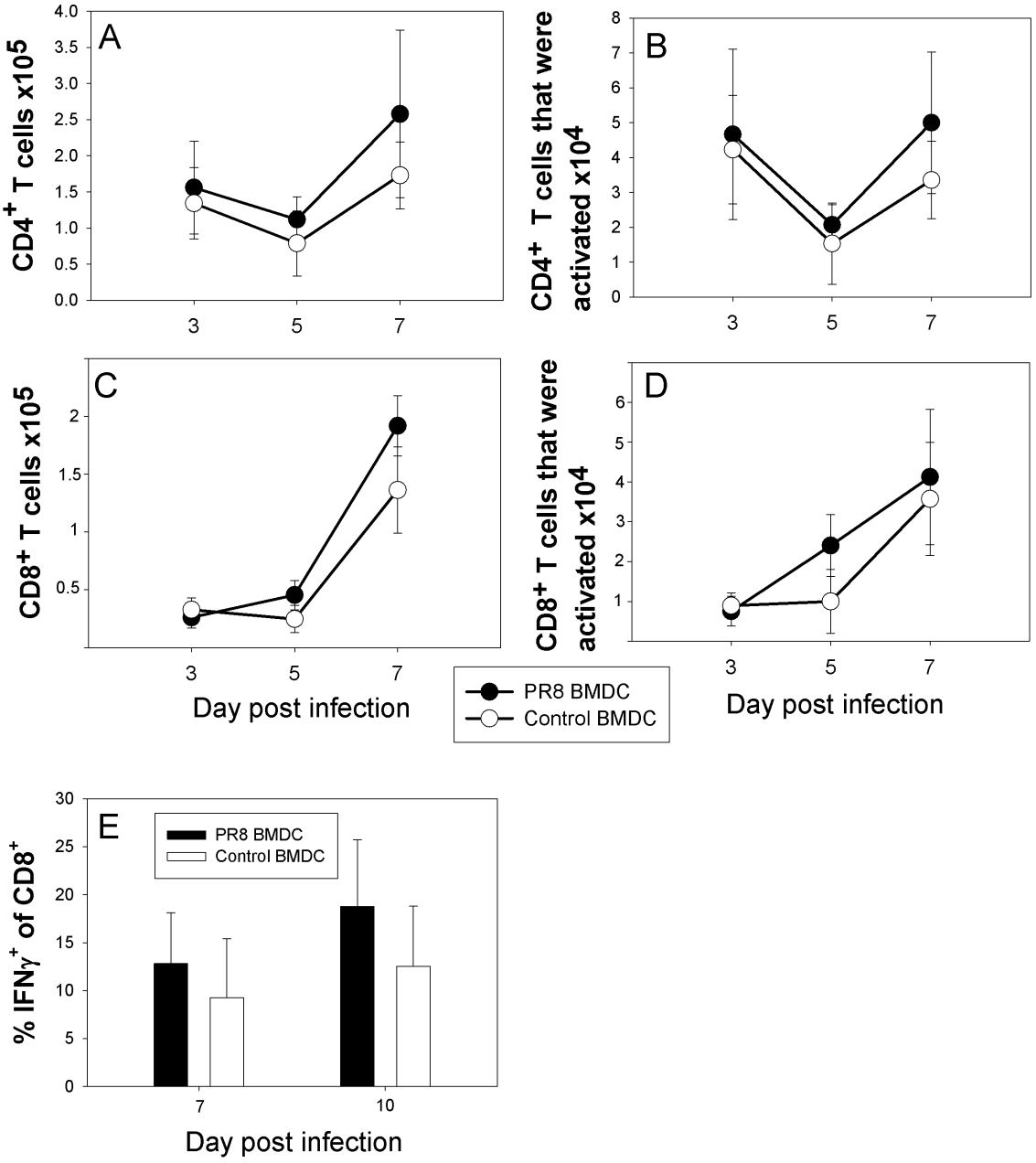
A
B
C
D
E
CD4+ T cells x10^5
CD4+ T cells that were activated x10^4
CD8+ T cells x10^5
CD8+ T cells that were activated x10^4
Day post infection
PR8 BMDC
Control BMDC
% IFNγ+ of CD8+

Figure 3.4 Adoptive transfer of PR8 infected BMDCs minimally effects overall T cell responses during influenza virus infection.

BMDCs were generated from C57Bl/6 adult female mice and treated with LPS before incubation with the PR8 strain of influenza virus or HBSS. Two-day-old C57BL/6 mice were injected with 10^4 BMDCs before being infected i.n. with an LD10 dose of the PR8 strain of influenza virus. Total $CD4^+$ (A), total activated ($CD44^+$ $CD62L^{lo}$) $CD4^+$ T cells (B), total $CD8^+$ (C), and total activated ($CD44^+$ $CD62L^{lo}$) $CD8^+$ T cells (D) were assessed by flow cytometry in the lungs of infected pups on day 3, 5 and 7 post-infection. Proportion of $CD8^+$ T cells that were also IFN_γ^+ was assessed by flow cytometry on day 7 and 10 post-infection (E). Data show mean ± SD for two separate experiments with at least 5 mice per group at each time point.

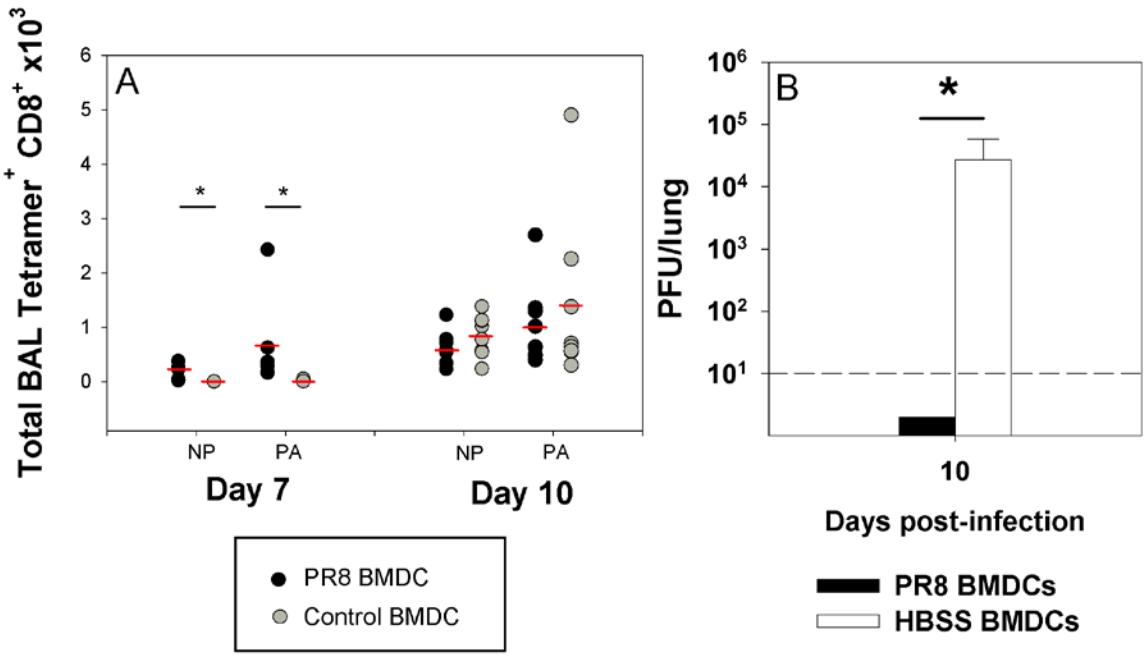

Figure 3.5 Adoptive transfer of PR8 infected BMDCs improves viral clearance and alters immunodominance during neonatal influenza virus infection.

BMDCs were generated from C57Bl/6 adult female mice and treated with LPS before incubation with the PR8 strain of influenza virus or HBSS. Two-day-old C57BL/6 mice were injected with 10^4 BMDCs before being infected i.n. with an LD10 dose of the PR8 strain of influenza virus. Total $CD8^+$ T cells that were NP or PA $tetramer^+$ in the BAL was assessed by flow cytometry on day 7 and 10 post-infection (A). Viral burden at day 10 post-infection was assessed by plaque assay (B). Data show individual mice and mean or mean ± SD for two separate experiments with at least 6 mice per group. Panel B used with permission of Samantha Jewell. * $p < 0.05$ by student's T test.

CHAPTER 4. PEPTIDE VACCINATION WITH $PA_{224-233}$ DOES NOT PROTECT PUPS DURING INFLUENZA VIRUS INFECTION

4.1 Introduction

Vaccinating infants is one of the most important early life health interventions in neonatal care (Rao et al., 2019). Vaccine schedules have been developed to optimize the intersection of the developing neonatal immune system with the observed outcomes of specific vaccines to maximize protection for infants as early in life as possible (Robinson et al., 2019). Unfortunately, effective responses to some vaccines require infants to be 6 months old or even a year old before protective immunity can be established, leaving a window of vulnerability to these diseases (Grohskopf et al., 2018).

Influenza virus is a significant cause of neonatal respiratory disease, and although a vaccine exists for this virus, it cannot be administered before 6 months of age (Antonova et al., 2012; Nelson et al., 2014; Neuzil et al., 2002). Additionally, it requires two injections for full protection as opposed to the one injection adults require (Grohskopf et al., 2018). On top of this, influenza virus vaccination is required yearly due to viral antigenic drift and even these efforts only result in protection rates approaching 60% on the most successful years (Doyle et al., 2019; Reed et al., 2015; Thommes et al., 2017).

Significant effort is devoted to trying to increase the protection of influenza virus vaccination by generating responses that are cross-reactive against more than one virus strain (Estrada et al., 2019; Gottlieb et al., 2014; Xiang et al., 2015). These methods stimulate B or T cell responses that target conserved regions of the virus such as the stalk

region of the influenza virus surface protein HA or conserved regions of internal components like NP (Krammer et al., 2019). Conserved regions of viral proteins typically cannot mutate readily without decreasing viral fitness, so it is difficult for viruses to escape immune responses to these epitopes (Kosik et al., 2019). Broadly neutralizing antibodies are generated by a small subset of influenza virus infected humans, and work is being done to replicate this phenomenon reliably in a vaccination setting (Morgan et al., 2016; van Doorn et al., 2017).

$CD8^+$ T cells are also the target of vaccination efforts for pathogens like influenza viruses. These vaccines are often considered in conjunction with B cell responses rather than as a standalone vaccine (Laidlaw et al., 2013; Lee et al., 2019). Studies of influenza disease severity in humans suggests that $CD8^+$ memory confers cross-reactive protection against severe morbidity and mortality but not the complete neutralizing protection antibody mediated vaccines provide against a single strain of influenza virus (Sridhar et al., 2013). This still leaves $CD8^+$ vaccination as a potentially important mechanism of reducing disease severity with protection that is resistant to antigenic drift. Reduced morbidity during influenza virus infection improves wellbeing among normal adult populations, but among vulnerable populations such as infants and the elderly, the difference in severity might significantly alter mortality risk.

If vaccines targeting $CD8^+$ T cells are added to conventional influenza vaccine strategies, it is important to ascertain whether they will function the same in neonates as in adults. Neonates have well-documented alterations in T cell function compared to adults. These include reduced numbers and infiltration into infected tissue, diminished cytotoxicity, and reduced cytokine production. We have also shown along with others

that neonates demonstrate an altered $CD8^+$ T cell immunodominance hierarchy (Cukalac et al., 2014; Malloy et al., 2017; Ruckwardt et al., 2011). These factors raise concerns for whether a $CD8^+$ T cell vaccine would work in neonates, especially if viral subunits are chosen for T cell stimulation that neonates cannot respond to.

In this study we show that pups can respond to the influenza virus peptide $PA_{224-233}$ delivered by adult bone marrow derived dendritic cells (BMDCs), but not to $NP_{366-374}$. This vaccine results in a $PA_{224-233}$-specific memory response during reinfection without significantly altering the overall level of inflammation. $PA_{224-233}$ vaccinated pups, however, are not protected during lethal influenza virus challenge. Pup lung tissue is able to stimulate adult $NP_{366-374}$ and $PA_{224-233}$-specific $CD8^+$ T cells. These data suggest that age is an important factor when considering targets for subunit vaccines targeting $CD8^+$ T cells.

4.2 Results

4.2.1 BMDC-peptide vaccination in pups generates $PA_{224-233}$ but not $NP_{366-374}$-specific $CD8^+$ T cells.

The neonatal $CD8^+$ immunodominance hierarchy is altered compared to the adult hierarchy in mice across several different infections (Malloy et al., 2017) including influenza virus infection. We wanted to test whether neonatal mice could respond to adult dominant epitopes when vaccinated with bone marrow derived dendritic cells (BMDCs) loaded with peptide. We incubated BMDCs generated from adult C57BL/6 bone marrow with both $PA_{224-233}$ and $NP_{366-374}$ or with each peptide separately. We then

injected peptide loaded-BMDCs I.P. into 2 day old pups or 8 week old adults and examined antigen specific cells in the spleen by flow cytometry 14 days after vaccination. Pups vaccinated with both peptides responded to $PA_{224-233}$, but not $NP_{366-374}$ (Figure 4.1 A). When pups were vaccinated with each peptide separately, the animals also responded to $PA_{224-233}$, but not $NP_{366-374}$ (Figure 4.1 B). Adults vaccinated with both peptides and CpG responded strongly to $PA_{224-233}$. $NP_{366-374}$-specific cells were present in vaccinated adults, but only at low levels (Figure 4.1 C). Adults vaccinated separately with BMDCs pulsed with $NP_{366-374}$ or $PA_{224-233}$ responded to the peptide they received (Figure 4.1 D). These data suggest that unlike adults, pups cannot respond to $NP_{366-374}$ by BMDC vaccination.

4.2.2 Pups vaccinated with peptide loaded BMDCs display memory responses to $PA_{224-233}$ but not $NP_{366-374}$.

Influenza virus specific memory $CD8^+$ T cell responses protect against subsequent infections resulting in reduced morbidity and mortality. To test whether peptide vaccinated pups would display a memory response upon viral challenge, we vaccinated 2 day old pups with $PA_{224-233}$ or $NP_{366-374}$ loaded BMDCs as described before. Fourteen days post vaccination, we infected the mice with an LD_{10} dose of PR8 influenza virus and assessed the response in the whole lung digest, bronchioalveolar lavage, and the tracheobronchial lymph node 7 days after infection. Reinfection at 14 days post infection was chosen to ensure that the mice were still less than 3 weeks of age at reinfection. Pups vaccinated with $PA_{224-233}$ displayed elevated levels of $PA_{224-233}$-specific $CD8^+$ T cells in the whole lung digest compared to unvaccinated or $NP_{366-374}$ vaccinated pups, but NP_{366-}

$_{374}$ vaccinated pups did not display elevated $NP_{366-374}$-specific $CD8^+$ T cells compared to unvaccinated or $PA_{224-233}$ vaccinated pups (Figure 4.2 A). There were not significant differences between $PA_{224-233}$ or $NP_{366-374}$-specific $CD8^+$ T cell populations in the TBLN (Figure 4.2 B). Vaccinated and unvaccinated pups displayed similar concentrations of IFNγ in the BAL regardless of the peptide used (Figure 4.2 C). Additionally, vaccination did not impact the proportion of $CD4^+$ or $CD8^+$ T cells that were activated ($CD44^+$ $CD62L^{lo}$) (Figure 4.2 D). Vaccinated animals had higher proportions (Figure 4.2 E) and total numbers (Figure 4.2 F) of IFNγ producing $CD8^+$ T cells than unvaccinated animals and $PA_{224-233}$ vaccinated pups had higher proportions of IFNγ producing $CD4^+$ T cells than unvaccinated animals (Figure 4.2 E). These data suggest that $PA_{224-233}$ vaccination results in a robust $PA_{224-233}$-specific memory response in pups.

4.2.3 $PA_{224-233}$ peptide vaccination does not provide protection during lethal challenge in neonatal mice.

Effective $CD8^+$ memory can offer protection from infectious doses that would be lethal for naïve animals, but simply producing antigen specific T cells does not guarantee effective protection. We wanted to determine whether $PA_{224-233}$ vaccinated pups were protected during lethal challenge. We vaccinated 2 day old pups with peptide loaded BMDCs by i.p. injection. Fourteen days post vaccination, vaccinated and naïve mice were infected with a lethal dose of influenza virus. All mice lost weight (Figure 4.3 A), and neither vaccinated nor naïve animals survived the infection (Figure 4.3 B). These data suggest $PA_{224-233}$ vaccination offers no protection to pups during PR8 infection.

4.2.4 Pup lung tissue is able to stimulate both $NP_{366-374}$ and $PA_{224-233}$ specific $CD8^+$ T cells.

Although peptide loaded BMDCs were unable to stimulate pup responses specific for $NP_{366-374}$, future vaccination methods may be able to stimulate reliable responses to adult antigens in neonates. We wanted to test whether neonatal lung tissue could stimulate $NP_{366-374}$ specific $CD8^+$ memory T cells. We infected adult C57Bl/6 mice with PR8 influenza and isolated splenic $CD8^+$ T cells by MACS cell separation at day 8 of infection. Adult T cells were co-cultured with lung digest from naïve pups or adults or BMDCs and either $NP_{366-374}$ or $PA_{224-233}$ peptide. Supernatants were collected at day 3 of culture and IFNγ concentration was measured by ELISA. Pup lung tissue stimulated both $NP_{366-374}$ and $PA_{224-233}$-specific adult $CD8^+$ T cells (Figure 4.4 A). BMDCs also stimulated both $NP_{366-374}$ and $PA_{224-233}$-specific $CD8^+$ T cells. These data suggest that pup lungs are able to stimulate an IFNγ response from $NP_{366-374}$ specific $CD8^+$ T cells if they are generated during an immune response.

4.3 Discussion

Subunit vaccines rely on immunodominance to predict likely antigens that will provoke effective B or T cell responses across a population (Crowe et al., 2005). Work done characterizing adult $CD8^+$ T cell responses to influenza virus has shown that adult C57Bl/6 mice respond to $NP_{366-374}$ and $PA_{224-233}$ during primary PR8 infection (Belz et al., 2000). We have shown that pups respond to $PA_{224-233}$ but not $NP_{366-374}$. Although these specific peptides primarily relate to this particular mouse infection, altered

immunodominance in neonatal $CD8^+$ T cell responses has been seen in other infections as well, suggesting this is a general feature of T cell responses in early life (Ruckwardt et al., 2011). Altered immunodominance in neonates could prevent neonates from responding to subunit vaccines targeting known adult-dominant epitopes. A potential solution for shifted immunodominance is simply to target age appropriate antigens for vaccines used in neonatal or adult populations. The success of this strategy relies on two things. First, neonates must be able to respond to a chosen antigen, and secondly, the response to that antigen must be protective.

We found that mouse pups respond to $PA_{224-233}$ but not $NP_{366-374}$ vaccination using peptide loaded BMDCs (Figure 4.1). $PA_{224-233}$ vaccinated pups also display a robust $PA_{224-233}$-specific $CD8^+$ T cell response during influenza virus challenge, although overall T cell responses were not significantly elevated (Figure 4.2). Despite $PA_{224-233}$-specific memory, vaccinated pups were not protected from lethal challenge (Figure 4.3). Finally, pup lung tissue was able to activate adult $NP_{366-374}$-specific $CD8^+$ T cells (Figure 4.4).

We wanted to establish whether pups could be vaccinated with both $NP_{366-374}$ and $PA_{224-233}$. Pilot studies with both peptides and CpG as an adjuvant in adult vaccinations showed strong PA dominance, although some $NP_{366-374}$-specific cells were present (Figure 4.1 C). This is consistent with previous studies that showed that $PA_{224-233}$ is strongly dominant over $NP_{366-374}$ when antigen quantities are similar. Adult $PA_{224-233}$-specific cells have higher affinity than adult $NP_{366-374}$-specific cells which possibly allows them to become dominant when antigen is not limited (Cukalac et al., 2014). In an influenza virion, the quantity of NP far surpasses the quantity of $PA_{224-233}$ (Zheng et al., 2013), suggesting that in a natural infection, antigen availability is possibly not the same

for NP and PA, allowing $NP_{366-374}$ to become co-dominant. This may be particularly true in dendritic cells, as no viable viral replication occurs and the quantity of antigen in the actual virion may represent the antigen available for presentation to T cells. Some NP and HA synthesis occurs in DCs providing further evidence that NP may be available in much greater quantities for presentation on DCs (Ioannidis et al., 2012) .

Pups responded only to $PA_{224-233}$ when vaccinated with both peptides and BMDCs (Figure 4.1 A). This is probably not due to suppression of a $NP_{366-374}$ response by the $PA_{224-233}$ response as pups also did not respond to $NP_{366-374}$ when vaccinated with only $NP_{366-374}$ or $PA_{224-233}$ (Figure 4.1 B). Adults vaccinated with only $NP_{366-374}$ or $PA_{224-233}$ responded well to whichever peptide they received (Figure 4.1 D). These data show that even when delivered with adult BMDCs, pups do not respond to $NP_{366-374}$ peptide.

The $PA_{224-233}$ specific response exhibited by neonates expands appropriately during subsequent challenge, suggesting that the peptide vaccine succeeds in establishing $CD8^+$ T cell memory in pups (Figure 4.2 A). $NP_{366-374}$ vaccinated pups did not display similar expansion of $NP_{366-374}$-specific cells, confirming that there was no response to the $NP_{366-374}$ vaccine. Neither vaccine increased T cell activation significantly (Figure 4.2 D), but both vaccinated groups had more IFNγ producing cells than unvaccinated animals. This was not different between $NP_{366-374}$ and $PA_{224-233}$ vaccination, suggesting that this increase is not related to the expansion of $PA_{224-233}$-specific cells in $PA_{224-233}$ vaccinated pups. This is consistent with other work showing that pups respond in a more Th1 manner when given adult dendritic cells (van Haren et al., 2016).

If pups can respond well to $PA_{224-233}$, vaccinating with this peptide could provide protection, even if they fail to respond to $NP_{366-374}$. Adult studies have shown, however,

that $PA_{224-233}$-specific cells are not effective for anti-influenza viral defense. Adult mice vaccinated against $PA_{224-233}$ respond robustly, but they suffer greater morbidity and mortality than even naïve animals (Crowe et al., 2005). Dendritic cells can present both $NP_{366-374}$ and $PA_{224-233}$ equally well, as has been shown before (Crowe et al., 2003) and in Figure 4.4 B. Respiratory epithelium, however, was shown to only present $NP_{366-374}$ (Chen et al., 2004a). $PA_{224-233}$-specific $CD8^+$ T cells may be generated, but they may not recognize and target infected respiratory epithelial cells.

In a normal adult infection, $NP_{366-374}$ and $PA_{224-233}$-specific cells are activated by dendritic cells, but only $NP_{366-374}$-specific cells are able to recognize and kill infected cells. This seems to lead to more robust $NP_{366-374}$-specific memory and $NP_{366-374}$ dominance in secondary infection as secondary responses are thought to rely on infected tissue rather than dendritic cells for activation and expansion (Crowe et al., 2003). We saw this phenomenon clearly in our heterosubtypic infections (Figure 3.3). However, when adults are vaccinated for $PA_{224-233}$, the $PA_{224-233}$-specific cells strongly dominate during a challenge reaction and prevent an $NP_{366-374}$ response (Crowe et al., 2005). This lack of protection calls into doubt whether the $PA_{224-233}$ specific $CD8^+$ response exhibited by pups provides any benefit during influenza virus infection.

We saw that $PA_{224-233}$ vaccination provided no defense against lethal influenza virus challenge in pups (Figure 4.3 B). Simply vaccinating against $PA_{224-233}$ will not resolve the problems with the neonatal $CD8^+$ T cell response to PR8 influenza. This agrees with findings that suggest neonatal primary infections can “lock in” suboptimal memory cells for subsequent infections (Rudd et al., 2013).

Finally, questions remain as to whether $NP_{366-374}$ specific cells are effective in pups. We saw that pup lung tissue was able to activate $NP_{366-374}$-specific and $PA_{224-233}$-specific $CD8^+$ T cells *in vitro* (Figure 4.4 A). This provides some evidence that pups could utilize $NP_{366-374}$-specific cells in the lungs if a vaccine manages to generate them, but it does not show which cells are activating the T cells. Ideally, we would want to check whether respiratory epithelial cells in pups present $NP_{366-374}$ and $PA_{224-233}$, but given the lack of protection in $PA_{224-233}$ vaccinated pups, it is likely that they follow the adult $NP_{366-374}$-only presentation pattern.

Given these results, we suggest that the goal of neonatal $CD8^+$ T cell vaccination should be to generate responses with adult specificity. Special consideration has been given to neonatal immune responses before when designing vaccines such as the development of conjugated vaccines that target polysaccharide antigens to protect against *Haemophilus influenza B* and *Neisseria meningitidis*. Neonates respond poorly to polysaccharide antigens without T cell help, so polysaccharide antigens were conjugated to protein carriers to provide T cell as well as B cell stimulation (D'Angio et al., 1995). Given that LPS stimulated PR8 infected BMDCs were able to generate an $NP_{366-374}$ specific response in neonatal primary infections (Figure 3.5 F), we hoped this would allow $NP_{366-374}$ peptide vaccination in pups, but it was evidently not sufficient to stimulate a $NP_{366-374}$-specific response. There are many vaccination strategies and adjuvants besides BMDCs loaded with peptide, and it is possible that one of these methods will be able to stimulate an $NP_{366-374}$-specific $CD8^+$ T cell response in neonates.

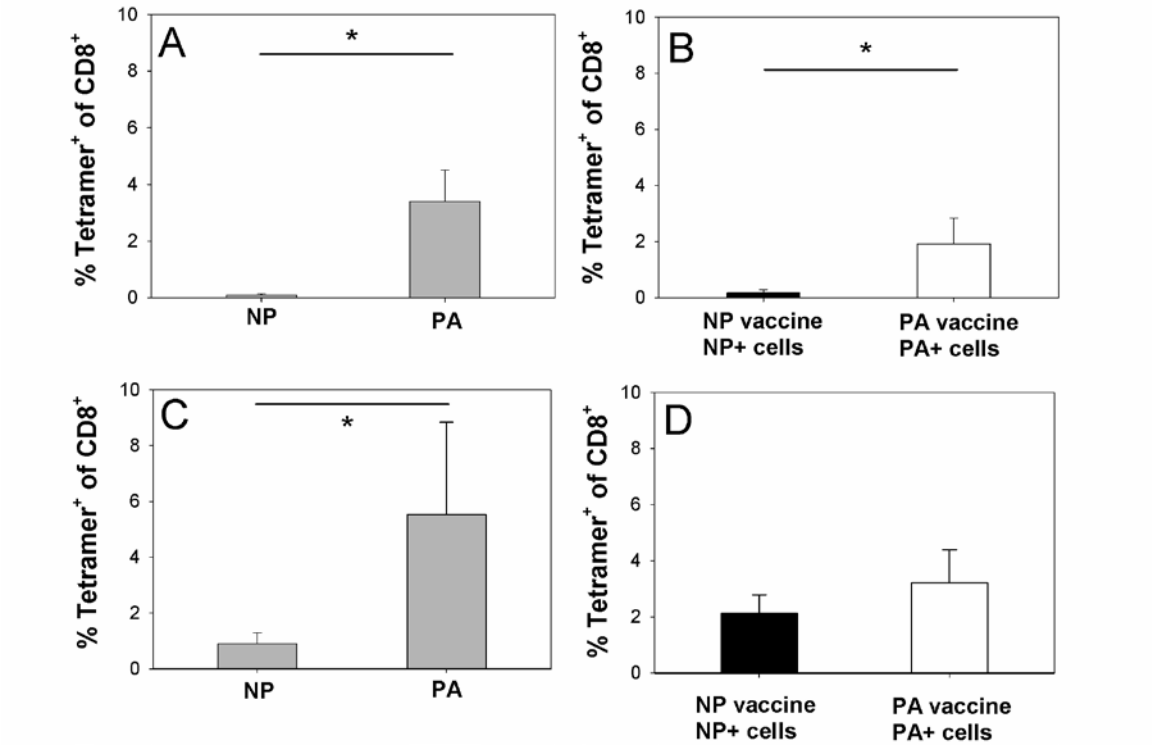

Figure 4.1 Pups respond to $PA_{224-233}$ peptide but not $NP_{366-374}$ peptide after BMDC vaccination.

BMDCs were generated from C57Bl/6 adult female mice and treated with LPS before incubation with $NP_{366-374}$ and $PA_{224-233}$ together or individually. 2 day old or adult mice were injected i.p. with 10^4 or 10^5 BMDCs respectively. At day 14 post vaccination, splenocytes were stained with anti-$CD8^+$ antibody as well as H-2D(b) $NP_{366-374}$ and H-2D(b) $PA_{224-233}$ tetramers. Tetramer+ cells were assessed in pups vaccinated with both $NP_{366-374}$ and $PA_{224-233}$ simultaneously by flow cytometry (A). After separate $NP_{366-374}$ or $PA_{224-233}$ vaccination, NP^+ cells in $NP_{366-374}$ vaccinated pups and PA^+ cells in $PA_{224-233}$ vaccinated pups (B) and adults (D) were assessed by flow cytometry at 14 days post vaccination. $Tetramer^+$ cells in adults vaccinated with $NP_{366-374}$ and $PA_{224-233}$ together was assessed by flow cytometry (C). Data show mean ± SD for two separate experiments with at least 4 mice per group. * $p < 0.05$ by student's T test.

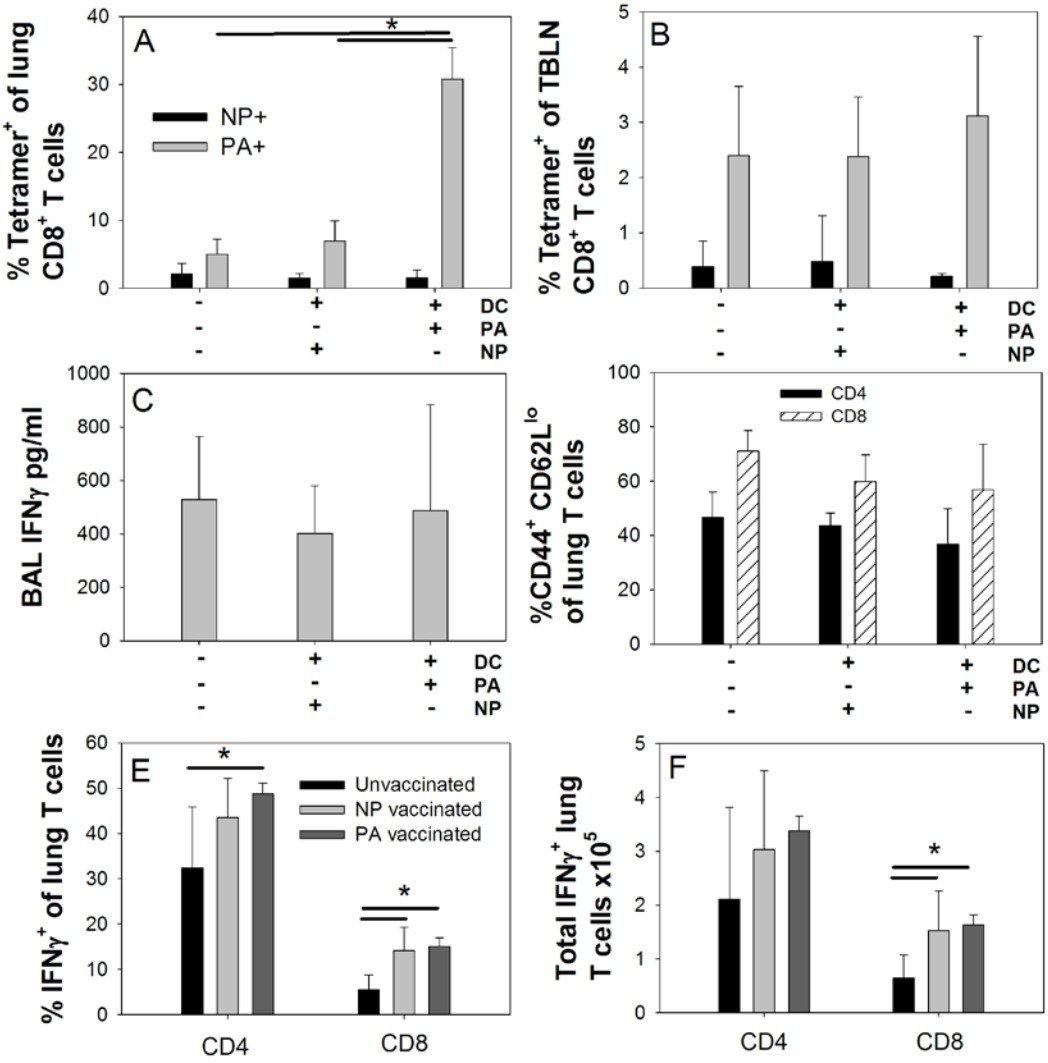
A
% Tetramer+ of lung CD8+ T cells
NP+
PA+
B
% Tetramer+ of TBLN CD8+ T cells
C
BAL IFNγ pg/ml
%CD44+ CD62Llo of lung T cells
CD4
CD8
DC
PA
NP
E
% IFNγ+ of lung T cells
Unvaccinated
NP vaccinated
PA vaccinated
F
Total IFNγ+ lung T cells x10^5
CD4
CD8

Figure 4.2 $PA_{224-233}$ vaccinated pups develop a $PA_{224-233}$-specific memory response when challenged with PR8 Influenza virus.

2 day old mice were injected i.p. with 10^4 BMDCs pulsed with $NP_{366-374}$ or $PA_{224-233}$ and then infected 14 days later with a LD_{10} dose of the PR8 strain of influenza virus. The percent NP or PA tetramer$^+$ of $CD8^+$ lymphocytes in the lung digest (A) and TBLN (B) at day 7 post infection was determined by flow cytometry. IFNγ was measured in the first wash of the BAL from day 7 post infection by ELISA (C). Percent activation ($CD44^{hi}$ $CD62L^{lo}$) of $CD4^+$ and $CD8^+$ lymphocytes on day 7 post infection was determined by flow cytometry (D). Proportion (E) and total numbers of $CD4^+$ and $CD8^+$ lymphocytes that are $IFN\gamma^+$ on day 7 post infection were determined by flow cytometry. Data show mean ± SD for two separate experiments with at least 4 mice per group. * $p< 0.05$ by one way ANOVA followed by Holm-Sidak *post-hoc* test for pairwise comparisons.

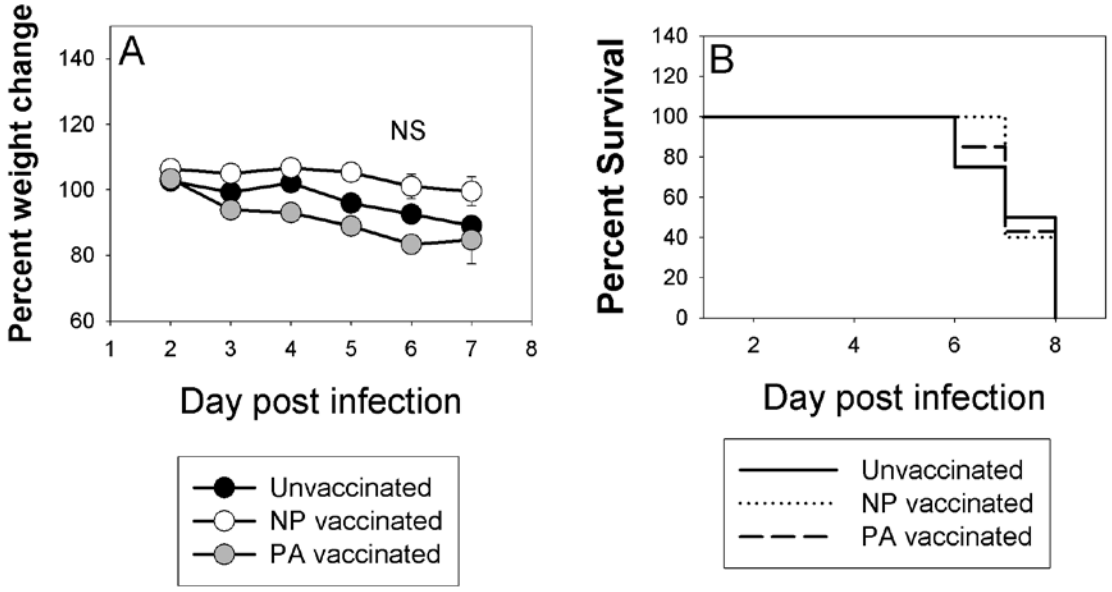

Figure 4.3 $PA_{224-233}$ peptide vaccination does not protect neonates during lethal infection. 2 day old mice were injected i.p. with 10^4 BMDCs pulsed with $NP_{366-374}$ or $PA_{224-233}$ and then infected i.n. 14 days later with an lethal dose of the PR8 strain of influenza virus. Mice were weighed daily (A). Pup survival was also determined daily (B). Weight data show mean ± SEM for two separate experiments with at least 4 mice per group. NS, Not significant by one way ANOVA followed by Holm-Sidak *post-hoc* test for pairwise comparisons.

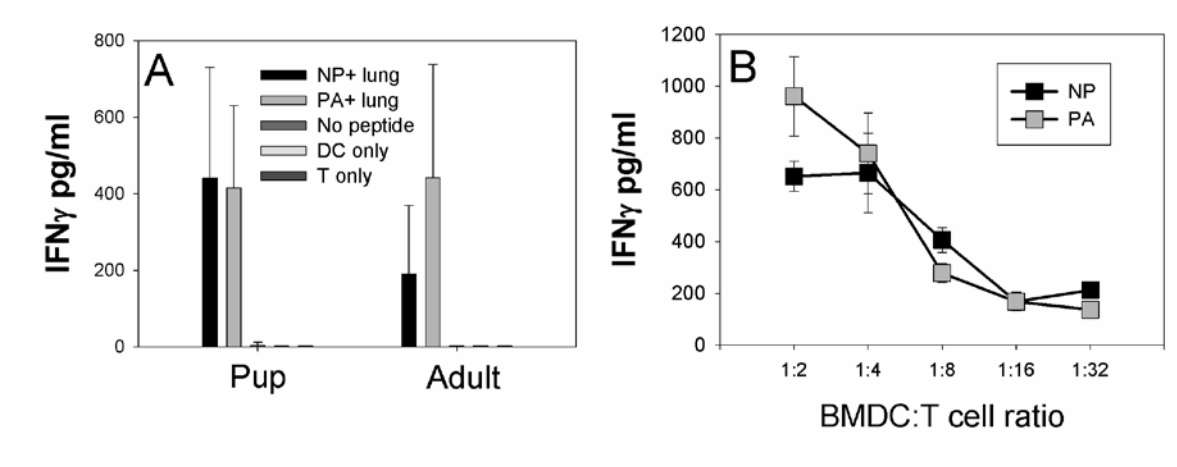

Figure 4.4 Pup lung tissue activates $NP_{366-374}$ and $PA_{224-233}$-specific adult memory $CD8^+$ T cells.

8 week old mice were infected with a LD_{10} dose of the PR8 strain of influenza virus and $CD8^+$ T cells were isolated from spleens on day 8 of infection. Lung digest from naïve 2 day old or adult mice (A) or BMDCs (B) were co-cultured with $CD8^+$ T cells and $NP_{366-374}$ or $PA_{224-233}$ peptides. Supernatants were collected on day 3 and IFNγ concentration was assessed by ELISA. Data represent mean ± SD (A) or SEM (B) for two separate experiments. n=4 mice per group (A) or 3 replicate wells (B).

CHAPTER 5. REGULATORY T CELLS DURING NEONATAL INFLUENZA VIRUS INFECTION.

5.1 Introduction

Immunopathology is a necessary consequence of immune responses that kill pathogens, but one that can be devastating to vulnerable populations such as neonates (Merkus, 2003). It has been proposed before that disease tolerance is an alternate strategy organisms may take to survive pathogen exposures (Medzhitov et al., 2012). The balance between response and tolerance to both pathogens and innocuous antigens is strongly skewed towards tolerance in neonates. This has been demonstrated in neonates' skewed T cell responses towards Th2 responses instead of Th1 (Adkins et al., 2002) as well as broad delays in activation and migration across multiple other cell types in response to infection (Lines et al., 2010). This results in increased susceptibility to many illnesses for neonates, but it is likely that the benefit of avoiding severe immunopathology is worth the weakened defense against pathogens.

Regulatory T cells (Tregs) are an important mediator of host tolerance both to self-antigens as well as pathogens. This is clearly demonstrated in scurfy mutant mice which lack Tregs due to a nonsense mutation in Foxp3, the master regulator transcription factor of Treg development. Scurfy mice develop massive autoimmunity and die by three weeks of age (Godfrey et al., 1991). Tregs suppress immune responses through a variety of mechanisms including expression of suppressive cytokines like TGFβ and IL-10. They also express high levels of CD25, the high affinity IL-2 receptor, allowing them to take up IL-2 and deny it to other T cells, limiting their growth (Vignali et al., 2008).

Tregs likely play a key role in neonatal immunosuppression. Tregs are crucial in protecting the fetus from maternal rejection during gestation (Michaelsson et al., 2006), and Treg levels remain high after birth (Hamza et al., 2015; Oliphant et al., 2015; Scharschmidt et al., 2015). Neonates also have a mechanism that promotes Treg differentiation as a sort of "default" outcome of T cell activation (Cheng et al., 2014; Wang et al., 2010).

Given that the bias towards tolerance diminishes neonatal defense against pathogens, elevated Tregs in neonates could underlie early life vulnerability to infection. Manipulating Tregs has been proposed as a way to augment immune responses; however efforts to remove Tregs to improve infection outcomes have revealed a much more complicated role for Tregs in infections of both adults and neonates than simple immunosuppression. Treg depletion unquestionably increases immunopathology during infection as expected (Lanteri et al., 2009; Lee et al., 2010; Suvas et al., 2004), but pathogen clearance is improved in adult mice for a wide variety of infections including tuberculosis (Scott-Browne et al., 2007), pneumocystis pneumonia (McKinley et al., 2006), and *Candida albicans* (Pandiyan et al., 2011). Conversely, Tregs are necessary or at least helpful for pathogen clearance in other adult infections including West Nile virus (Graham et al., 2014), lymphocytic choriomeningitis virus (LCMV) (Lund et al., 2008), and *Citrobacter* (Wang et al., 2014).

Little work has been done to characterize the role of Tregs in neonatal infections, but just as in adults, Treg depletion or absence enhances pathogen clearance for some infections and compromises it for others. Treg depletion magnifies neonatal responses to HIV (Legrand et al., 2006), and HSV-2 (Fernandez et al., 2008a), but neonates can

struggle to survive the increased inflammation produced by Treg depletion (Tucker et al., 2013). Partially depleting Tregs in pups that over-express them improves viral clearance (Jaligama et al., 2017), but we have previously shown that mouse pups without Tregs have diminished influenza virus clearance (Oliphant et al., 2015). Intriguingly, Treg depletion in adults during flu infection has been shown to result in diminished germinal center formation and decreased antibody production (Leon et al., 2014) although this did not alter the course of influenza infection in these animals (Betts et al., 2011). It is possible that Tregs perform a unique function in neonates or that the role they play in influenza viral defense is more essential in neonates.

Tbet and Gata3 are transcription factors associated with Th1 and Th2 $CD4^+$ T cell subsets, respectively, but they have functions beyond these cells. Tregs can express these proteins transiently along with the Treg master regulator transcription factor Foxp3. In Tregs, Tbet and Gata3 contribute to the maintenance of Treg function in inflammatory environments where Tregs are at risk for downregulation of Foxp3 and loss of suppressive function (Wohlfert et al., 2011). Tbet and Gata3 are partially redundant in this role, as single Tbet or Gata3 Treg specific knockout mice do not develop broad autoimmunity while double knockouts develop scurfy-like autoimmunity at around 6 weeks of life (Yu et al., 2015). While the role of Tbet and Gata3 is being studied in the context of suppressive function in adults, their role in neonatal Tregs as well as their potential role in neonatal influenza virus infections is unknown.

In this study we show that neonates lacking Tregs display compromised antibody production during influenza virus infection. This correlates with diminished lymph node size and a concurrent decrease in T follicular helper cells (Figure 5.1). We also show that

a large proportion of neonatal Tregs express Gata3 compared to adult Tregs, especially during influenza virus infection (Figure 5.2). Finally, we show that Treg specific Gata3 knockout pups are grossly abnormal (Figure 5.3), display elevated Tbet expression in Tregs as well as conventional T cells during influenza infection (Figure 5.4), and suffer increased morbidity during infection despite increased Tfh populations (Figure 5.5).

5.2 Results

5.2.1 Treg deficient mice have compromised influenza virus neutralizing antibody production

We have previously shown that neonatal mice that lack Tregs cannot clear influenza virus. Other studies have shown that Treg depleted adult mice have diminished germinal center formation during influenza virus infection although this does not alter the course of adult infection. We wanted to assess whether Treg deficient pups display decreased antibody production as this could be responsible for the diminished anti-influenza response observed in these animals. We infected 2 day old pups or adult mice and harvested the tracheobronchial lymph node (TBLN) at day 10 post infection. Infected pups and adults had more total $CXCR5^+$ $PD\text{-}1^+$ T follicular helper cells (Tfh) than uninfected age-matched controls, but infected adults also had significantly more Tfhs than infected pups (Figure 5.1 A). We then depleted Tregs from $Foxp3^{DTR}$ pups and infected them with an LD_{10} dose of influenza virus. We evaluated the TBLN at day 10 of infection by flow cytometry. Depleted pups had decreased proportions of $CD4^+$ T cells, $CD19^+$ B cells, and $Foxp3^+$ Tregs. The proportion of Tfh cells was not different between depleted and undepleted pups (Figure 5.1 C). The TBLNs of depleted pups were much

smaller than undepleted pups and this correlated with lower cell counts in depleted animals. Depleted pups had fewer total $CD4^+$ T cells, $CD19^+$ B cells, $Foxp3^+$ Tregs, and Tfhs (Figure 5.1 D). We then infected scurfy pups and WT littermates with an LD_{10} dose of influenza and collected serum at day 9 of infection to evaluate anti-influenza antibody production by hemagglutinin inhibition assay. Scurfy pups had diminished antibody production compared to WT littermates (Figure 5.1 E). As expected, Treg depleted animals had elevated numbers of $CD4^+$ T cells (Figure 5.1 F) and elevated IFNγ producing $CD4^+$ T cells in the lungs (Figure 5.1 G). These data suggest that loss of Tregs significantly increases lung inflammation while diminishing effective TBLN responses and compromising hemagglutinin neutralizing antibody production in pups.

5.2.2 Pup and adult Tregs express Tbet during influenza virus infection and Pup Tregs express Gata3.

Tregs often display diverse functions in pathologic and steady state conditions and may express different transcriptional programs depending on their environment. We wanted to determine whether neonatal Tregs expressed conventional T cell transcription factors Tbet and Gata3 during influenza virus infection. We infected 2 day old pups and adults with an LD_{10} dose of influenza and evaluated Tbet, Gata3 and Foxp3 expression in the whole lung digest at days 7, 11, and 14 post-infection (Figure 5.2 A-D). Few Tregs express Tbet in uninfected pups or adults at early or late in the infection in infected animals, but an elevated proportion of $Foxp3^+$ cells co-express Tbet at day 11 of infection in both pups and adults (Figure 5.2 E). Infected and uninfected pups display a higher proportion of Tregs co-expressing Foxp3 and Gata3, especially during infection. Adult Treg expression of Gata3 remains low in uninfected animals as well as during influenza

virus infection (Figure 5.2 F). These data suggest that Gata3 is often expressed by pup Tregs and Tbet is upregulated in Tregs during influenza virus infection.

5.2.3 Treg specific knockout of Gata3 results in small mice with no overt disease

Previous work has suggested that Gata3 performs a role in suppressive Treg physiology by promoting the maintenance of Foxp3 expression in inflammatory conditions. Given the high number of $Gata3^+$ Tregs in neonates, we wanted to determine whether Gata3 performs an important role in Treg function during influenza virus infection. We generated Treg specific Gata3 knockout mice by crossing $Foxp3^{cre}$ mice with mice expressing Gata3 flanked by loxp ($Foxp3^+Gata3^-$). Expression of Gata3 in $Foxp3^+$ cells is significantly diminished in these animals (Figure 5.3 A), but not littermate controls (Figure 5.3 B). $Foxp3^+Gata3^-$ animals are not phenotypically normal on gross examination (Figure 5.3 C). They have small litters with a decreased proportion of $Foxp3^+Gata3^-$ pups (Figure 5.3 D) and $Foxp3^+Gata3^-$ pups are substantially smaller than littermate controls (Figure 5.3 E). These data suggest that $Foxp3^+Gata3^-$ mice are viable but that loss of Gata3 in Tregs reduces fitness substantially.

5.2.4 Treg specific Gata3 KO pups express higher levels of Tbet without an increase of IFNγ producing cells

The precise effects of knocking out Gata3 in Tregs is debated, and the consequences of this KO in pup anti-influenza virus immune responses has not been established. We infected $Foxp3^+Gata3^-$ pups and littermate controls with an LD_{10} dose of influenza virus and evaluated lung digest and lymph node cell populations by flow cytometry. $Foxp3^+Gata3^-$ pups had a higher proportion of Foxp3+ cells in the TBLN compared to littermate controls (Figure 5.4 A), although total numbers of Tregs did not

differ between Foxp3^{+}Gata3^{-} and littermate control TBLNs (Figure 5.4 B). There was no difference in proportion or total numbers of Foxp3^{+} CD4^{+} T cells in the lung digest of Foxp3^{+}Gata3^{-} pups or littermate controls (Figure 5.4 C, D). Proportions of IFNγ producing CD4^{+} T cells were higher in the lung digest of Foxp3^{+}Gata3^{-} pups (Figure 5.4 E), but proportions of IFNγ producing CD8^{+} T cells did not differ between lung digests (Figure 5.4 F). Higher proportions of both Tregs (Figure 5.4 G) and conventional CD4^{+} T cells (Figure 5.4 H) expressed Tbet in Foxp3^{+}Gata3^{-} pups. These data suggest that loss of Gata3 in Tregs increases Tbet expression, and this shift is correlated with an increase of IFNγ producing CD4^{+} T cells.

5.2.5 Foxp3^{+}Gata3^{-} pups have no significant decrease in hemagglutinin neutralizing antibody, but suffer greater morbidity during infection than littermate controls

As Tregs are important for germinal center formation and antibody production during influenza virus infection, we wanted to test whether Treg-specific Gata3 loss would alter this function in pups. We infected Foxp3^{+}Gata3^{-} pups and littermates with an LD_{10} dose of influenza virus and evaluated lymph node cell populations by flow cytometry. Foxp3^{+}Gata3^{-} pups and littermates displayed similar proportions (Figure 5.5 A) and total numbers (Figure 5.5 B) of CD19^{+} cells in the TBLN at day 13 of infection. Despite displaying normal levels of CD19^{+} cells, Treg Gata3 pups had lower influenza virus neutralizing antibody titers in the serum compared with WT pups with a p value of 0.15 (Figure 5.5 C). Finally, Foxp3^{+}Gata3^{-} pups suffer greater morbidity than littermate controls during influenza infection with weight loss late in the infection rather than just the slowed growth WT pups experience when infected (Figure 5.5 D, E). These data

suggest that Treg specific loss of Gata3 detrimentally influences the influenza disease course in mouse pups, possibly by decreasing influenza neutralizing antibody production.

5.3 Discussion

Tregs clearly suppress pathogen-specific immune responses in pups and adults (Fernandez et al., 2008a), but simply diminishing their function or removing some of them is not a solution for improving outcomes, especially in neonates who manage the increased inflammation that accompanies decreased Treg function poorly (Tucker et al., 2013). So poorly, in fact, that performing experiments examining neonatal immunity after Treg depletion is often challenging due to high pup mortality. We have shown that neonatal mice lacking Tregs cannot clear influenza with the same kinetics as WT mice, adding to the complex picture of Treg function during infections (Oliphant et al., 2015). Determining the specific nature of these functions is of paramount importance for safely manipulating anti-pathogen and vaccine responses.

In this study, we found that Treg depletion compromises influenza virus neutralizing antibody production in pups (Figure 5.1). We also saw that a large proportion of Tregs in pups co-expressed Gata3 and Foxp3 while both pups and adults co-expressed Tbet and Foxp3 during infection (Figure 5.2). Treg specific knockout of Gata3 produces abnormally small mice (Figure 5.3). We found that these mice have altered Tbet expression but minimally altered T cell responses in their lungs (Figure 5.4). Finally, we showed that Treg Gata3 KO pups experience greater morbidity during influenza virus infection correlated with decreased influenza virus neutralizing antibody.

Immune regulation is essential in both steady state and infection conditions, but the nature of that regulation differs between adult and neonatal animals according to the divergent needs of those phases. The ability to mount a protective response in neonates is superseded by immunosuppressive programming, but partially effective antiviral responses are still present in neonates (Harbeson, Ben-Othman, et al., 2018; Medzhitov et al., 2012). This response conveys substantially reduced protection from influenza virus infection compared to adult responses, but the limited functionality of the response leaves pups vulnerable to perturbations of the system.

Other studies have shown that Tregs support germinal center formation and thus high quality antibody production by taking up excess IL-2 that would otherwise inhibit the generation of T follicular helper cells (Tfh) (Leon et al., 2014). Treg depletion was not shown to have any effect on disease course in adults (Betts et al., 2011), suggesting that the diminished antibody production did not compromise viral defense in these animals. We showed that a similarly diminished Tfh response occurs after Treg depletion in pups (Figure 5.1 C). However, pups already display a diminished Tfh response to influenza virus compared to adults (Figure 5.1 A). Additionally, others have reported that under normal circumstances pups have disordered germinal center formation and decreased B-T cell interaction leading to lower production of high quality antibodies during infection (Mastelic et al., 2012; Munguía-Fuentes et al., 2017). Decreasing the already dangerously low lymph node response in pups may compromise antibody production to a greater extent than in adults (Figure 5.1 D). This could explain the inability of scurfy pups to clear influenza infection within the 14 days WT pups require to clear.

While Foxp3 is the defining transcription factor in Treg development and function, conventional T cell transcription factors like Tbet and Gata3 are also expressed by Tregs (Yu et al., 2015). These factors appear to perform a variety of roles in supporting Treg function, especially in inflammatory conditions, but their role in neonatal Tregs is unknown as is their role in promoting influenza virus defense. Expression of Gata3 and Tbet is known to be volatile in Tregs, and we see that in neonatal Tregs during influenza virus infection as well. Pup Tregs upregulate Tbet during infection which is consistent with what has been reported with adult Tregs (Bedoya et al., 2013). Bedoya et al (2013) associated Tbet expression with the development of adult influenza-specific Tregs. These Tregs are partially responsible for restraining anti-viral immunity and preventing extensive pathology. Given that pup $CD4^+$ T cells are predisposed to differentiate into Tregs upon antigen stimulation (Wang et al., 2010), it is likely that many Tregs during influenza virus infection are influenza-specific. The elevated proportion of $Tbet^+$ Tregs during infection may represent these virus-specific cells in pups as well as in adults (Figure 5.2 A).

Gata3 is thought to help Tregs maintain suppressive activity during inflammatory conditions. Foxp3 expression is not stable and Tregs can de-differentiate if proper stimulation is not maintained (Wohlfert et al., 2011). This is important for maintaining the proper level of suppression at steady state and during inflammation as balance must be maintained between the effectiveness of the response and potential pathology. We found that a high proportion of Tregs in pups expressed Gata3 (Figure 5.2), compared to adults, suggesting that this transcription factor may have a significant role in neonatal Treg function.

A simple explanation for the elevated Gata3 could be that Gata3 can be upregulated in Tregs during IL-4 stimulation (Abdel Aziz et al., 2018). Given the Th2 bias in neonatal T cell responses and the elevated IL-4 produced during neonatal influenza infection (Lines et al., 2010), the high expression of Gata3 in neonatal Tregs may be driven by IL-4. Using IL-4 KO mice could help determine if this is an important mechanism in promoting Gata3 expression in neonatal Tregs.

Global knock out of Gata3 is embryonic lethal, but cell specific Gata3 KO mice are feasible. We generated $Foxp3^{+}Gata3^{-}$ mice by crossing mice expressing Gata3 flanked by loxp with Foxp3 cre mice. This has been done before, and other groups report that these mice are phenotypically normal or that they develop autoimmunity several months into life (Wohlfert et al., 2011; Yu et al., 2015). Losing Gata3 does not totally compromise Treg function, but one study reported that elimination of both Tbet and Gata3 led to a scufy like phenotype in affected mice including multi-organ autoimmunity and early death (Yu et al., 2015).

We saw that $Foxp3^{+}Gata3^{-}$ mice were not phenotypically normal, with reduced body mass compared to WT animals and difficulties breeding (Figure 5.3). Of our breeding pairs, only $Foxp3^{+}Gata3^{-}$ females successfully bred. $Foxp3^{+}Gata3^{-}$ males produced no offspring (data not shown). This was despite the small size of $Foxp3^{+}Gata3^{-}$ females. The poor breeding went beyond infrequent litters. By simple genetics, litters should have been half $Foxp3^{+}Gata3^{-}$ pups and half littermate controls, but we consistently observed that fewer than 1 in 3 pups from any litter were $Foxp3^{+}Gata3^{-}$ (Figure 5.3 D). This could have been due to death shortly after birth, destruction by the mother, or resorption in utero. Tregs are essential for maintaining tolerance to the fetus during

pregnancy, and disruption of Treg function often results in loss of pups (La Rocca et al., 2014). Regardless, other than these factors, the Foxp3^{+}Gata3^{-} mice showed no overt signs of disease or development of autoimmunity during the course of these studies.

Others have reported that Treg specific deletion of Gata3 does not impair Treg function, but they were assessing the ability of Foxp3^{+}Gata3^{-} Tregs to prevent colitis in an adoptive transfer model (Yu et al., 2015). There is other evidence that Gata3 may play a role in promoting Treg function specifically during inflammatory conditions (Abdel Aziz et al., 2018; Wohlfert et al., 2011). Our findings suggest that loss of Gata3 does not result in a total loss of suppressive function, but that does not mean there is no effect. Infected Foxp3^{+}Gata3^{-} pups had higher proportions of Tbet^{+} conventional T cells and Tregs (Figure 5.4 E, F). The higher proportion of Tbet expressing Tregs is not surprising given that Gata3 expression suppresses Tbet expression under normal conditions (Kanhere et al., 2012). The increase in conventional T cell Tbet expression, however, indicates a possible loss of suppression and induction of a Th1 phenotype in CD4^{+} T cells. IFNγ expression is increased in CD4^{+} T cells but not CD8^{+} T cells of Foxp3^{+}Gata3^{-} mice suggesting that this increase in Tbet expression is promoting a Th1 phenotype in Foxp3^{+}Gata3^{-} mice. Although this decrease is suppressive function during infection is consistent with previous studies (Wohlfert et al., 2011), we did not see a defect in Treg accumulation in the lungs (Figure 5.4 C, D). Wohlfert et al. (2011) examined Treg accumulation during gut inflammation, so it is possible that Gata3 effects targeting to that tissue but not the lung, or that Treg accumulation in inflamed tissues occurs through different mechanisms in neonates.

If Treg suppressive function is impacted by elimination of Gata3, it is likely that pups will experience greater morbidity during infection. Indeed, $Foxp3^{+}Gata3^{-}$ pups suffered increased morbidity during infection than littermate controls, especially later in infection. $Foxp3^{+}Gata3^{-}$ pups actually lost weight for two days during the infection, which typically only happens in severely ill pups (Figure 5.5 E). Small size may be a contributing factor to the decreased weight gain experienced by KO pups, but we cannot determine how much initial weight factored into morbidity.

Finally, $Foxp3^{+}Gata3^{-}$ pups also had slightly diminished influenza neutralizing antibody in their serum than littermate controls (Figure 5.5 C). This is not the severely compromised antibody response seen in scurfy pups, but it may contribute to the increase morbidity in $Foxp3^{+}Gata3^{-}$ pups. Total numbers of TBLN B cells were not altered in $Foxp3^{+}Gata3^{-}$ pups (Figure 5.5 A, B), but this says nothing about the function of those cells.

While Tregs do suppress neonatal immune reactions, they are certainly not purely detrimental during influenza virus infection. Manipulating Treg function is a tempting target for improving neonatal outcomes, but we have demonstrated that these cells are likely required for optimal responses. More sophisticated manipulations might be able to separate the suppressive and pro-immune functions of Tregs, but this must be done with care.

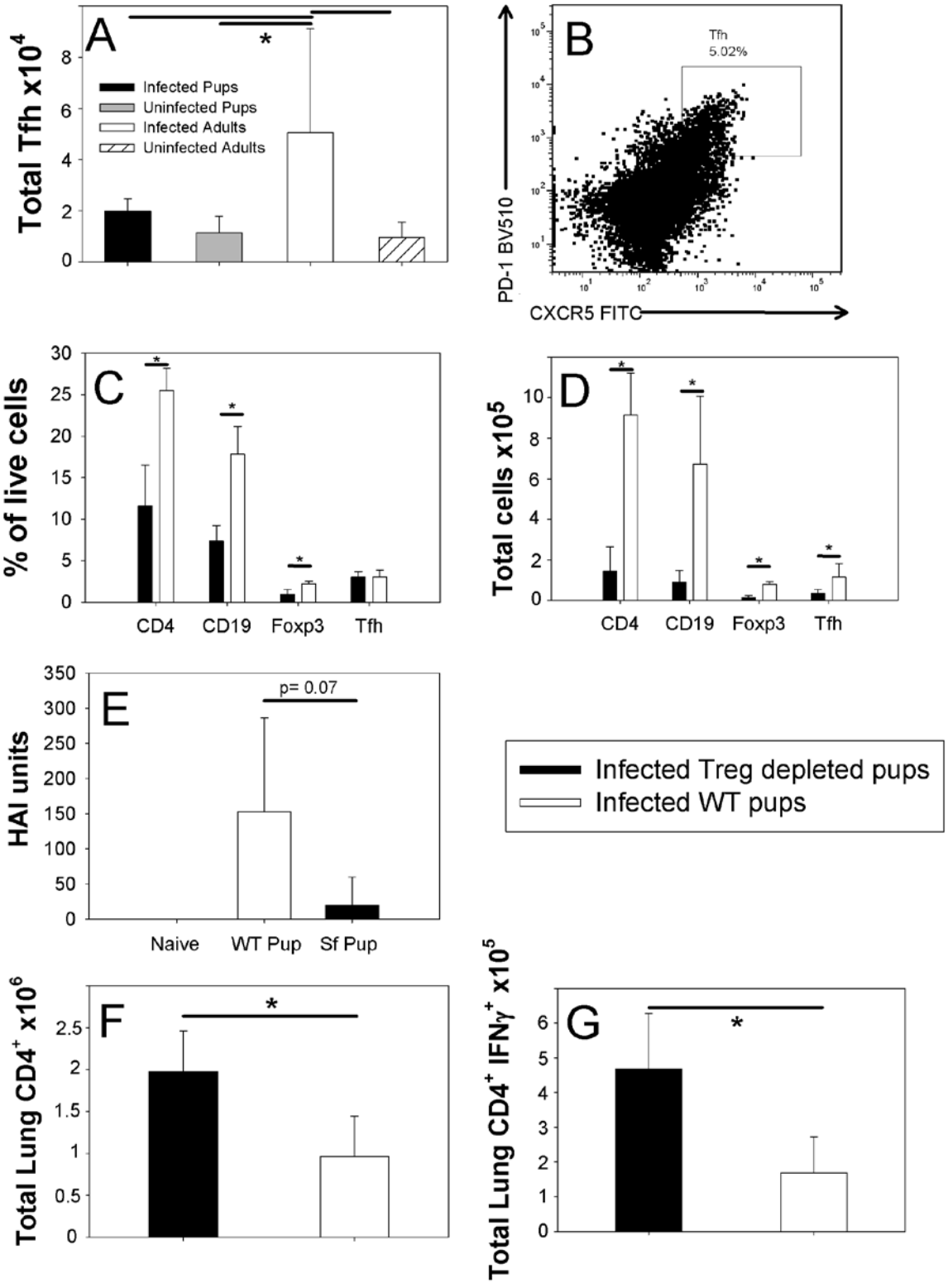
A
Total Tfh x10^4
Infected Pups
Uninfected Pups
Infected Adults
Uninfected Adults
*
B
Tfh
5.02%
PD-1 BV510
CXCR5 FITC
C
% of live cells
CD4
CD19
Foxp3
Tfh
D
Total cells x10^5
CD4
CD19
Foxp3
Tfh
E
HAI units
p= 0.07
Naive
WT Pup
Sf Pup
Infected Treg depleted pups
Infected WT pups
F
Total Lung CD4+ x10^6
G
Total Lung CD4+ IFNγ+ x10^5

Figure 5.1 Loss of Tregs compromises anti-influenza virus antibody production. 2 day old or adult mice were infected i.n. with an LD_{10} dose of the PR8 strain of influenza virus. TBLNs were collected at day 10 of infection and total numbers of Tfhs were determined by flow cytometry (A). Tfhs were defined by expression of PD-1 and CXCR5 (B). 2 day old $Foxp3^{DTR}$ mice were injected with 50μg/kg diphtheria toxin or HBSS i.p. and then infected i.n. with an LD_{10} dose of the PR8 strain of influenza virus. Proportion (C) and total numbers (D) of $CD4^+$, $CD19^+$, $Foxp3^+$ and Tfh cells were determined in the TBLN of infected animals at day 10 of infection. 2 day old scurfy and WT littermates were infected i.n. with an LD_{10} dose of the PR8 strain of influenza virus and serum was collected at day 10 of infection. Hemagglutinin neutralizing antibodies were measured by hemagglutinin inhibition assay (E). Total lung $CD4^+$ (F) and $CD4^+$ $IFN\gamma^+$ (G) T cells were assessed in scurfy mice by flow cytometry. Data show mean ± SD for two separate experiments with at least 4 mice per group. * $p< 0.05$ by student's T test or one way ANOVA followed by Holm-Sidak *post-hoc* test for pairwise comparisons.

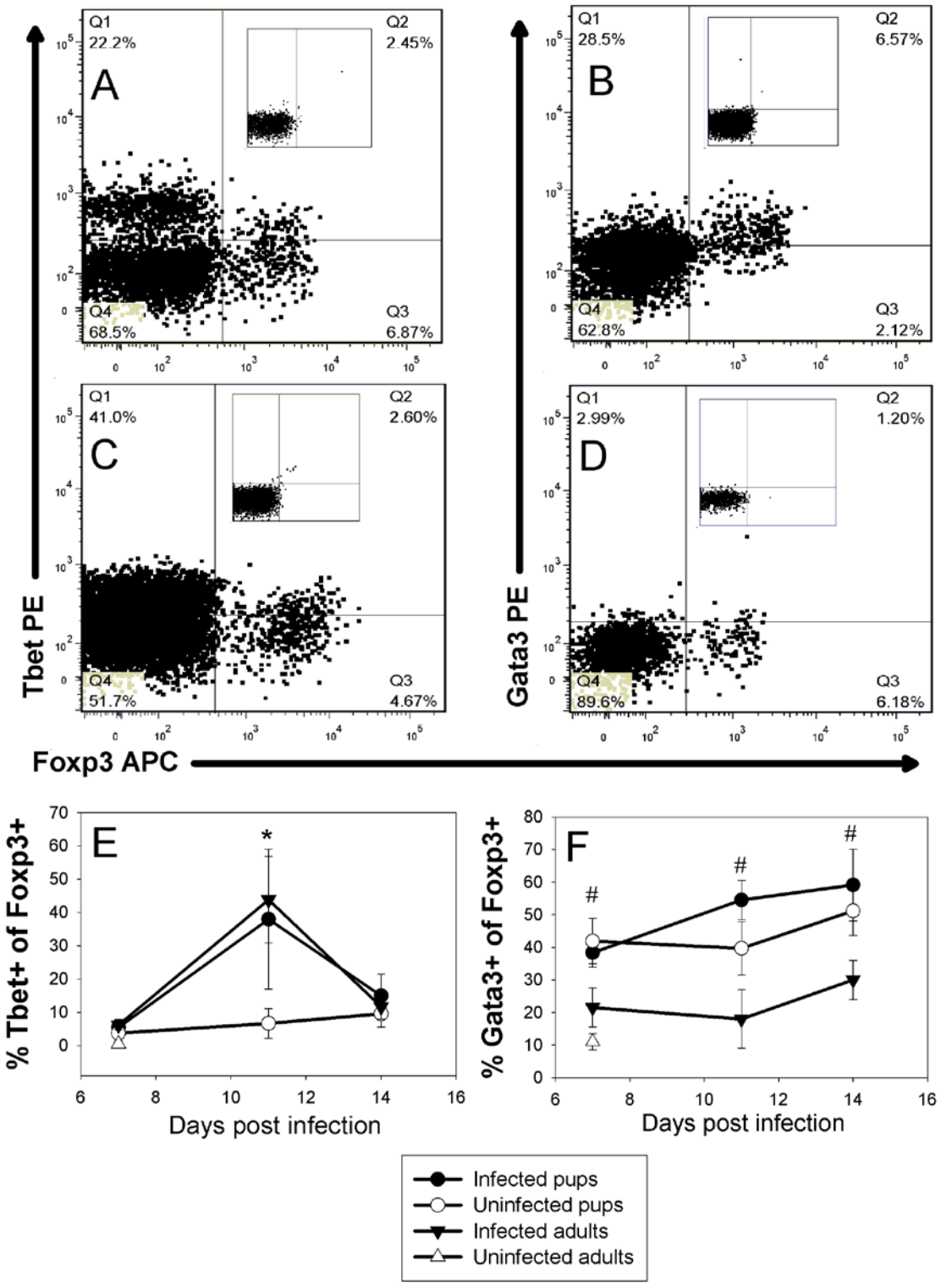
A
Q1
22.2%
Q2
2.45%
Q4
68.5%
Q3
6.87%
B
Q1
28.5%
Q2
6.57%
Q4
62.8%
Q3
2.12%
C
Q1
41.0%
Q2
2.60%
Q4
51.7%
Q3
4.67%
D
Q1
2.99%
Q2
1.20%
Q4
89.6%
Q3
6.18%
Tbet PE
Gata3 PE
Foxp3 APC
E
% Tbet+ of Foxp3+
Days post infection
F
% Gata3+ of Foxp3+
Days post infection
Infected pups
Uninfected pups
Infected adults
Uninfected adults

Figure 5.2 Pup Tregs express Gata3 and both pup and adult Tregs express Tbet during influenza virus infection.

2 day old or adult mice were infected i.n. with an LD_{10} dose of the PR8 strain of influenza virus. Representative dot plots gated on $CD4^+$ T cells are shown of Foxp3 and Tbet (A) or Gata3 (B) expression at day 11 of infection in pups. Representative dot plots are shown of Foxp3 and Tbet (C) and Gata3 (D) expression at day 11 of infection in adults. Insets show isotype control. Proportion $Tbet^+$ (E) or $Gata3^+$ (F) of $Foxp3^+$ $CD4^+$ lymphocytes in the lung digest at days 7, 11, and 14 of infection was determined by flow cytometry. Data show mean ± SD for two separate experiments with at least 6 mice per group. * $p<0.05$ compared to uninfected animals. # $p<0.05$ compared to adult mice at the same day. Significance calculated by one-way ANOVA followed by Holm-Sidak *post-hoc* test for pairwise comparisons.

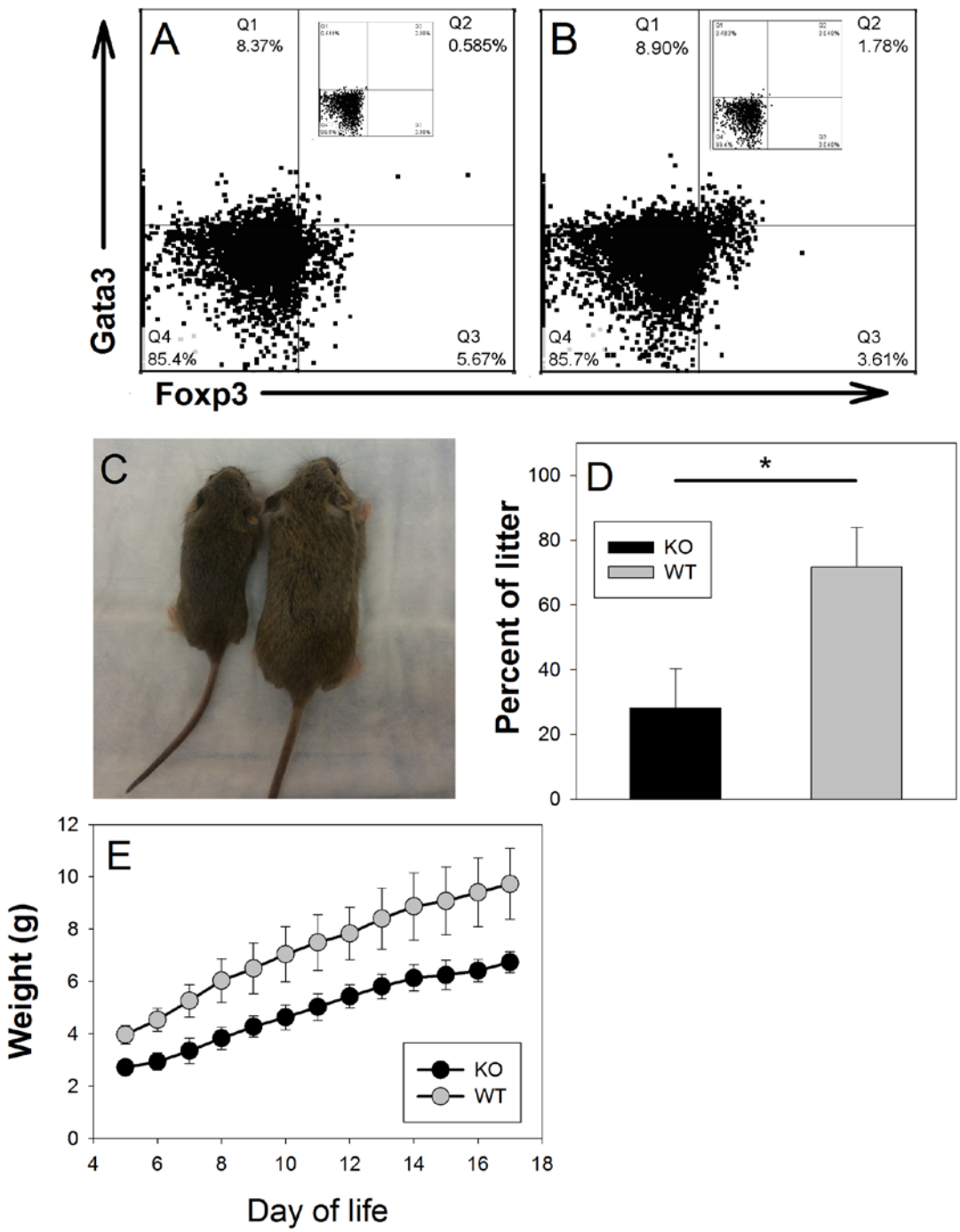
A
Q1
8.37%
Q2
0.585%
Q4
85.4%
Q3
5.67%
B
Q1
8.90%
Q2
1.78%
Q4
85.7%
Q3
3.61%
Gata3
Foxp3
C
D
*
Percent of litter
100
80
60
40
20
0
KO
WT
E
Weight (g)
12
10
8
6
4
2
0
4
6
8
10
12
14
16
18
Day of life
KO
WT

Figure 5.3 Treg specific knock out of Gata3 results in small mice that breed poorly but show no sign of overt disease.

$Foxp3^{+}Gata3^{-}$ mice were generated by crossing mice expressing Gata3 flanked by loxp with $Foxp3^{cre}$ mice. Proportion $Gata3^{+}$ of $Foxp3^{+}$ $CD4^{+}$ lymphocytes in the lung digest for $Foxp3^{+}Gata3^{-}$ (A) and littermate controls (B) was determined by flow cytometry. $Foxp3^{+}Gata3^{-}$ and littermate controls were photographed at day 15 of life (C). $Foxp3^{+}Gata3^{-}$ pups and littermates were counted in each litter (D) and were weighed daily (E). Data show mean ± SD. * $p< 0.05$ by student's T test.

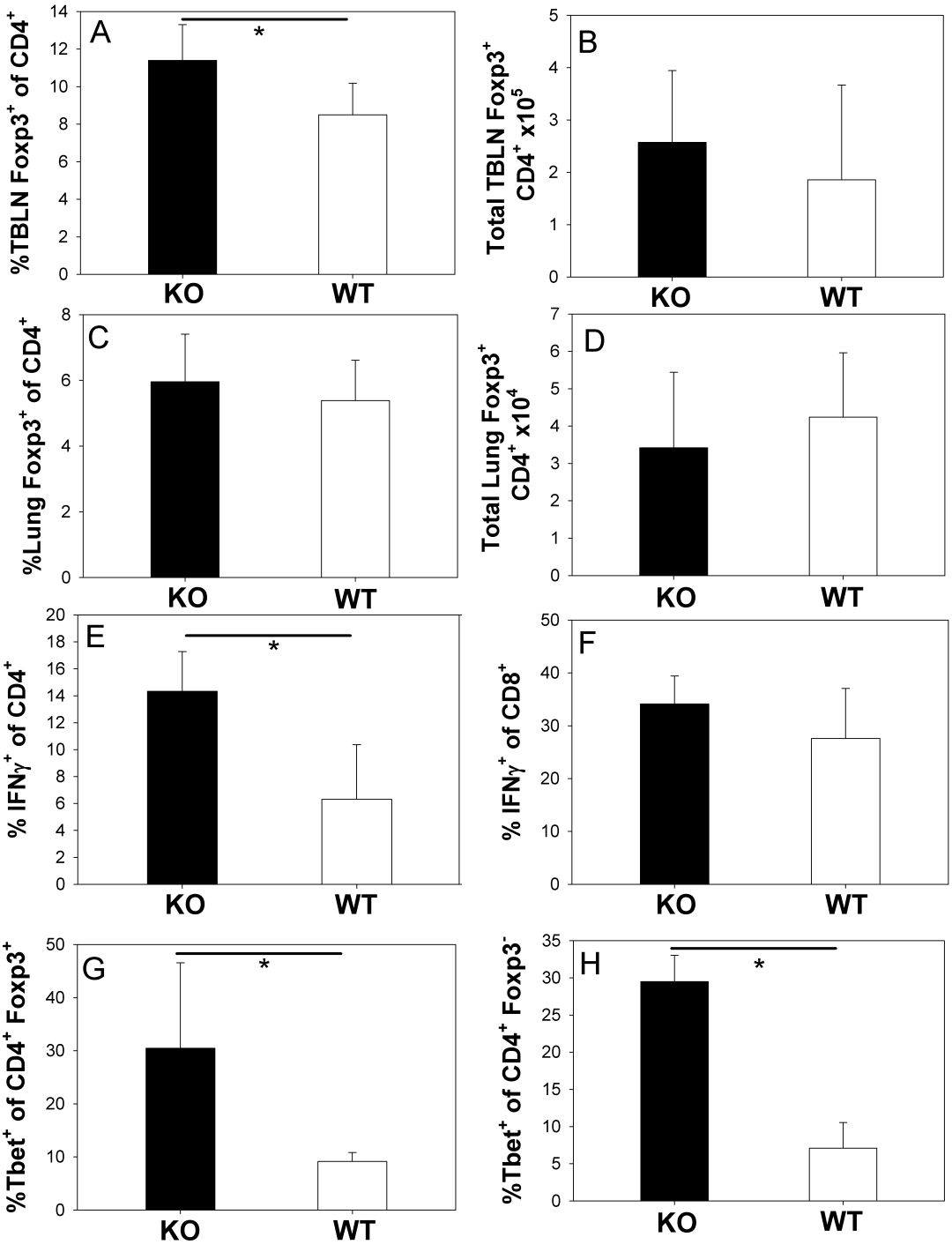
A
%TBLN Foxp3+ of CD4+
*
KO
WT
B
Total TBLN Foxp3+ CD4+ x10^5
KO
WT
C
%Lung Foxp3+ of CD4+
KO
WT
D
Total Lung Foxp3+ CD4+ x10^4
KO
WT
E
% IFNγ+ of CD4+
*
KO
WT
F
% IFNγ+ of CD8+
KO
WT
G
%Tbet+ of CD4+ Foxp3+
*
KO
WT
H
%Tbet+ of CD4+ Foxp3-
*
KO
WT

Figure 5.4 Foxp3^{+}Gata3^{-} pups express higher levels of Tbet without an increase of IFNγ producing cells.

2 day old Foxp3^{+}Gata3^{-} mice and littermate controls were infected with an LD_{10} dose of the PR8 strain of influenza virus. TBLN were obtained from infected Foxp3^{+}Gata3^{-} mice and littermate controls at day 13 of infection and proportion (A) and total numbers (B) of Foxp3+ CD4^{+} T cells were determined by flow cytometry. Proportion of CD4^{+} (C) and CD8^{+} (D) cells that were IFNγ+ were enumerated in lung digest of infected Foxp3^{+}Gata3^{-} mice and littermate controls at day 13 of infection by flow cytometry. Proportion Tbet+ of Foxp3+ (E) and CD4^{+} (F) cells was determined for TBLN of infected Foxp3^{+}Gata3^{-} mice and littermate controls at day 13 of infection by flow cytometry. Data show mean ± SD for two separate experiments. KO n=3. WT n= 9. * $p<0.05$ by student's T test.

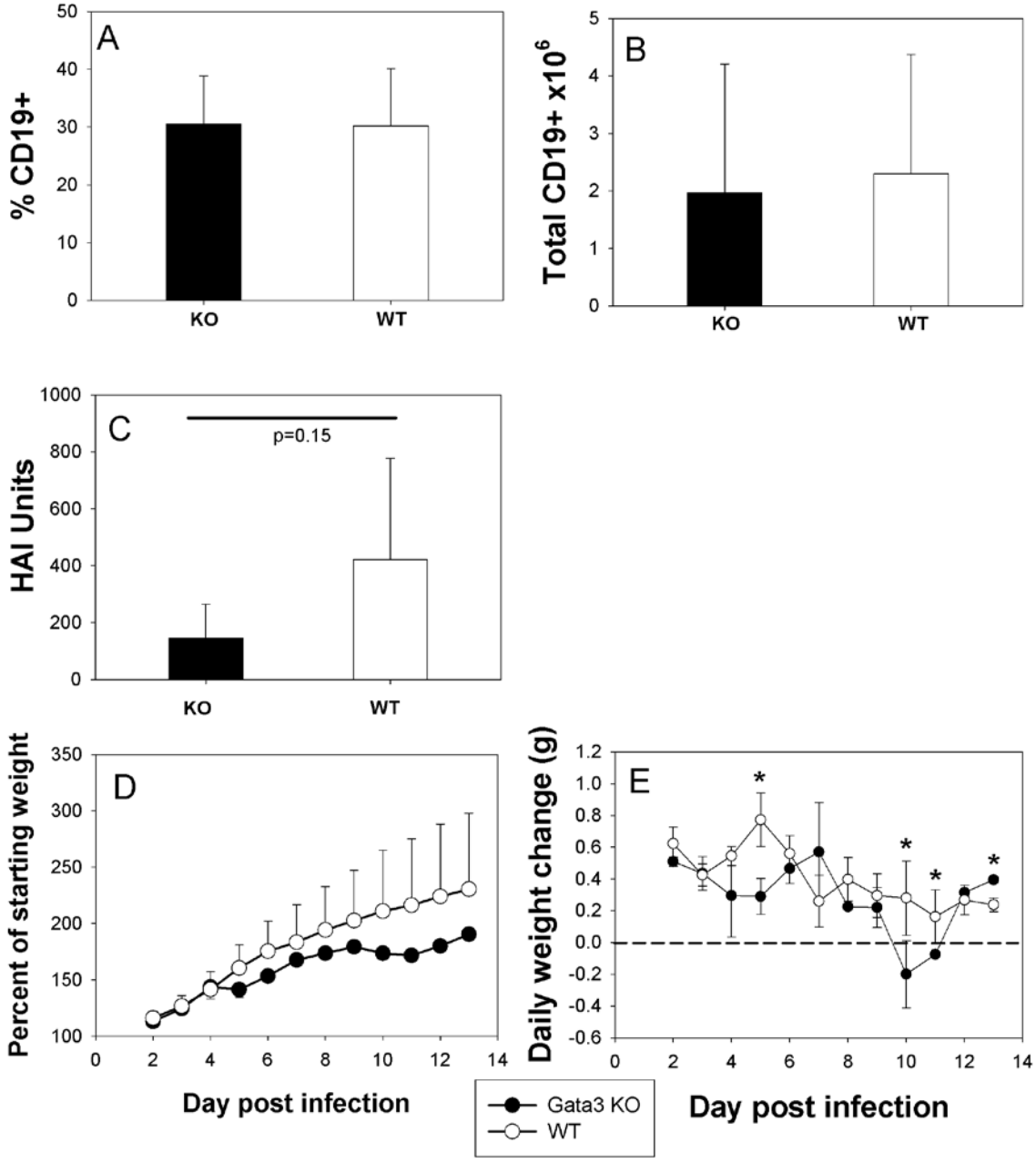
A
% CD19+
50
40
30
20
10
0
KO
WT
B
Total CD19+ x10^6
5
4
3
2
1
0
KO
WT
C
HAI Units
p=0.15
1000
800
600
400
200
0
KO
WT
D
Percent of starting weight
350
300
250
200
150
100
0
2
4
6
8
10
12
14
Day post infection
E
Daily weight change (g)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
*
0
2
4
6
8
10
12
14
Day post infection
Gata3 KO
WT

Figure 5.5 Treg specific KO of Gata3 did not reduce hemagglutinin neutralizing antibody and increased morbidity during infection.

2 day old $Foxp3^{+}Gata3^{-}$ mice and WT littermate controls were infected with an LD_{10} dose of the PR8 strain of influenza virus. TBLN were obtained from infected $Foxp3^{+}Gata3^{-}$ mice and littermate controls at day 13 of infection and proportion (A) and total numbers (B) of $CD19^{+}$ cells were determined by flow cytometry. Hemagglutinin neutralizing antibody titer in the serum of infected $Foxp3^{+}Gata3^{-}$ mice and littermate controls was determined at day 13 post infection by hemagglutinin inhibition assay (C). Mice were weighed daily (D) and daily weight change was measured (E). Data show mean ± SD for two separate experiments. KO n=3. WT n= 9. * $p<0.05$ compared to littermate controls at the same timepoint by one-way ANOVA followed by Holm-Sidak *post-hoc* test for pairwise comparisons.

CHAPTER 6. OVERALL DISCUSSION

The neonatal vulnerability to infection poses a difficult challenge for potential immunological intervention. Significant evidence and thought suggests that the globally diminished function of the neonatal immune system is required to avoid devastating immunopathology and ensure effective development of tolerance to innocuous antigens (Harbeson, Francis, et al., 2018). Nevertheless, interventions can and should be made to improve neonatal health. This usually takes the form of vaccines, and the field of vaccinology includes an impressive list of successes for human health. There are, however, warnings as well. Vaccinations can provoke unintended or harmful responses as was the case with early efforts at RSV vaccination. These vaccines resulted in significant morbidity and even mortality among treated infants (Delgado et al., 2009). Beyond this, many vaccines function poorly in neonates, requiring older age or multiple doses to stimulate effective protection (Robinson et al., 2019).

It has become clear that sophisticated new methods of immune manipulation and vaccination are sometimes required to safely improve outcomes in neonates. Vaccines often try to mimic natural infections. This follows the reasonable logic that when faced with the complexity of a protective immune response, it may be best to allow the immune system to operate as naturally as possible during a vaccine response for the best chance of establishing protective immunity. This becomes problematic, however, when the natural response to an antigen or a pathogen provides sub-optimal protection. Such is the case with conventional attempts at HIV vaccination, and even conventional influenza vaccination. Recent work demonstrated the feasibility of designing a vaccine that would preferentially activate and expand a specific germ-line B cell clone that could produce

broadly neutralizing antibody against HIV after somatic hyper-mutation and affinity maturation (Abbott et al., 2018). These B cells are not dominant during normal viral responses, but through novel antigen stimulation, they were able to provoke a significant response from these subdominant cells.

I have shown in chapter 3 that neonates display an altered immunodominance hierarchy. I have also shown in chapter 4 that the dominant $PA_{224-233}$-specific cells pups express are not protective during influenza virus infection. This is consistent with adult studies that showed that $PA_{224-233}$ vaccinated adults are not protected during influenza infection. $PA_{224-233}$ and $NP_{366-374}$ are both presented by dendritic cells, but infected respiratory epithelial cells only present $NP_{366-374}$ (Chen et al., 2004a; Crowe et al., 2003). This likely shapes the strongly $NP_{366-374}$ dominant hierarchy of adult secondary infections as it is thought that infected epithelial cells are the principal activators of memory $CD8^+$ T cells during influenza virus infection. This agrees with what we saw in the strongly $NP_{366-374}$ dominant adult secondary infections (Figure 3.3 A).

Mice first infected as pups, however, demonstrated an onset of antigen specific cells more characteristic of adult primary infections. It is possible that the mice first infected as pups had no significant memory population of $NP_{366-374}$-specific cells to quickly activate during secondary infection, and the memory $PA_{224-233}$ cells they did possess were not activated by infected epithelium. The antigen specific cell expansion in these mice may represent dendritic cell stimulation in the lymph node as if this was a primary infection. Even without a rapid $CD8^+$ T cell response, these mice survived a lethal infection. It is possible that the increased protection of mice first infected as pups is due to $CD4^+$ T cell memory. Although some of the dominant $CD4^+$ T cell epitopes are

within HA, others are in NP and would probably offer protection during heterosubtypic secondary infections (Crowe et al., 2005).

In the case of $CD8^+$ T cell vaccination, the failure of generating protection from $PA_{224-233}$ vaccination in pups and adults suggests that simply vaccinating neonates with antigens they can respond to naturally may not be an effective strategy for improving outcomes. A more sophisticated manipulation is required to promote adult-like immunodominance in neonates. I was not able to stimulate a $NP_{366-374}$-specific response in neonates with a vaccine, but I was able to detect $NP_{366-374}$-specific cells in infected mice that received adult BMDCs. Therefore, at least in the context of PR8 infection of C57Bl/6 mice, the neonatal immunodominance hierarchy can be changed with proper stimulation. Formulating the specifics of that stimulation and how they relate to human infant responses will require much more work, but theories can be made based on current knowledge.

Immunodominance of $CD8^+$ T cells is fundamentally a product of four primary factors. These are: the peptides that the MHC-I of a particular individual can bind for presentation to $CD8^+$ T cells, the efficiency of processing and presentation of potential epitopes by APCs, the T cells that are available to respond to the presented peptides, and the suppressive effect of dominant $CD8^+$ T cells on T cells of another specificity (Chen et al., 2000). These factors have been extensively dissected in adult C57Bl/6 mice infected with PR8 influenza virus, but they are only now being understood in pups. We have provided data that will help fill in a few holes in this body of knowledge with the hope that by illuminating basic functions of neonatal mouse immunodominance, future efforts

will be able to impact neonatal human immune reactions in highly specific and effective ways to improve vaccination or acute care outcomes.

MHC haplotype is the most important factor in determining which epitopes can be part of the immunodominance hierarchy for a particular infection in a particular individual, as only peptides that can bind to MHC class I have any chance of interacting with $CD8^+$ T cells. It is also a direct outcome of the genes an individual possesses and should not differ between an adult and a neonate of the same MHC haplotype. This is demonstrated in our mice by the observation that pups respond to the same PA epitope (224-233) as adults. They also respond to the same NP epitope as adults when they are able (Figure 3.5 A). We did not test whether pups responded to different epitopes than those that appear in the adult hierarchy, but if there were significant differences between what peptides MHC class I was presenting in pups and adults, then we would likely not see the same set of peptides making up the hierarchy of pups and adults within our inbred C57Bl/6 system. Theoretically different subclasses of MHC class I could be expressed in different levels in pups and adults, but both $NP_{366\text{-}374}$ and $PA_{224\text{-}233}$ are presented by $H2D^b$, making it unlikely that this is a driving mechanism of the altered neonatal responses to these two epitopes. Humans, of course, have much more diverse MHC class I and as such have diverse immunodominance hierarchies. We do not predict, however, that the peptides presented by a particular human haplotype will differ between infants and adults.

Acquisition, processing, and presentation of different viral peptides by antigen presenting cells such as dendritic cells as well as infected respiratory epithelial cells may be a central mediator of altered neonatal immunodominance in primary infections.

Dendritic cells can acquire antigen through direct infection or phagocytosis of infected materials (Bachmann et al., 1996; Ho et al., 2011). During adult infections, it is known that $NP_{366-374}$ is presented through direct presentation by influenza infected DCs without requiring immunoproteosome processing, while $PA_{224-233}$ requires processing in the immunoproteosome and cross-presentation by DCs that ingest infected cellular debris (Chen et al., 2004b). There is some debate over whether neonatal dendritic cells can cross-present phagocytosed material effectively with human data suggesting no defect (Gold et al., 2007), and mouse data showing that neonatal DCs are unable to cross-present soluble antigens effectively (Kollmann et al., 2004). The strongly $PA_{224-233}$-dominant neonatal response suggests that neonates have APCs capable of processing $PA_{224-233}$ with the immunoproteosome and cross-presenting $PA_{224-233}$ to $CD8^+$ T cells. The lack of an $NP_{366-374}$ response, on the other hand, suggests several other possibilities for neonatal dendritic cell function.

First, there may be a defect in direct presentation of viral peptides to MHC class I in neonatal dendritic cells, but I have not found any published evidence of this. Second, neonatal dendritic cells are less prevalent than adult DCs which may cause problems in and of itself. It is possible that few DCs are directly infected early in the course of influenza virus infection and as a result must obtain viral antigen from infected epithelial cells. Finally, neonatal DCs may present $NP_{366-374}$ in some fashion, but for some reason, such as the poor stimulatory capacity of neonatal DCs, $NP_{366-374}$-specific naïve $CD8^+$ T cells cannot be activated by these APCs. Adult $NP_{366-374}$-specific T cells have slightly lower affinity than $PA_{224-233}$-specific cells which may require a higher threshold of stimulation for proper activation (Cukalac et al., 2014; Luciani et al., 2013).

It is now known that BMDCs generated by GM-CSF do not represent any natural dendritic cell population (Helft et al., 2015). They do, however, collect and present antigens well to T cells, especially after LPS stimulation (Boonnak et al., 2013; Ebrahimi-Nik et al., 2018). As such, they represent a sort of activation ideal and can help us bypass some of the limitations of neonatal immunity and examine whether those factors played a role in the outcomes observed in infected mice. During the adoptive transfers performed for Figures 3.4 and 3.5, the influenza virus infected BMDCs would be able to present antigen directly to $CD8^+$ T cells with adult levels of stimulation (Herter et al., 2005). Transfer of these BMDCs did promote $NP_{366-374}$-specific cells in pups, but the transferred cells were enough to promote the generation of $NP_{366-374}$-specific cells with or without prior infection of the BMDCs (Figure 3.5). It is not clear whether the uninfected BMDCs become infected once transferred to the pups. As such, I cannot determine whether the superior activation potential of BMDCs or the access to infected DCs is responsible for the shift in immunodominance observed in these animals. If the infection of DCs is important, however, pre-infected BMDCs would likely offer an advantage in the speed of the response. This is supported by the earlier onset of antigen specific cells and improved viral clearance in animals that received infected BMDCs (Figure 3.5).

Notably, we found a lack of response to $NP_{366-374}$ loaded BMDC vaccination (Figure 4.1 B). As I mentioned before, it may be important to consider the role of $CD4^+$ T cells in this process. Adult BMDCs will be able to stimulate $CD4^+$ as well as $CD8^+$ T cell responses from whole virus, but not from $CD8^+$ T cell restricted peptide epitopes. It is well recognized that $CD4^+$ T cell help is an important component of robust $CD8^+$ T cell

memory (Bedoui et al., 2016; Epstein et al., 1998), and it is possible that activated $CD4^+$ T cells provide some essential help to $NP_{366-374}$-specific reactions in neonates that allows them to respond to live virus and BMDCs, but not peptide and BMDCs.

Differential precursor frequency of $NP_{366-374}$ and $PA_{224-233}$ specific $CD8^+$ T cells is linked to immunodominance in adults (Cukalac et al., 2014), but nothing is known about neonatal precursors for these epitopes. Studies in RSV have suggested that neonates have altered ratios of precursors for different epitopes within RSV compared to adults, but this is an analysis of 2-4 cells per animal and it is difficult to assess how significant these differences actually are (Ruckwardt et al., 2011). Ruckwardt et al. reported that the altered precursor frequency did not predict the immunodominance hierarchy of RSV infection in pups. Regardless, precursors for a specific epitope appear to be exceptionally rare in neonates even by the standards of naïve T cell precursors, and this rarity may in and of itself impact the altered hierarchy observed in pups. In fact, if the inability to respond to $NP_{366-374}$ was more absolute, it could suggest that neonates simply lack $NP_{366-374}$-specific T cell precursors early in life. Studies have shown that neonates have reduced TCR variability compared to older pups and adults (Carey et al., 2016). However, we argue that this is not likely as we did see pups responding to $NP_{366-374}$ under certain circumstances suggesting that pups must have $NP_{366-374}$- specific T cell precursors (Figure 3.5 A).

The existence of precursors is not the only T cell intrinsic factor for determining an immunodominance hierarchy. Studies have shown that $PA_{224-233}$-specific T cells have higher TCR binding affinity than $NP_{366-374}$-specific cells (Cukalac et al., 2014; La Gruta et al., 2004). Adult DCs can stimulate both $NP_{366-374}$ and $PA_{224-233}$-specific $CD8^+$ T cells,

but it is possible that the lower stimulatory capacity of neonatal DCs cannot reach the activation threshold of naïve $NP_{366-374}$-specific cells. This could explain why adult BMDCs were able to stimulate $NP_{366-374}$ responses in neonates. As mentioned before, this may require $CD4^+$ help as peptide loaded BMDCs did not stimulate an $NP_{366-374}$ response. The observation that pup lung tissue can activate $NP_{366-374}$-specific memory cells is consistent with this theory as memory cells have a lower threshold of activation than naïve cells (Kumar et al., 2011; Slifka et al., 2001). This phenomenon has been suggested in neonatal RSV infections. T cells specific for the adult dominant epitope have lower affinity than subdominant epitopes and pups display a reduced response to this low affinity dominant epitope (Ruckwardt et al., 2018).

The final major determinant of $CD8^+$ T cell immunodominance is suppression of subdominant epitopes by dominant ones. Suppression of a dominant specificity of $CD8^+$ T cell by a subdominant specificity is the result of competition between T cells for access to stimulation by antigen presenting cells (Chen et al., 2000; Willis et al., 2006). We saw that simultaneous vaccination with $NP_{366-374}$ and $PA_{224-233}$ in adults resulted in $PA_{224-233}$ dominance (Figure 4.1 C). This is consistent with published findings that $PA_{224-233}$ dominates over $NP_{366-374}$ when antigen doses are similar (Cukalac et al., 2014). It is interesting to note that when adults are vaccinated for $PA_{224-233}$, it not only fails to provide protection during a challenge infection, but the $PA_{224-233}$ dominant secondary response prevents any appreciable $NP_{366-374}$ response from developing, leading to worse outcomes (Crowe et al., 2005). The $PA_{224-233}$ dominant neonatal response to primary infection, however, does not prevent an $NP_{366-374}$-specific response in later secondary infections (Figure 3.3 A). It is possible that the poor survival and function of memory

$CD8^+$ T cells generated early in life (Connors et al., 2018; Smith et al., 2014) partially alleviates the "locking in" of suboptimal $CD8^+$ T cell specificities for subsequent infections that has been observed with neonatal responses before (Rudd et al., 2013). It is not clear whether this phenomenon extends to peptide vaccination as while $PA_{224-233}$ vaccinated mice had a detectable $NP_{366-374}$ response when challenged, we did not follow them long enough to see if the $PA_{224-233}$ vaccinated mice would develop a significant $NP_{366-374}$- specific response later in the infection. It is also possible that the two months between primary and secondary infections in Figures 3.2 and 3.3 result in a less "locked in" memory immunodominance hierarchy than the challenge infections two weeks after vaccination in Figure 4.2.

The majority of published data on the $NP_{366-374}$ and $PA_{224-233}$ hierarchy in adult mice suggests that $NP_{366-374}$-specific cells are not dominant because they have a T cell intrinsic advantage over $PA_{224-233}$ cells, but instead they likely dominate because $PA_{224-233}$ requires immunoproteasome processing to be resented, it is much easier to present $NP_{366-374}$ than $PA_{224-233}$ both in DCs and infected target cells (Crowe et al., 2003). Although we have not tested $NP_{366-374}$ and $PA_{224-233}$ presentation in neonatal tissues, these findings suggest that differential presentation of $NP_{366-374}$ and $PA_{224-233}$ is less likely to be the major driving force behind the dominant $PA_{224-233}$ response in pups. Instead, I propose that the magnitude of DC stimulation provides the crucial difference between neonate and adult $CD8^+$ T cell responses and that $CD8^+$ T cells can be a promising target for neonatal vaccination if increased DC stimulation is provided.

To conclude the discussion of immunodominance in neonatal infections, I want to address one more question. Why would the immunodominance hierarchy in neonates

favor an epitope that cannot generate effective $CD8^+$ T cell responses? It is possible that this is an adaptation by influenza virus to diminish effective $CD8^+$ T cell immunity, although this seems to have little effect on $CD8^+$ T cell mediated clearance of virus during normal adult infections. It is also possible that altered immunodominance protects neonates from immune pathology during infection. An adult study with RSV infection has shown that shifting the hierarchy to favor the RSV epitope pups preferentially respond to reduces inflammation and morbidity (Ruckwardt et al., 2010). They, however also show that mice with shifted hierarchies have no defect in viral clearance. It is not clear if altered immunodominance is beneficial in avoiding severe immunopathology in neonatal influenza virus infections, but our data suggests that this may not be the case. Inducing $NP_{366-374}$-specific cells with PR8 loaded BMDCs improved viral clearance without increasing morbidity (Figure 3.5). Further study is needed to assess whether shifted immunodominance exists in neonatal responses to other common mild early life infections such as rhinovirus to determine if this may be an important mechanism of pathology sparing in infants.

Manipulating Tregs is often discussed in the context of decreasing suppressive function to bypass the immunosuppressive solid tumor environment or increasing suppressive function to combat autoimmunity, but these cells have been fairly extensively studied in the context of infection as well. This is less true for neonatal infections, but some things are known. Others have shown that a reduction of Tregs in neonates that overexpress them improves clearance of influenza virus (Jaligama et al., 2017), and we have shown that elimination of Tregs decreases viral clearance (Oliphant et al., 2015).

Unsurprisingly, studies have also shown that elimination of Tregs can dramatically increase morbidity in neonatal infections (Tucker et al., 2013). These three findings encapsulate the central factors surrounding Treg manipulation.

Manipulation of Tregs during infection must decrease suppression just enough to improve responses without allowing intolerable levels of immune pathology, all the while avoiding compromising the essential pro-immune function of Tregs in influenza virus defense. Our data presents some evidence that it is possible to decrease suppressive function of Tregs without compromising some pro-immune function, but all manipulations we attempted still resulted in increased morbidity. In fact, neonates may be fragile enough that a safe, therapeutic range of Treg intervention might not exist in this population.

I will present three theories that may explain why Treg depletion is detrimental to viral clearance. The first was proposed in adult mice and suggests that Tregs promote T follicular helper (Tfh) cell function, and therefore antibody production, by taking up IL-2 that would otherwise inhibit the generation of Tfh cells (Leon et al., 2014). The second suggests that without Tregs, competition between virus specific $CD4^+$ T cells and autoimmune $CD4^+$ T cells prevents adequate Tfh generation and interferes with effective immunity. Third, Tregs may perform some direct pro-immune effector function such as production of conventional Th cytokines.

Leon et al. demonstrated that adult mice depleted of Tregs had low numbers of influenza specific Tfhs in their lymph nodes along with diminished germinal center B cells (Leon et al., 2014). We saw a decrease in Tfh and anti-influenza virus antibody titre in scurfy neonates. We know from previous work that viral clearance is significantly

diminished in scurfy pups (Oliphant et al., 2015), and although these mice were not followed long enough to establish whether survival was possible, Treg depletion reliably resulted in extensive morbidity and mortality during all experiments performed (data not shown). In contrast to this, reports have suggested that Treg depletion does not change the course of influenza disease progression in adults (Betts et al., 2011). This was a partial depletion with anti-CD25 antibody, but depletions I performed in adult Foxp3^{DTR} mice, survived better than similarly depleted pups (data not shown). This is far from conclusive, but it suggests that Treg depletion in adult mice either does not compromise viral defense as significantly in adults as in pups, or that the increased inflammation from Treg depletion is much more lethal in pups than adults.

Neonates have poor Tfh function (Mastelic et al., 2012) and low Tfh numbers (Figure 5.1 A) may be especially sensitive to disruptions of this essential population. It is not currently clear if the decreased Tfh cell population in Treg depleted animals is responsible for the diminished antibody production scurfy pups exhibit, but decreased antibody production is a likely cause of diminished viral clearance. B cell deficient mice are essentially unable to clear highly pathogenic influenza strains like PR8 (Mozdzanowska et al., 1997; Mozdzanowska et al., 2000).

Polyclonal autoimmunity is characteristic of Treg deficient animals (Kim et al., 2007). Scurfy pups and Treg depleted mice develop lymphadenopathy from massive autoimmune activation of lymphocytes. Somewhat paradoxically, during influenza infection, Treg depleted animals develop smaller lymph nodes than undepleted infected animals. This diminished size could be related to the poor development of Tfh cells as I mentioned before, but it could also result from the extensive exodus of activated

autoimmune and virus-specific lymphocytes to the infected lung tissue. We showed that Treg depleted pups had an increase in $CD4^+$ T cells and $IFN\gamma^+$ $CD4^+$ T cells in the lungs during infection, possibly indicating that this mass migration has occurred (Figure 5.1 G, H).

Previous work has suggested that influenza specific $CD4^+$ T cell populations in the lymph node are split between Tfh and non-Tfh cells. These populations compete in the lymph node and extensive non-Tfh $CD4^+$ populations can suppress effective Tfh responses (Olson et al., 2016). During Treg depletion in infected animals, the significant polyclonal expansion of T cell populations may interfere with effective anti-influenza responses as well as anti-influenza Tfh generation. It is not clear if autoreactive cells are able to compete with influenza virus-specific $CD4^+$ T cells in the germinal center, but if competition reduces Tfh generation or results in non-influenza specific Tfh generation, this could have a serious impact on effective anti-influenza virus antibody production, as it has been clearly demonstrated that Tfh viral specificity is required for effective anti-viral B cell help (Deenick et al., 2010; Qi et al., 2014).

Tregs have well recognized proinflamatory functions under certain circumstances, although this is usually appreciated in the setting of autoimmune disease. It is possible that Tregs are required to perform a Th-like function in neonates, although they do have conventional $CD4^+$ T cells that respond during infections. We do not have any evidence of Tregs producing conventional effector cytokines in neonatal influenza virus infections, but it may be worth examining in the future. We did, however, assess expression of the conventional $CD4^+$ T cell associated transcription factors Tbet and Gata3 in neonatal Tregs.

While several studies suggest that Gata3 plays a role in Treg function, the scope of that involvement is poorly defined. We showed that a high proportion of neonatal Tregs express Gata3 (Figure 5.2 B), but it is unclear if this is evidence of some age specific role of Gata3 in Treg function or simply a consequence of Tregs existing in a neonatal environment. IL-4 stimulation is known to upregulate Gata3 in Tregs (Noval Rivas et al., 2015), so it is possible that Gata3 expression in neonatal Tregs is related to the Th2 bias and IL-4 production neonatal mice experience during many infections (Adkins et al., 1992; Coutinho et al., 1994). Uninfected pups also have elevated proportions of $Gata3^+$ Tregs, so this may not be a key mechanism. Asthmatic patients have a Th2 biased environment, and these individuals also have high proportions of Gata3 expressing Tregs (Chen, Hou, et al., 2018). Once again it is not clear if this is the product of T helper cells biased towards a Th2 response in an atopic individual or the direct result of the IL-4.

Several studies suggest that the primary role of Gata3 in Tregs is to support Foxp3 expression and therefore maintain suppressive function (Abdel Aziz et al., 2018; Wohlfert et al., 2011). Other studies suggest that Tregs can express transcription factors like Gata3 and Tbet to dedifferentiate into poorly suppressive cells that produce proinflammatory cytokines (Xu, Yang, et al., 2018). This raises the question of whether $Gata3^+$ Tregs in neonates are transitioning to non-suppressive cells or if the Gata3 expression is part of their suppressive function. Treg populations remain high during infection in neonates and other studies have suggested that $CD4^+$ T cells in neonates are biased towards becoming Tregs after stimulation rather than the reverse (Wang et al., 2010). Additionally, Leon et al. demonstrated that Tfhs in adult mice did not

differentiate from Treg precursors (Leon et al., 2014). We did not demonstrate that de-differentiation of Foxp3^{+} Tregs to Foxp3^{-} conventional T cells is not occurring in infected pups, but there is also no evidence of this occurring at this time.

We generated mice with Treg specific loss of Gata3 to examine the role this transcription factor might be playing in neonatal infections. We did not see a significant decrease in B cells or influenza virus neutralizing antibody production in Foxp3^{+} Gata3^{-} pups, suggesting that Gata3 is not responsible for the pro-antibody function of Tregs. Instead, Foxp3^{+}Gata3^{-} mice exhibited a phenotype consistent with diminished but not absent suppressive function both before and during infection.

There is debate about whether loss of Gata3 in Tregs results in a significant defect in Treg suppressive function. Some have reported no effect (Yu et al., 2015) and others have suggested that these animals develop autoimmunity several months into life (Wohlfert et al., 2011; Xu, Yang, et al., 2018). The small size of our Foxp3^{+}Gata3^{-} pups and their reduced representation in litters suggests that they are less healthy than Gata3^{+} littermates. Additionally, during infection, Foxp3^{+}Gata3^{-} pups have an increase in Tbet expression and IFNγ producing CD4^{+} T cells in the lung (Figure 5.4 E). This is similar to the increase in cell number and Th1 function that scurfy pups exhibit, and as such possibly indicates a decrease in suppressive function (Oliphant et al., 2015). The increase in IFNγ expression correlates with increased morbidity in Foxp3^{+}Gata3^{-} pups (Figure 5.5 E). Foxp3^{+}Gata3^{-} pups did not experience the high lethality of a full Treg depletion, but they do exhibit some of the increased morbidity associated with other studies of Treg depletion in infection (Lee et al., 2010; Suvas et al., 2004; Tucker et al., 2013).

I do not think that reducing Treg function is currently a worthwhile goal in the process of improving neonatal influenza virus vaccination or infection. Neonates are too intolerant of increased inflammation and the adaptive immune response to influenza virus is too specific to support reducing Treg function in this age group.

6.1 Limitations and further directions

While peptide vaccination is undoubtedly useful for personalized immunotherapy for cancer, it is likely not an ideal strategy for generating antiviral responses in a broad population. The immunodominant epitopes of PR8 infection in C57Bl/6 mice are known and predicable, but human infections with a diverse array of influenza virus strains and a broad range of HLA haplotypes are not as consistent. While human $CD8^+$ T cells responses are often reliably stimulated by a few viral subunits, peptides are too specific. As such, we do not know if human infants experience altered immunodominance during influenza virus infection, nor what form such a shift would take. The altered immunodominance of neonatal mice to RSV infection suggests that this is not a phenomenon of a single infection, but we do not have confirmation that this is a human phenomenon as well. In a practical sense, any vaccine tested for use in humans will be tested in neonates before being used extensively in this population, and this will ascertain the response to any antigens used in a particular vaccine. Further work understanding the determinants of neonatal immunodominance in mice will hopefully help resolve whatever difficulties arise in the use of subunit T cell vaccines in human neonates.

I have now outlined many questions regarding dendritic cell function and I believe understanding this cell population better lies at the heart of any efforts to improve

neonatal T cell responses. While others have pointed to the immaturity of the T cell receptor repertoire (Carey et al., 2016), we have shown that BMDCs could alter the activation of these immature cells. Beyond this, manipulating dendritic cells is likely a much more feasible and safe goal than trying to alter the TCR repertoire of infants. I will propose what I believe are the most immediate goals of future work regarding understanding neonatal immunodominance during influenza virus infection.

First, we must determine whether neonatal dendritic cells can present both $NP_{366-374}$ and $PA_{224-233}$ during influenza virus infection. If they cannot, we should investigate whether direct presentation and cross-presentation mechanisms are intact in neonatal dendritic cells. This may involve examining components of the peptide processing pathways and expression of MHC class I. Also, while work has characterized defects in the stimulatory capacity of neonatal DCs, we will need to ascertain whether this poor stimulation prevents adequate activation of $NP_{366-374}$ specific cells.

Second, we need to find a way to safely mature neonatal dendritic cells in vivo. While it is known that administering LPS (Malloy et al., 2017), GM-CSF (Qureshi et al., 2005) or Flt3 ligand (Remot et al., 2016) can improve neonatal DC maturity, perhaps the most crucial task will be determining if this maturation can be done in a specific and limited manner to avoid excess activation. While vaccination with autologous GM-CSF derived dendritic cells is performed for cancer immunotherapy, it is hardly a desirable procedure for routine vaccination. Conventional adjuvants are needed.

Third, while we showed evidence of the role immunodominance plays in neonatal $CD8^+$ T cell responses to influenza virus, we did very little to characterize the $CD4^+$ T cell immunodominance hierarchy and the role of $CD4^+$ T cells in subunit vaccination.

$CD4^+$ T cell immunodominance is challenging to study, as the reagents are less developed and the hierarchy is typically much broader than $CD8^+$ T cells hierarchy (Crowe et al., 2006), but effective use of subunit vaccines in neonates will require understanding whether the neonatal $CD4^+$ T cell hierarchy is shifted as well and whether the different T cell specificities in that hierarchy are able to provide adequate protection. As $CD4^+$ and $CD8^+$ T cells do not share peptide epitopes, this relates more to subunit vaccination than peptide vaccination, but similar principles apply. Our data suggest that $CD4^+$ T cell memory may be playing an important role in T cell memory established as a pup as mice first infected as pups were protected from lethal heterosubtypic influenza virus infection (Figure 3.2 A) despite not displaying an early onset of $NP_{366-374}$-specific cells (Figure 3.3 A).

To revisit a significant concern with the methodology, I spoke in chapter 3 about the possibility of tetramers underestimating antigen specific populations (Rius et al., 2018). This is an important concern, especially as I suggested that the lower affinity of $NP_{366-374}$ cells may be related to their lack of expansion in neonates. I believe the data obtained here is accurate for two reasons. First, we detected $NP_{366-374}$-specific cells by tetramer both in the few pups that naturally respond to $NP_{366-374}$ and in the pups that received adult BMDCs, suggesting that tetramers can detect $NP_{366-374}$-specific cells produced by pups. Second, pups repeatedly demonstrated significantly lower IFNγ production when stimulated with $NP_{366-374}$ and BMDCs than adults stimulated with $NP_{366-374}$ and BMDCs or pups stimulated with $PA_{224-233}$ and BMDCs. BMDC stimulation is more sensitive than tetramers and should have detected a significant $NP_{366-374}$-specific

population if it existed. ELISPOT is even more specific and sensitive and would give the most certain answer as to whether this population is really absent.

Our data on the role of Tregs in influenza infection is particularly limited due to the high mortality rate of Treg depleted mice. Additionally, the total loss of Tregs results in extremely atypical immunity that makes it difficult to isolate single causes for a given phenomenon. In part because of these difficulties, many questions remain about the role of Tregs in neonatal influenza virus infections. We showed a decrease in Tfh cells after Treg depletion, but it is not clear how functional the remaining Tfh cells are. IL-21 production and CD40 expression could demonstrate these cells' capacity to help B cells during infection.

Neonates have disorganized germinal center morphology under normal circumstances (Munguía-Fuentes et al., 2017), but it would be useful to know if Treg depletion disrupts these structures further. Microscopy of Treg depleted TBLNs would help address this question. If IL-2 inhibits Tfh generation in Treg depleted adults, we should examine lymph nodes of Treg depleted pups to see if IL-2 levels are increased.

Additionally, I did little to characterize B cell function in the lymph node. Examining class switching would help illuminate whether B cells are receiving adequate help. Finally, more sensitive anti-influenza antibody screening in the lavage by ELISA could help ascertain whether scurfy pups lack an antibody response or if it is just diminished. Hemagglutination inhibition assays are not as sensitive.

The full role of Gata3 in Tregs remains unclear. The increased Tbet expression in KO pups could suggest that KO Tregs are less suppressive than WT cells, although an *in vitro* suppressive assay is needed to fully demonstrate this. Loss of Gata3 does not reduce Treg ability to suppress ulcerative colitis (Yu et al., 2015), but the gross phenotypic changes we observed in KO mice suggests that there may be more subtle defects, especially in pups (Figure 5.3). While the increase in Tbet expression seems consistent, more trials are needed to determine whether there is a change in antibody production. Future studies should also assess the function of the increased Tfh population in $Foxp3^{+}Gata3^{-}$ mice and determine whether these cells are virus specific.

As with Treg depletion studies, low sample size limits the conclusions that can be drawn from the $Foxp3^{+}Gata3^{-}$ mice. There were not enough pups to examine uninfected $Foxp3^{+}Gata3^{-}$ mice, but assessing the immune response of these animals is essential to fully understand the effect of infection on these dysregulated neonates.

6.2 Summary

We showed that immunodominance is altered in neonatal pups and that the differences in the hierarchy have potentially serious consequences for $CD8^{+}$ T cell vaccination in neonates. Our data suggests that responses to adult-dominant epitopes can be generated in neonates with proper stimulation by dendritic cells. Additionally, we showed that Tregs likely have important suppressive and anti-viral roles in influenza infection by decreasing Th1 responses and supporting antibody generation respectively. I propose that efforts to improve neonatal immune T cell reactions should focus on altering dendritic cell function without tampering with Treg suppression. Overall, it appears that

although neonatal immunity is different from adult immunity, at least some of these differences are not absolute and can be altered by future therapies.

LIST OF ABBREVIATIONS

A/HKx31	HKx31
A/Puerto Rico8/1934 H1N1	PR8
Acid polymerase (amino acids 224-233)	$PA_{224-233}$
Antigen presenting cell	APC
B-cell lymphoma 6 protein	BCL-6
Bone marrow-derived dendritic cells	BMDCs
Bovine serum albumin	BSA
Bronchial alveolar lavage	BAL
$CD103^{+}$ Dendritic cell	cDC1
CD11b+ Dendritic cell	cDC2
Chicken red blood cell	CRBC
CpG oligodeoxynucleotides	CpG
Diphtheria toxin receptor	DTR
Ethylenediaminetetraacetic acid	EDTA\
Fetal Calf Serum	FCS
FMS-related tyrosine kinase 3 ligand	Flt3L
Forkhead box p3	Foxp3
GATA binding protein 3	Gata3
Granulocyte-Monocyte Colony Stimulating Factor	GM-CSF
Hank’s Buffered Salt Solution	HBSS
Hemagglutinin	HA
Hemagglutinin Inhibition Assay	HAI
Hemagglutinin Inhibition Unit	HAU
Herpes simplex virus	HSV
Human rhinovirus	HRV
Immunoglobulin	Ig
Induced bronchial associated lymph tissue	iBALT
Interleukin	IL

Intranasal	i.n.
Intraperitoneal	i.p.
Lethal dose in 10% of mice	LD_{10}
Lipopolysaccharide	LPS
Major histocompatibility complex	MHC
Matrix protein 1	M1
Matrix protein 2	M2
Neuraminidase	NA
Nonstructural protein 1	NS1
Nonstructural protein 2	NEP
Nucleoprotein (amino acids 366-374)	$NP_{366-374}$
Pathogen associated molecular patterns	PAMPs
Pattern recognition receptors	PRRs
Plasmacytoid dendritic cell	pDC
Phorbol 12-myristate 13-acetate	PMA
Polymerase basic protein 1 frame 2	PB1-F2
Polymerase basic protein 1	PB1
Polymerase basic protein 2	PB2
Protein blocking agent	PMA
RAR-related orphan receptor gamma	RORγ
Respiratory syncytial virus	RSV
Ribonucleoprotein particles	RNPs
Roswell Park Memorial Institute	RPMI
T cell receptor	TCR
T follicular helper cell	Tfh
T helper	Th
T regulatory cells	Treg
T-box transcription factor TBX21	Tbet
Toll-like receptors	TLR
Tracheal-bronchial lymph node	TBLN

Transforming growth factor beta	TGFβ
Tumor Necrosis Factor alpha	TNFα
Type I interferon alpha	IFNα
Type II interferon gamma	IFNγ

REFERENCES

Abbott, R. K., Lee, J. H., Menis, S., Skog, P., Rossi, M., Ota, T., . . . Crotty, S. (2018). Precursor Frequency and Affinity Determine B Cell Competitive Fitness in Germinal Centers, Tested with Germline-Targeting HIV Vaccine Immunogens. *Immunity, 48*(1), 133-146.e136.

Abdel Aziz, N., Nono, J. K., Mpotje, T., & Brombacher, F. (2018). The Foxp3+ regulatory T-cell population requires IL-4Ralpha signaling to control inflammation during helminth infections. *PLoS Biol, 16*(10), e2005850.

Adkins, B. (2005). Neonatal T cell function. *J Pediatr Gastroenterol Nutr, 40 Suppl 1*, S5-7.

Adkins, B., Bu, Y., & Guevara, P. (2002). Murine neonatal CD4+ lymph node cells are highly deficient in the development of antigen-specific Th1 function in adoptive adult hosts. *J Immunol, 169*(9), 4998-5004.

Adkins, B., & Du, R. Q. (1998). Newborn mice develop balanced Th1/Th2 primary effector responses in vivo but are biased to Th2 secondary responses. *J Immunol, 160*(9), 4217-4224.

Adkins, B., Ghanei, A., & Hamilton, K. (1994). Up-regulation of murine neonatal T helper cell responses by accessory cell factors. *J Immunol, 153*(8), 3378-3385.

Adkins, B., & Hamilton, K. (1992). Freshly isolated, murine neonatal T cells produce IL-4 in response to anti-CD3 stimulation. *J Immunol, 149*(11), 3448-3455.

Afkarian, M., Sedy, J. R., Yang, J., Jacobson, N. G., Cereb, N., Yang, S. Y., . . . Murphy, K. M. (2002). T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nat Immunol, 3*(6), 549-557.

Akondy, R. S., Fitch, M., Edupuganti, S., Yang, S., Kissick, H. T., Li, K. W., . . . Ahmed, R. (2017). Origin and differentiation of human memory CD8 T cells after vaccination. *Nature, 552*(7685), 362-367.

Alejandre-Alcazar, M. A., Michiels-Corsten, M., Vicencio, A. G., Reiss, I., Ryu, J., de Krijger, R. R., . . . Morty, R. E. (2008). TGF-beta signaling is dynamically regulated during the alveolarization of rodent and human lungs. *Dev Dyn, 237*(1), 259-269.

Angeletti, D., & Yewdell, J. W. (2018). Is It Possible to Develop a "Universal" Influenza Virus Vaccine? Outflanking Antibody Immunodominance on the Road to Universal Influenza Vaccination. *Cold Spring Harb Perspect Biol, 10*(7).

Antonova, E. N., Rycroft, C. E., Ambrose, C. S., Heikkinen, T., & Principi, N. (2012). Burden of paediatric influenza in Western Europe: a systematic review. *BMC Public Health, 12*, 968-968.

Ascough, S., Paterson, S., & Chiu, C. (2018). Induction and Subversion of Human Protective Immunity: Contrasting Influenza and Respiratory Syncytial Virus. *Frontiers in Immunology, 9*, 323.

Bacchetta, R., Barzaghi, F., & Roncarolo, M. G. (2016). From IPEX syndrome to FOXP3 mutation: a lesson on immune dysregulation. *Ann N Y Acad Sci*.

Bachmann, M. F., Lutz, M. B., Layton, G. T., Harris, S. J., Fehr, T., Rescigno, M., & Ricciardi-Castagnoli, P. (1996). Dendritic cells process exogenous viral proteins and virus-like particles for class I presentation to CD8+ cytotoxic T lymphocytes. *Eur J Immunol, 26*(11), 2595-2600.

Baharom, F., Thomas, S., Bieder, A., Hellmer, M., Volz, J., Sandgren, K. J., . . . Smed-Sorensen, A. (2015). Protection of human myeloid dendritic cell subsets against influenza A virus infection is differentially regulated upon TLR stimulation. *J Immunol, 194*(9), 4422-4430.

Ballesteros-Tato, A., Leon, B., Lee, B. O., Lund, F. E., & Randall, T. D. (2014). Epitope-specific regulation of memory programming by differential duration of antigen presentation to influenza-specific CD8(+) T cells. *Immunity, 41*(1), 127-140.

Banerjee, I., Miyake, Y., Nobs, S. P., Schneider, C., Horvath, P., Kopf, M., . . . Yamauchi, Y. (2014). Influenza A virus uses the aggresome processing machinery for host cell entry. *Science, 346*(6208), 473-477.

Basha, S., Surendran, N., & Pichichero, M. (2014). Immune responses in neonates. *Expert Rev Clin Immunol, 10*(9), 1171-1184.

Bedoui, S., Heath, W. R., & Mueller, S. N. (2016). CD4(+) T-cell help amplifies innate signals for primary CD8(+) T-cell immunity. *Immunol Rev, 272*(1), 52-64.

Bedoya, F., Cheng, G. S., Leibow, A., Zakhary, N., Weissler, K., Garcia, V., . . . Caton, A. J. (2013). Viral antigen induces differentiation of Foxp3+ natural regulatory T cells in influenza virus-infected mice. *J Immunol, 190*(12), 6115-6125.

Belongia, E. A., Kieke, B. A., Donahue, J. G., Greenlee, R. T., Balish, A., Foust, A., . . . Shay, D. K. (2009). Effectiveness of inactivated influenza vaccines varied substantially with antigenic match from the 2004-2005 season to the 2006-2007 season. *J Infect Dis, 199*(2), 159-167.

Belz, G. T., Stevenson, P. G., & Doherty, P. C. (2000). Contemporary analysis of MHC-related immunodominance hierarchies in the CD8+ T cell response to influenza A viruses. *J Immunol, 165*(5), 2404-2409.

Bender, A., Albert, M., Reddy, A., Feldman, M., Sauter, B., Kaplan, G., . . . Bhardwaj, N. (1998). The distinctive features of influenza virus infection of dendritic cells. *Immunobiology, 198*(5), 552-567.

Bettelli, E., Sullivan, B., Szabo, S. J., Sobel, R. A., Glimcher, L. H., & Kuchroo, V. K. (2004). Loss of T-bet, But Not STAT1, Prevents the Development of Experimental Autoimmune Encephalomyelitis. *The Journal of Experimental Medicine, 200*(1), 79-87.

Betts, R. J., Ho, A. W., & Kemeny, D. M. (2011). Partial depletion of natural CD4(+)CD25(+) regulatory T cells with anti-CD25 antibody does not alter the course of acute influenza A virus infection. *PLoS One, 6*(11), e27849.

Bhattacharya, P., Gopisetty, A., Ganesh, B. B., Sheng, J. R., & Prabhakar, B. S. (2011). GM-CSF-induced, bone-marrow-derived dendritic cells can expand natural Tregs and induce adaptive Tregs by different mechanisms. *Journal of Leukocyte Biology, 89*(2), 235-249.

Boonnak, K., Vogel, L., Orandle, M., Zimmerman, D., Talor, E., & Subbarao, K. (2013). Antigen-activated dendritic cells ameliorate influenza A infections. *J Clin Invest, 123*(7), 2850-2861.

Bouvier, N. M., & Lowen, A. C. (2010). Animal Models for Influenza Virus Pathogenesis and Transmission. *Viruses, 2*(8), 1530-1563.

Braciale, T. J., & Hahn, Y. S. (2013). Immunity to viruses. *Immunol Rev, 255*(1), 5-12.

Brankston, G., Gitterman, L., Hirji, Z., Lemieux, C., & Gardam, M. (2007). Transmission of influenza A in human beings. *Lancet Infect Dis, 7*(4), 257-265.

Carey, A. J., Gracias, D. T., Thayer, J. L., Boesteanu, A. C., Kumova, O. K., Mueller, Y. M., . . . Katsikis, P. D. (2016). Rapid Evolution of the CD8+ TCR Repertoire in Neonatal Mice. *J Immunol*.

Carey, A. J., Hope, J. L., Mueller, Y. M., Fike, A. J., Kumova, O. K., van Zessen, D. B. H., . . . Katsikis, P. D. (2017). Public Clonotypes and Convergent Recombination Characterize the Naive CD8(+) T-Cell Receptor Repertoire of Extremely Preterm Neonates. *Front Immunol, 8*, 1859.

Carrat, F., Vergu, E., Ferguson, N. M., Lemaitre, M., Cauchemez, S., Leach, S., & Valleron, A. J. (2008). Time lines of infection and disease in human influenza: a review of volunteer challenge studies. *Am J Epidemiol, 167*(7), 775-785.

Chen, T., Hou, X., Ni, Y., Du, W., Han, H., Yu, Y., & Shi, G. (2018). The Imbalance of FOXP3/GATA3 in Regulatory T Cells from the Peripheral Blood of Asthmatic Patients. *J Immunol Res, 2018*, 3096183.

Chen, W., Anton, L. C., Bennink, J. R., & Yewdell, J. W. (2000). Dissecting the multifactorial causes of immunodominance in class I-restricted T cell responses to viruses. *Immunity, 12*(1), 83-93.

Chen, W., Pang, K., Masterman, K.-A., Kennedy, G., Basta, S., Dimopoulos, N., . . . Yewdell, J. W. (2004a). Reversal in the Immunodominance Hierarchy in Secondary $CD8^+$ T Cell Responses to Influenza A Virus: Roles for Cross-Presentation and Lysis-Independent Immunodomination. *The Journal of Immunology, 173*(8), 5021-5027.

Chen, W., Pang, K., Masterman, K.-A., Kennedy, G., Basta, S., Dimopoulos, N., . . . Yewdell, J. W. (2004b). Reversal in the Immunodominance Hierarchy in Secondary CD8⁺ T Cell Responses to Influenza A Virus: Roles for Cross-Presentation and Lysis-Independent Immunodomination. *The Journal of Immunology, 173*(8), 5021-5027.

Chen, X., Liu, S., Goraya, M. U., Maarouf, M., Huang, S., & Chen, J.-L. (2018). Host Immune Response to Influenza A Virus Infection. *Frontiers in Immunology, 9*, 320-320.

Cheng, C., Wang, S., Ye, P., Huang, X., Liu, Z., Wu, J., . . . Xia, J. (2014). 'Default' generated neonatal regulatory T cells are hypomethylated at conserved non-coding sequence 2 and promote long-term cardiac allograft survival. *Immunology, 143*(4), 618-630.

Chevalier, N. (2015). Quantifying helper cell function of human TFH cells in vitro. *Methods Mol Biol, 1291*, 209-226.

Chevrier, S., Kratina, T., Emslie, D., Tarlinton, D. M., & Corcoran, L. M. (2017). IL4 and IL21 cooperate to induce the high Bcl6 protein level required for germinal center formation. *Immunol Cell Biol, 95*(10), 925-932.

Ciancanelli, M. J., Abel, L., Zhang, S. Y., & Casanova, J. L. (2016). Host genetics of severe influenza: from mouse Mx1 to human IRF7. *Curr Opin Immunol, 38*, 109-120.

Clay, C. C., Reader, J. R., Gerriets, J. E., Wang, T. T., Harrod, K. S., & Miller, L. A. (2014). Enhanced viral replication and modulated innate immune responses in infant airway epithelium following H1N1 infection. *J Virol, 88*(13), 7412-7425.

Connors, T. J., Baird, J. S., Yopes, M. C., Zens, K. D., Pethe, K., Ravindranath, T. M., . . . Farber, D. L. (2018). Developmental Regulation of Effector and Resident

Memory T Cell Generation during Pediatric Viral Respiratory Tract Infection. *The Journal of Immunology*, *201*(2), 432.

Cook, P. C., & MacDonald, A. S. (2016). Dendritic cells in lung immunopathology. *Seminars in immunopathology*, *38*(4), 449-460.

Cottey, R., Rowe, C. A., & Bender, B. S. (2001). Influenza virus. *Curr Protoc Immunol*, *Chapter 19*, Unit 19.11.

Coutinho, G. C., Delassus, S., Kourilsky, P., Bandeira, A., & Coutinho, A. (1994). Developmental shift in the patterns of interleukin production in early post-natal life. *Eur J Immunol*, *24*(8), 1858-1862.

Crowe, S. R., Miller, S. C., Brown, D. M., Adams, P. S., Dutton, R. W., Harmsen, A. G., . . . Woodland, D. L. (2006). Uneven distribution of MHC class II epitopes within the influenza virus. *Vaccine*, *24*(4), 457-467.

Crowe, S. R., Miller, S. C., Shenyo, R. M., & Woodland, D. L. (2005). Vaccination with an acidic polymerase epitope of influenza virus elicits a potent antiviral T cell response but delayed clearance of an influenza virus challenge. *J Immunol*, *174*(2), 696-701.

Crowe, S. R., Turner, S. J., Miller, S. C., Roberts, A. D., Rappolo, R. A., Doherty, P. C., . . . Woodland, D. L. (2003). Differential Antigen Presentation Regulates the Changing Patterns of CD8(+) T Cell Immunodominance in Primary and Secondary Influenza Virus Infections. *The Journal of Experimental Medicine*, *198*(3), 399-410.

Cruz, F. M., Colbert, J. D., Merino, E., Kriegsman, B. A., & Rock, K. L. (2017). The Biology and Underlying Mechanisms of Cross-Presentation of Exogenous Antigens on MHC-I Molecules. *Annu Rev Immunol*, *35*, 149-176.

Cukalac, T., Chadderton, J., Zeng, W., Cullen, J. G., Kan, W. T., Doherty, P. C., . . . La Gruta, N. L. (2014). The Influenza Virus–Specific CTL Immunodominance Hierarchy in Mice Is Determined by the Relative Frequency of High-Avidity T Cells. *The Journal of Immunology*, *192*(9), 4061-4068.

D'Angio, C. T., Maniscalco, W. M., & Pichichero, M. E. (1995). Immunologic response of extremely premature infants to tetanus, Haemophilus influenzae, and polio immunizations. *Pediatrics*, *96*(1 Pt 1), 18-22.

Dakic, A., Shao, Q. X., D'Amico, A., O'Keeffe, M., Chen, W. F., Shortman, K., & Wu, L. (2004). Development of the dendritic cell system during mouse ontogeny. *J Immunol*, *172*(2), 1018-1027.

Das, T., Sargan, A., Yagmur, G., Yildirim, M., Topal, C. S., Gurler, A. S., . . . Ozdemirel, R. (2016). Viral Pneumonias in Forensic Autopsies: Evaluation and Classification of Histopathologic Changes With Microbiologic Correlation. *Am J Forensic Med Pathol*, *37*(4), 255-263.

Day, E. B., Charlton, K. L., La Gruta, N. L., Doherty, P. C., & Turner, S. J. (2011). Effect of MHC class I diversification on influenza epitope-specific CD8+ T cell precursor frequency and subsequent effector function. *J Immunol*, *186*(11), 6319-6328.

de Kleer, I. M., Kool, M., de Bruijn, M. J., Willart, M., van Moorleghem, J., Schuijs, M. J., . . . Lambrecht, B. N. (2016). Perinatal Activation of the Interleukin-33 Pathway Promotes Type 2 Immunity in the Developing Lung. *Immunity*, *45*(6), 1285-1298.

De Wit, D., Olislagers, V., Goriely, S., Vermeulen, F., Wagner, H., Goldman, M., & Willems, F. (2004). Blood plasmacytoid dendritic cell responses to CpG oligodeoxynucleotides are impaired in human newborns. *Blood, 103*(3), 1030-1032.

Debock, I., Jaworski, K., Chadlaoui, H., Delbauve, S., Passon, N., Twyffels, L., . . . Flamand, V. (2013). Neonatal follicular Th cell responses are impaired and modulated by IL-4. *J Immunol, 191*(3), 1231-1239.

Deenick, E. K., Chan, A., Ma, C. S., Gatto, D., Schwartzberg, P. L., Brink, R., & Tangye, S. G. (2010). Follicular helper T cell differentiation requires continuous antigen presentation that is independent of unique B cell signaling. *Immunity, 33*(2), 241-253.

Delgado, M. F., Coviello, S., Monsalvo, A. C., Melendi, G. A., Hernandez, J. Z., Batalle, J. P., . . . Polack, F. P. (2009). Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. *Nat Med, 15*(1), 34-41.

Devaud, C., Darcy, P. K., & Kershaw, M. H. (2014). Foxp3 expression in T regulatory cells and other cell lineages. *Cancer Immunol Immunother, 63*(9), 869-876.

Dhamne, C., Chung, Y., Alousi, A. M., Cooper, L. J. N., & Tran, D. Q. (2013). Peripheral and Thymic Foxp3(+) Regulatory T Cells in Search of Origin, Distinction, and Function. *Frontiers in Immunology, 4*, 253.

Diebold, S. S., Montoya, M., Unger, H., Alexopoulou, L., Roy, P., Haswell, L. E., . . . Reis e Sousa, C. (2003). Viral infection switches non-plasmacytoid dendritic cells into high interferon producers. *Nature, 424*(6946), 324-328.

Dinter, J., Gourdain, P., Lai, N. Y., Duong, E., Bracho-Sanchez, E., Rucevic, M., . . . Le Gall, S. (2014). Different antigen-processing activities in dendritic cells, macrophages, and monocytes lead to uneven production of HIV epitopes and affect CTL recognition. *J Immunol, 193*(9), 4322-4334.

Doherty, P. C., Topham, D. J., Tripp, R. A., Cardin, R. D., Brooks, J. W., & Stevenson, P. G. (1997). Effector CD4+ and CD8+ T-cell mechanisms in the control of respiratory virus infections. *Immunol Rev, 159*, 105-117.

Doyle, J. D., Chung, J. R., Kim, S. S., Gaglani, M., Raiyani, C., Zimmerman, R. K., . . . Flannery, B. (2019). Interim Estimates of 2018-19 Seasonal Influenza Vaccine Effectiveness - United States, February 2019. *MMWR Morb Mortal Wkly Rep, 68*(6), 135-139.

Durbin, R. K., Kotenko, S. V., & Durbin, J. E. (2013). Interferon induction and function at the mucosal surface. *Immunol Rev, 255*(1), 25-39.

Ebrahimi-Nik, H., Corwin, W. L., Shcheglova, T., Das Mohapatra, A., Mandoiu, II, & Srivastava, P. K. (2018). CD11c(+) MHCII(lo) GM-CSF-bone marrow-derived dendritic cells act as antigen donor cells and as antigen presenting cells in neoepitope-elicited tumor immunity against a mouse fibrosarcoma. *Cancer Immunol Immunother, 67*(9), 1449-1459.

Edinger, T. O., Pohl, M. O., & Stertz, S. (2014). Entry of influenza A virus: host factors and antiviral targets. *J Gen Virol, 95*(Pt 2), 263-277.

Epstein, S. L., Lo, C. Y., Misplon, J. A., & Bennink, J. R. (1998). Mechanism of protective immunity against influenza virus infection in mice without antibodies. *J Immunol, 160*(1), 322-327.

Estrada, L. D., & Schultz-Cherry, S. (2019). Development of a Universal Influenza Vaccine. *The Journal of Immunology, 202*(2), 392-398.

Fadel, S. A., Cowell, L. G., Cao, S., Ozaki, D. A., Kepler, T. B., Steeber, D. A., & Sarzotti, M. (2006). Neonate-primed CD8+ memory cells rival adult-primed memory cells in antigen-driven expansion and anti-viral protection. *Int Immunol, 18*(2), 249-257.

Feldman, A. S., He, Y., Moore, M. L., Hershenson, M. B., & Hartert, T. V. (2015). Toward primary prevention of asthma. Reviewing the evidence for early-life respiratory viral infections as modifiable risk factors to prevent childhood asthma. *American journal of respiratory and critical care medicine, 191*(1), 34-44.

Fensterl, V., Chattopadhyay, S., & Sen, G. C. (2015). No Love Lost Between Viruses and Interferons. *Annu Rev Virol, 2*(1), 549-572.

Fernandez, M. A., Puttur, F. K., Wang, Y. M., Howden, W., Alexander, S. I., & Jones, C. A. (2008a). T Regulatory Cells Contribute to the Attenuated Primary CD8+ and CD4+ T Cell Responses to Herpes Simplex Virus Type 2 in Neonatal Mice. *The Journal of Immunology, 180*(3), 1556-1564.

Fernandez, M. A., Puttur, F. K., Wang, Y. M., Howden, W., Alexander, S. I., & Jones, C. A. (2008b). T regulatory cells contribute to the attenuated primary CD8+ and CD4+ T cell responses to herpes simplex virus type 2 in neonatal mice. *J Immunol, 180*(3), 1556-1564.

Fike, A. J., Kumova, O. K., Tardif, V. J., & Carey, A. J. (2018). Neonatal influenza-specific effector CTLs retain elevated CD31 levels at the site of infection and have decreased IFN-gamma production. *J Leukoc Biol*.

Foster, J. J., Goss, K. L., George, C. L., Bangsund, P. J., & Snyder, J. M. (2006). Galectin-1 in secondary alveolar septae of neonatal mouse lung. *Am J Physiol Lung Cell Mol Physiol, 291*(6), L1142-1149.

Galani, V., Papadatos, S. S., Alexiou, G., Galani, A., & Kyritsis, A. P. (2017). In Vitro and In Vivo Preclinical Effects of Type I IFNs on Gliomas. *J Interferon Cytokine Res, 37*(4), 139-146.

Ge, M. Q., Ho, A. W., Tang, Y., Wong, K. H., Chua, B. Y., Gasser, S., & Kemeny, D. M. (2012). NK cells regulate CD8+ T cell priming and dendritic cell migration during influenza A infection by IFN-gamma and perforin-dependent mechanisms. *J Immunol, 189*(5), 2099-2109.

Gervassi, A. L., & Horton, H. (2014). Is Infant Immunity Actively Suppressed or Immature? *Virology (Auckl), 2014*(5), 1-9.

Geurts van Kessel, C. H., Willart, M. A., van Rijt, L. S., Muskens, F., Kool, M., Baas, C., . . . Lambrecht, B. N. (2008). Clearance of influenza virus from the lung depends on migratory langerin+CD11b- but not plasmacytoid dendritic cells. *J Exp Med, 205*(7), 1621-1634.

Godfrey, V. L., Wilkinson, J. E., & Russell, L. B. (1991). X-linked lymphoreticular disease in the scurfy (sf) mutant mouse. *Am J Pathol, 138*(6), 1379-1387.

Gold, M. C., Robinson, T. L., Cook, M. S., Byrd, L. K., Ehlinger, H. D., Lewinsohn, D. M., & Lewinsohn, D. A. (2007). Human neonatal dendritic cells are competent in MHC class I antigen processing and presentation. *PLoS One, 2*(9), e957.

Gondek, D. C., Lu, L. F., Quezada, S. A., Sakaguchi, S., & Noelle, R. J. (2005). Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a

granzyme B-dependent, perforin-independent mechanism. *J Immunol, 174*(4), 1783-1786.

Gottlieb, T., & Ben-Yedidia, T. (2014). Epitope-based approaches to a universal influenza vaccine. *J Autoimmun, 54*, 15-20.

Graham, B. S., Bunton, L. A., Wright, P. F., & Karzon, D. T. (1991). Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. *J Clin Invest, 88*(3), 1026-1033.

Graham, J. B., Da Costa, A., & Lund, J. M. (2014). Regulatory T cells shape the resident memory T cell response to virus infection in the tissues. *J Immunol, 192*(2), 683-690.

Graham, M. B., Braciale, V. L., & Braciale, T. J. (1994). Influenza virus-specific CD4+ T helper type 2 T lymphocytes do not promote recovery from experimental virus infection. *J Exp Med, 180*(4), 1273-1282.

Grayson, M. H., Ramos, M. S., Rohlfing, M. M., Kitchens, R., Wang, H. D., Gould, A., . . . Holtzman, M. J. (2007). Controls for lung dendritic cell maturation and migration during respiratory viral infection. *J Immunol, 179*(3), 1438-1448.

Grohskopf, L. A., Sokolow, L. Z., Broder, K. R., Walter, E. B., Fry, A. M., & Jernigan, D. B. (2018). Prevention and Control of Seasonal Influenza with Vaccines: Recommendations of the Advisory Committee on Immunization Practices-United States, 2018-19 Influenza Season. *MMWR Recomm Rep, 67*(3), 1-20.

Guarner, J., Paddock, C. D., Shieh, W.-J., Packard, M. M., Patel, M., Montague, J. L., . . . Zaki, S. R. (2006). Histopathologic and Immunohistochemical Features of Fatal Influenza Virus Infection in Children during the 2003–2004 Season. *Clinical Infectious Diseases, 43*(2), 132-140.

Habibi, M. S., Jozwik, A., Makris, S., Dunning, J., Paras, A., DeVincenzo, J. P., . . . Mechanisms of Severe Acute Influenza Consortium, I. (2015). Impaired Antibody-mediated Protection and Defective IgA B-Cell Memory in Experimental Infection of Adults with Respiratory Syncytial Virus. *American journal of respiratory and critical care medicine, 191*(9), 1040-1049.

Hamza, E., Mirkovitch, J., Steinbach, F., & Marti, E. (2015). Regulatory T cells in early life: comparative study of CD4+CD25high T cells from foals and adult horses. *PLoS One, 10*(3), e0120661.

Harbeson, D., Ben-Othman, R., Amenyogbe, N., & Kollmann, T. R. (2018). Outgrowing the Immaturity Myth: The Cost of Defending From Neonatal Infectious Disease. *Front Immunol, 9*, 1077.

Harbeson, D., Francis, F., Bao, W., Amenyogbe, N. A., & Kollmann, T. R. (2018). Energy Demands of Early Life Drive a Disease Tolerant Phenotype and Dictate Outcome in Neonatal Bacterial Sepsis. *Front Immunol, 9*, 1918.

Hardy, R. R., & Hayakawa, K. (2001). B cell development pathways. *Annu Rev Immunol, 19*, 595-621.

Heit, A., Gebhardt, F., Lahl, K., Neuenhahn, M., Schmitz, F., Anderl, F., . . . Kastenmuller, K. (2008). Circumvention of regulatory CD4(+) T cell activity during cross-priming strongly enhances T cell-mediated immunity. *Eur J Immunol, 38*(6), 1585-1597.

Helft, J., Bottcher, J., Chakravarty, P., Zelenay, S., Huotari, J., Schraml, B. U., . . . Reis e Sousa, C. (2015). GM-CSF Mouse Bone Marrow Cultures Comprise a

Heterogeneous Population of CD11c(+)MHCII(+) Macrophages and Dendritic Cells. *Immunity, 42*(6), 1197-1211.

Hemann, E. A., Sjaastad, L. E., Langlois, R. A., & Legge, K. L. (2016). Plasmacytoid Dendritic Cells Require Direct Infection To Sustain the Pulmonary Influenza A Virus-Specific CD8 T Cell Response. *Journal of Virology, 90*(6), 2830-2837.

Herter, S., Osterloh, P., Hilf, N., Rechtsteiner, G., Hohfeld, J., Rammensee, H. G., & Schild, H. (2005). Dendritic cell aggresome-like-induced structure formation and delayed antigen presentation coincide in influenza virus-infected dendritic cells. *J Immunol, 175*(2), 891-898.

Hilty, M., Burke, C., Pedro, H., Cardenas, P., Bush, A., Bossley, C., . . . Cookson, W. O. C. (2010). Disordered microbial communities in asthmatic airways. *PLoS One, 5*(1), e8578-e8578.

Hinton, H. J., Jegerlehner, A., & Bachmann, M. F. (2008). Pattern recognition by B cells: the role of antigen repetitiveness versus Toll-like receptors. *Curr Top Microbiol Immunol, 319*, 1-15.

Ho, A. W., Prabhu, N., Betts, R. J., Ge, M. Q., Dai, X., Hutchinson, P. E., . . . Kemeny, D. M. (2011). Lung CD103+ dendritic cells efficiently transport influenza virus to the lymph node and load viral antigen onto MHC class I for presentation to CD8 T cells. *J Immunol, 187*(11), 6011-6021.

Holbrook, B. C., Hayward, S. L., Blevins, L. K., Kock, N., Aycock, T., Parks, G. D., & Alexander-Miller, M. A. (2015). Nonhuman primate infants have an impaired respiratory but not systemic IgG antibody response following influenza virus infection. *Virology, 476*, 124-133.

Hsia, C. C., Hyde, D. M., & Weibel, E. R. (2016). Lung Structure and the Intrinsic Challenges of Gas Exchange. *Compr Physiol, 6*(2), 827-895.

Hussell, T., & Bell, T. J. (2014). Alveolar macrophages: plasticity in a tissue-specific context. *Nat Rev Immunol, 14*(2), 81-93.

Ibricevic, A., Pekosz, A., Walter, M. J., Newby, C., Battaile, J. T., Brown, E. G., . . . Brody, S. L. (2006). Influenza virus receptor specificity and cell tropism in mouse and human airway epithelial cells. *J Virol, 80*(15), 7469-7480.

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., . . . Steinman, R. M. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med, 176*(6), 1693-1702.

Inoue, T., Moran, I., Shinnakasu, R., Phan, T. G., & Kurosaki, T. (2018). Generation of memory B cells and their reactivation. *Immunol Rev, 283*(1), 138-149.

Ioannidis, L. J., Verity, E. E., Crawford, S., Rockman, S. P., & Brown, L. E. (2012). Abortive replication of influenza virus in mouse dendritic cells. *J Virol, 86*(10), 5922-5925.

Jackson, M. L., Chung, J. R., Jackson, L. A., Phillips, C. H., Benoit, J., Monto, A. S., . . . Flannery, B. (2017). Influenza Vaccine Effectiveness in the United States during the 2015-2016 Season. *N Engl J Med, 377*(6), 534-543.

Jaligama, S., Saravia, J., You, D., Yadav, N., Lee, G. I., Shrestha, B., & Cormier, S. A. (2017). Regulatory T cells and IL10 suppress pulmonary host defense during early-life exposure to radical containing combustion derived ultrafine particulate matter. *Respir Res, 18*(1), 15.

Jones, G. S., Smith, V. C., & D'Orazio, S. E. F. (2017). Listeria monocytogenes Replicate in Bone Marrow-Derived CD11c(+) Cells but Not in Dendritic Cells Isolated from the Murine Gastrointestinal Tract. *J Immunol, 199*(11), 3789-3797.

Kaji, T., Ishige, A., Hikida, M., Taka, J., Hijikata, A., Kubo, M., . . . Takemori, T. (2012). Distinct cellular pathways select germline-encoded and somatically mutated antibodies into immunological memory. *J Exp Med, 209*(11), 2079-2097.

Kandasamy, M., Ying, P. C., Ho, A. W., Sumatoh, H. R., Schlitzer, A., Hughes, T. R., . . . Sivasankar, B. (2013). Complement mediated signaling on pulmonary CD103(+) dendritic cells is critical for their migratory function in response to influenza infection. *PLoS Pathog, 9*(1), e1003115.

Kanhere, A., Hertweck, A., Bhatia, U., Gökmen, M. R., Perucha, E., Jackson, I., . . . Jenner, R. G. (2012). T-bet and GATA3 orchestrate Th1 and Th2 differentiation through lineage-specific targeting of distal regulatory elements. *Nature Communications, 3*, 1268.

Kim, A., & Sadegh-Nasseri, S. (2015). Determinants of immunodominance for CD4 T cells. *Curr Opin Immunol, 34*, 9-15.

Kim, J. M., Rasmussen, J. P., & Rudensky, A. Y. (2007). Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol, 8*(2), 191-197.

Kluger, M. A., Meyer, M. C., Nosko, A., Goerke, B., Luig, M., Wegscheid, C., . . . Steinmetz, O. M. (2016). RORgammat+Foxp3+ Cells are an Independent Bifunctional Regulatory T Cell Lineage and Mediate Crescentic GN. *J Am Soc Nephrol, 27*(2), 454-465.

Knuschke, T., Bayer, W., Rotan, O., Sokolova, V., Wadwa, M., Kirschning, C. J., . . . Westendorf, A. M. (2014). Prophylactic and therapeutic vaccination with a nanoparticle-based peptide vaccine induces efficient protective immunity during acute and chronic retroviral infection. *Nanomedicine, 10*(8), 1787-1798.

Koch, Meghan A., Thomas, Kerri R., Perdue, Nikole R., Smigiel, Kate S., Srivastava, S., & Campbell, Daniel J. (2012). T-bet+ Treg Cells Undergo Abortive Th1 Cell Differentiation due to Impaired Expression of IL-12 Receptor β2. *Immunity, 37*(3), 501-510.

Kollmann, T. R., Way, S. S., Harowicz, H. L., Hajjar, A. M., & Wilson, C. B. (2004). Deficient MHC class I cross-presentation of soluble antigen by murine neonatal dendritic cells. *Blood, 103*(11), 4240-4242.

Kosik, I., Angeletti, D., Gibbs, J. S., Angel, M., Takeda, K., Kosikova, M., . . . Yewdell, J. W. (2019). Neuraminidase inhibition contributes to influenza A virus neutralization by anti-hemagglutinin stem antibodies. *J Exp Med, 216*(2), 304-316.

Krammer, F., & Palese, P. (2019). Universal influenza virus vaccines that target the conserved hemagglutinin stalk and conserved sites in the head domain. *J Infect Dis*.

Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K., & Muller, W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. *Cell, 75*(2), 263-274.

Kumar, R., Ferez, M., Swamy, M., Arechaga, I., Rejas, M. T., Valpuesta, J. M., . . . van Santen, H. M. (2011). Increased sensitivity of antigen-experienced T cells through

the enrichment of oligomeric T cell receptor complexes. *Immunity, 35*(3), 375-387.

La Gruta, N. L., Rothwell, W. T., Cukalac, T., Swan, N. G., Valkenburg, S. A., Kedzierska, K., . . . Turner, S. J. (2010). Primary CTL response magnitude in mice is determined by the extent of naive T cell recruitment and subsequent clonal expansion. *J Clin Invest, 120*(6), 1885-1894.

La Gruta, N. L., Turner, S. J., & Doherty, P. C. (2004). Hierarchies in cytokine expression profiles for acute and resolving influenza virus-specific CD8+ T cell responses: correlation of cytokine profile and TCR avidity. *J Immunol, 172*(9), 5553-5560.

La Rocca, C., Carbone, F., Longobardi, S., & Matarese, G. (2014). The immunology of pregnancy: regulatory T cells control maternal immune tolerance toward the fetus. *Immunol Lett, 162*(1 Pt A), 41-48.

Laidlaw, B. J., Decman, V., Ali, M. A., Abt, M. C., Wolf, A. I., Monticelli, L. A., . . . Wherry, E. J. (2013). Cooperativity between CD8+ T cells, non-neutralizing antibodies, and alveolar macrophages is important for heterosubtypic influenza virus immunity. *PLoS Pathog, 9*(3), e1003207.

Lakdawala, S. S., Fodor, E., & Subbarao, K. (2016). Moving On Out: Transport and Packaging of Influenza Viral RNA into Virions. *Annual Review of Virology, 3*(1), 411-427.

Langston, C. (1983). Normal and abnormal structural development of the human lung. *Prog Clin Biol Res, 140*, 75-91.

Lanka, G. K. K., Yu, J.-J., Gong, S., Gupta, R., Mustafa, S. B., Murthy, A. K., . . . Arulanandam, B. P. (2016). IgA modulates respiratory dysfunction as a sequela to pulmonary chlamydial infection as neonates. *Pathogens and disease, 74*(3), ftv121.

Lanteri, M. C., O'Brien, K. M., Purtha, W. E., Cameron, M. J., Lund, J. M., Owen, R. E., . . . Norris, P. J. (2009). Tregs control the development of symptomatic West Nile virus infection in humans and mice. *J Clin Invest, 119*(11), 3266-3277.

Larsson, M., Messmer, D., Somersan, S., Fonteneau, J. F., Donahoe, S. M., Lee, M., . . . Bhardwaj, N. (2000). Requirement of mature dendritic cells for efficient activation of influenza A-specific memory CD8+ T cells. *J Immunol, 165*(3), 1182-1190.

Lau, L. L., Cowling, B. J., Fang, V. J., Chan, K. H., Lau, E. H., Lipsitch, M., . . . Leung, G. M. (2010). Viral shedding and clinical illness in naturally acquired influenza virus infections. *J Infect Dis, 201*(10), 1509-1516.

Lee, D. C., Harker, J. A., Tregoning, J. S., Atabani, S. F., Johansson, C., Schwarze, J., & Openshaw, P. J. (2010). CD25+ natural regulatory T cells are critical in limiting innate and adaptive immunity and resolving disease following respiratory syncytial virus infection. *J Virol, 84*(17), 8790-8798.

Lee, N., Chan, P. K., Hui, D. S., Rainer, T. H., Wong, E., Choi, K. W., . . . Sung, J. J. (2009). Viral loads and duration of viral shedding in adult patients hospitalized with influenza. *J Infect Dis, 200*(4), 492-500.

Lee, S. Y., Kang, J. O., & Chang, J. (2019). Nucleoprotein vaccine induces cross-protective cytotoxic T lymphocytes against both lineages of influenza B virus. *Clin Exp Vaccine Res, 8*(1), 54-63.

Legge, K. L., & Braciale, T. J. (2003). Accelerated migration of respiratory dendritic cells to the regional lymph nodes is limited to the early phase of pulmonary infection. *Immunity, 18*(2), 265-277.

Legrand, F. A., Nixon, D. F., Loo, C. P., Ono, E., Chapman, J. M., Miyamoto, M., . . . Kallas, E. G. (2006). Strong HIV-1-specific T cell responses in HIV-1-exposed uninfected infants and neonates revealed after regulatory T cell removal. *PLoS One, 1*, e102.

Leon, B., Bradley, J. E., Lund, F. E., Randall, T. D., & Ballesteros-Tato, A. (2014). FoxP3+ regulatory T cells promote influenza-specific Tfh responses by controlling IL-2 availability. *Nat Commun, 5*, 3495.

Liang, S., Mozdzanowska, K., Palladino, G., & Gerhard, W. (1994). Heterosubtypic immunity to influenza type A virus in mice. Effector mechanisms and their longevity. *J Immunol, 152*(4), 1653-1661.

Lines, J. L., Hoskins, S., Hollifield, M., Cauley, L. S., & Garvy, B. A. (2010). The migration of T cells in response to influenza virus is altered in neonatal mice. *J Immunol, 185*(5), 2980-2988.

Liu, K., Victora, G. D., Schwickert, T. A., Guermonprez, P., Meredith, M. M., Yao, K., . . . Nussenzweig, M. (2009). In vivo analysis of dendritic cell development and homeostasis. *Science, 324*(5925), 392-397.

Luciani, F., Sanders, M. T., Oveissi, S., Pang, K. C., & Chen, W. (2013). Increasing viral dose causes a reversal in CD8+ T cell immunodominance during primary influenza infection due to differences in antigen presentation, T cell avidity, and precursor numbers. *J Immunol, 190*(1), 36-47.

Lund, J. M., Hsing, L., Pham, T. T., & Rudensky, A. Y. (2008). Coordination of Early Protective Immunity to Viral Infection by Regulatory T Cells. *Science (New York, N.Y.), 320*(5880), 1220-1224.

Mahmoud, A. B., Tu, M. M., Wight, A., Zein, H. S., Rahim, M. M., Lee, S. H., . . . Makrigiannis, A. P. (2016). Influenza Virus Targets Class I MHC-Educated NK Cells for Immunoevasion. *PLoS Pathog, 12*(2), e1005446.

Malloy, A. M. W., Ruckwardt, T. J., Morabito, K. M., Lau-Kilby, A. W., & Graham, B. S. (2017). Pulmonary dendritic cell subsets shape the respiratory syncytial virus-specific CD8(+) T cell immunodominance hierarchy in neonates. *Journal of immunology (Baltimore, Md. : 1950), 198*(1), 394-403.

Mastelic, B., Kamath, A. T., Fontannaz, P., Tougne, C., Rochat, A. F., Belnoue, E., . . . Siegrist, C. A. (2012). Environmental and T cell-intrinsic factors limit the expansion of neonatal follicular T helper cells but may be circumvented by specific adjuvants. *J Immunol, 189*(12), 5764-5772.

McKinley, L., Logar, A. J., McAllister, F., Zheng, M., Steele, C., & Kolls, J. K. (2006). Regulatory T cells dampen pulmonary inflammation and lung injury in an animal model of pneumocystis pneumonia. *J Immunol, 177*(9), 6215-6226.

Medzhitov, R., Schneider, D. S., & Soares, M. P. (2012). Disease tolerance as a defense strategy. *Science, 335*(6071), 936-941.

Merkus, P. J. (2003). Effects of childhood respiratory diseases on the anatomical and functional development of the respiratory system. *Paediatr Respir Rev, 4*(1), 28-39.

Michaelsson, J., Mold, J. E., McCune, J. M., & Nixon, D. F. (2006). Regulation of T cell responses in the developing human fetus. *J Immunol, 176*(10), 5741-5748.

Miller, E. K., Edwards, K. M., Weinberg, G. A., Iwane, M. K., Griffin, M. R., Hall, C. B., . . . Williams, J. V. (2009). A novel group of rhinoviruses is associated with asthma hospitalizations. *J Allergy Clin Immunol, 123*(1), 98-104.e101.

Miller, E. K., Gebretsadik, T., Carroll, K. N., Dupont, W. D., Mohamed, Y. A., Morin, L. L., . . . Williams, J. V. (2013). Viral etiologies of infant bronchiolitis, croup and upper respiratory illness during 4 consecutive years. *Pediatr Infect Dis J, 32*(9), 950-955.

Miller, E. K., Williams, J. V., Gebretsadik, T., Carroll, K. N., Dupont, W. D., Mohamed, Y. A., . . . Hartert, T. V. (2011). Host and viral factors associated with severity of human rhinovirus-associated infant respiratory tract illness. *J Allergy Clin Immunol, 127*(4), 883-891.

Mintern, J. D., & Villadangos, J. A. (2015). Antigen-presenting cells look within during influenza infection. *Nature Medicine, 21*, 1123.

Montecino-Rodriguez, E., & Dorshkind, K. (2011). Formation of B-1 B cells from neonatal B-1 transitional cells exhibits NF-kappaB redundancy. *J Immunol, 187*(11), 5712-5719.

Mordstein, M., Kochs, G., Dumoutier, L., Renauld, J. C., Paludan, S. R., Klucher, K., & Staeheli, P. (2008). Interferon-lambda contributes to innate immunity of mice against influenza A virus but not against hepatotropic viruses. *PLoS Pathog, 4*(9), e1000151.

Morgan, S. B., Hemmink, J. D., Porter, E., Harley, R., Shelton, H., Aramouni, M., . . . Tchilian, E. (2016). Aerosol Delivery of a Candidate Universal Influenza Vaccine Reduces Viral Load in Pigs Challenged with Pandemic H1N1 Virus. *J Immunol, 196*(12), 5014-5023.

Moyron-Quiroz, J. E., Rangel-Moreno, J., Kusser, K., Hartson, L., Sprague, F., Goodrich, S., . . . Randall, T. D. (2004). Role of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity. *Nat Med, 10*(9), 927-934.

Mozdzanowska, K., Furchner, M., Maiese, K., & Gerhard, W. (1997). CD4+ T cells are ineffective in clearing a pulmonary infection with influenza type A virus in the absence of B cells. *Virology, 239*(1), 217-225.

Mozdzanowska, K., Maiese, K., & Gerhard, W. (2000). Th cell-deficient mice control influenza virus infection more effectively than Th- and B cell-deficient mice: evidence for a Th-independent contribution by B cells to virus clearance. *J Immunol, 164*(5), 2635-2643.

Munguía-Fuentes, R., Yam-Puc, J. C., Silva-Sánchez, A., Marcial-Juárez, E., Gallegos-Hernández, I. A., Calderón-Amador, J., . . . Flores-Romo, L. (2017). Immunization of Newborn Mice Accelerates the Architectural Maturation of Lymph Nodes, But AID-Dependent IgG Responses Are Still Delayed Compared to the Adult. *Frontiers in Immunology, 8*, 13.

Murugesan, S., Hong, J., Yi, J., Li, D., Beach, J. R., Shao, L., . . . Hammer, J. A. (2016). Formin-generated actomyosin arcs propel T cell receptor microcluster movement at the immune synapse. *J Cell Biol, 215*(3), 383-399.

Nakanishi, H., Sugiura, T., Streisand, J. B., Lonning, S. M., & Roberts, J. D., Jr. (2007). TGF-beta-neutralizing antibodies improve pulmonary alveologenesis and

vasculogenesis in the injured newborn lung. *Am J Physiol Lung Cell Mol Physiol, 293*(1), L151-161.

Nakatsu, S., Sagara, H., Sakai-Tagawa, Y., Sugaya, N., Noda, T., & Kawaoka, Y. (2016). Complete and Incomplete Genome Packaging of Influenza A and B Viruses. *MBio, 7*(5), e01248-01216.

Nelson, E. A. S., Ip, M., Tam, J. S., Mounts, A. W., Chau, S. L., Law, S. K., . . . Chan, P. K. S. (2014). Burden of influenza infection in hospitalised children below 6 months of age and above in Hong Kong from 2005 to 2011(). *Vaccine, 32*(49), 6692-6698.

Neuzil, K. M., Zhu, Y., Griffin, M. R., Edwards, K. M., Thompson, J. M., Tollefson, S. J., & Wright, P. F. (2002). Burden of Interpandemic Influenza in Children Younger than 5 Years: A 25-Year Prospective Study. *Journal of Infectious Diseases, 185*(2), 147-152.

Neyt, K., & Lambrecht, B. N. (2013). The role of lung dendritic cell subsets in immunity to respiratory viruses. *Immunol Rev, 255*(1), 57-67.

Nguyen, H. H., Moldoveanu, Z., Novak, M. J., van Ginkel, F. W., Ban, E., Kiyono, H., . . . Mestecky, J. (1999). Heterosubtypic immunity to lethal influenza A virus infection is associated with virus-specific CD8(+) cytotoxic T lymphocyte responses induced in mucosa-associated tissues. *Virology, 254*(1), 50-60.

Nicholson, K. G. (1992). Clinical features of influenza. *Semin Respir Infect, 7*(1), 26-37.

Nolz, J. C. (2015). Molecular mechanisms of CD8(+) T cell trafficking and localization. *Cellular and molecular life sciences : CMLS, 72*(13), 2461-2473.

Noval Rivas, M., Burton, Oliver T., Wise, P., Charbonnier, L.-M., Georgiev, P., Oettgen, Hans C., . . . Chatila, Talal A. (2015). Regulatory T Cell Reprogramming toward a Th2-Cell-like Lineage Impairs Oral Tolerance and Promotes Food Allergy. *Immunity, 42*(3), 512-523.

O'Hagan, K. L., Miller, S. D., & Phee, H. (2017). Pak2 is essential for the function of Foxp3+ regulatory T cells through maintaining a suppressive Treg phenotype. *Sci Rep, 7*(1), 17097.

Oliphant, S., Lines, J. L., Hollifield, M. L., & Garvy, B. A. (2015). Regulatory T Cells Are Critical for Clearing Influenza A Virus in Neonatal Mice. *Viral Immunol, 28*(10), 580-589.

Olson, M. R., Chua, B. Y., Good-Jacobson, K. L., Doherty, P. C., Jackson, D. C., & Turner, S. J. (2016). Competition within the virus-specific CD4 T cell pool limits the T follicular helper response after influenza infection. *Immunol Cell Biol*.

Ortigoza, M. B., Blaser, S. B., Zafar, M. A., Hammond, A. J., & Weiser, J. N. (2018). An Infant Mouse Model of Influenza Virus Transmission Demonstrates the Role of Virus-Specific Shedding, Humoral Immunity, and Sialidase Expression by Colonizing Streptococcus pneumoniae. *MBio, 9*(6).

Oshansky, C. M., & Thomas, P. G. (2012). The human side of influenza. *Journal of Leukocyte Biology, 92*(1), 83-96.

Ostler, T., Davidson, W., & Ehl, S. (2002). Virus clearance and immunopathology by CD8(+) T cells during infection with respiratory syncytial virus are mediated by IFN-gamma. *Eur J Immunol, 32*(8), 2117-2123.

Oymans, J., & Te Velthuis, A. J. W. (2018). A Mechanism for Priming and Realignment during Influenza A Virus Replication. *J Virol, 92*(3).

Palese, P., Tobita, K., Ueda, M., & Compans, R. W. (1974). Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. *Virology, 61*(2), 397-410.

Pandiyan, P., Conti, H. R., Zheng, L., Peterson, A. C., Mathern, D. R., Hernandez-Santos, N., . . . Lenardo, M. J. (2011). CD4(+)CD25(+)Foxp3(+) regulatory T cells promote Th17 cells in vitro and enhance host resistance in mouse Candida albicans Th17 cell infection model. *Immunity, 34*(3), 422-434.

Pang, I. K., Ichinohe, T., & Iwasaki, A. (2013). IL-1R signaling in dendritic cells replaces pattern-recognition receptors in promoting CD8(+) T cell responses to influenza A virus. *Nat Immunol, 14*(3), 246-253.

Papaioannou, N. E., Pasztoi, M., & Schraml, B. U. (2019). Understanding the Functional Properties of Neonatal Dendritic Cells: A Doorway to Enhance Vaccine Effectiveness? *Frontiers in Immunology, 9*, 3123-3123.

Peteranderl, C., Herold, S., & Schmoldt, C. (2016). Human Influenza Virus Infections. *Semin Respir Crit Care Med, 37*(4), 487-500.

Plantinga, M., Guilliams, M., Vanheerswynghels, M., Deswarte, K., Branco-Madeira, F., Toussaint, W., . . . Lambrecht, B. N. (2013). Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity, 38*(2), 322-335.

Qi, H., Chen, X., Chu, C., Lu, P., Xu, H., & Yan, J. (2014). Follicular T-helper cells: controlled localization and cellular interactions. *Immunol Cell Biol, 92*(1), 28-33.

Qureshi, M. H., Empey, K. M., & Garvy, B. A. (2005). Modulation of proinflammatory responses to Pneumocystis carinii f. sp. muris in neonatal mice by granulocyte-macrophage colony-stimulating factor and IL-4: role of APCs. *J Immunol, 174*(1), 441-448.

Rai, D., Martin, M. D., & Badovinac, V. P. (2014). The longevity of memory CD8 T cell responses after repetitive antigen stimulations. *J Immunol, 192*(12), 5652-5659.

Rao, S., Alishahi, M., De Crescenzo, L., & Dempsey, A. F. (2019). A population-based study of maternal and infant factors influencing influenza vaccination among young children born in Colorado from 2008 to 2016. *Vaccine, 37*(10), 1293-1298.

Reed, C., Chaves, S. S., Daily Kirley, P., Emerson, R., Aragon, D., Hancock, E. B., . . . Finelli, L. (2015). Estimating Influenza Disease Burden from Population-Based Surveillance Data in the United States. *PLoS One, 10*(3), e0118369.

Remot, A., Descamps, D., Jouneau, L., Laubreton, D., Dubuquoy, C., Bouet, S., . . . Riffault, S. (2016). Flt3 ligand improves the innate response to respiratory syncytial virus and limits lung disease upon RSV reexposure in neonate mice. *Eur J Immunol, 46*(4), 874-884.

Ren, J., & Li, B. (2017). The Functional Stability of FOXP3 and RORgammat in Treg and Th17 and Their Therapeutic Applications. *Adv Protein Chem Struct Biol, 107*, 155-189.

Rius, C., Attaf, M., Tungatt, K., Bianchi, V., Legut, M., Bovay, A., . . . Sewell, A. K. (2018). Peptide–MHC Class I Tetramers Can Fail To Detect Relevant Functional T Cell Clonotypes and Underestimate Antigen-Reactive T Cell Populations. *The Journal of Immunology, 200*(7), 2263-2279.

Robinson, C. L., Bernstein, H., Romero, J. R., & Szilagyi, P. (2019). Advisory Committee on Immunization Practices Recommended Immunization Schedule for

Children and Adolescents Aged 18 Years or Younger - United States, 2019. *MMWR Morb Mortal Wkly Rep, 68*(5), 112-114.

Ruckwardt, T. J., Bonaparte, K. L., Nason, M. C., & Graham, B. S. (2009). Regulatory T cells promote early influx of CD8+ T cells in the lungs of respiratory syncytial virus-infected mice and diminish immunodominance disparities. *J Virol, 83*(7), 3019-3028.

Ruckwardt, T. J., Luongo, C., Malloy, A. M., Liu, J., Chen, M., Collins, P. L., & Graham, B. S. (2010). Responses against a subdominant CD8+ T cell epitope protect against immunopathology caused by a dominant epitope. *J Immunol, 185*(8), 4673-4680.

Ruckwardt, T. J., Malloy, A. M., Morabito, K. M., & Graham, B. S. (2014). Quantitative and qualitative deficits in neonatal lung-migratory dendritic cells impact the generation of the CD8+ T cell response. *PLoS Pathog, 10*(2), e1003934.

Ruckwardt, T. J., Malloy, A. M. W., Gostick, E., Price, D. A., Dash, P., McClaren, J. L., . . . Graham, B. S. (2011). Neonatal CD8 T-cell Hierarchy Is Distinct from Adults and Is Influenced by Intrinsic T cell Properties in Respiratory Syncytial Virus Infected Mice. *PLoS Pathog, 7*(12), e1002377.

Ruckwardt, T. J., Morabito, K. M., Bar-Haim, E., Nair, D., & Graham, B. S. (2018). Neonatal mice possess two phenotypically and functionally distinct lung-migratory CD103(+) dendritic cell populations following respiratory infection. *Mucosal Immunol, 11*(1), 186-198.

Rudd, B. D., Luker, G. D., Luker, K. E., Peebles, R. S., & Lukacs, N. W. (2007). Type I interferon regulates respiratory virus infected dendritic cell maturation and cytokine production. *Viral Immunol, 20*(4), 531-540.

Rudd, B. D., Venturi, V., Smith, N. L., Nzingha, K., Goldberg, E. L., Li, G., . . . Davenport, M. P. (2013). Acute Neonatal Infections 'Lock-In' a Suboptimal CD8+ T Cell Repertoire with Impaired Recall Responses. *PLoS Pathog, 9*(9), e1003572.

Rueda, C. M., Moreno-Fernandez, M. E., Jackson, C. M., Kallapur, S. G., Jobe, A. H., & Chougnet, C. A. (2015). Neonatal regulatory T cells have reduced capacity to suppress dendritic cell function. *Eur J Immunol*.

Sakaguchi, S., Miyara, M., Costantino, C. M., & Hafler, D. A. (2010). FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol, 10*(7), 490-500.

Sallusto, F., Lanzavecchia, A., Araki, K., & Ahmed, R. (2010). From vaccines to memory and back. *Immunity, 33*(4), 451-463.

Samji, T. (2009). Influenza A: understanding the viral life cycle. *The Yale journal of biology and medicine, 82*(4), 153-159.

Scharschmidt, T. C., Vasquez, K. S., Truong, H. A., Gearty, S. V., Pauli, M. L., Nosbaum, A., . . . Rosenblum, M. D. (2015). A Wave of Regulatory T Cells into Neonatal Skin Mediates Tolerance to Commensal Microbes. *Immunity, 43*(5), 1011-1021.

Schmidt, M. E., Knudson, C. J., Hartwig, S. M., Pewe, L. L., Meyerholz, D. K., Langlois, R. A., . . . Varga, S. M. (2018). Memory CD8 T cells mediate severe immunopathology following respiratory syncytial virus infection. *PLoS Pathog, 14*(1), e1006810.

Schoggins, J. W., Wilson, S. J., Panis, M., Murphy, M. Y., Jones, C. T., Bieniasz, P., & Rice, C. M. (2011). A diverse range of gene products are effectors of the type I interferon antiviral response. *Nature, 472*(7344), 481-485.

Schone, D., Hrycak, C. P., Windmann, S., Lapuente, D., Dittmer, U., Tenbusch, M., & Bayer, W. (2017). Immunodominance of Adenovirus-Derived CD8(+) T Cell Epitopes Interferes with the Induction of Transgene-Specific Immunity in Adenovirus-Based Immunization. *J Virol, 91*(20).

Scott-Browne, J. P., Shafiani, S., Tucker-Heard, G., Ishida-Tsubota, K., Fontenot, J. D., Rudensky, A. Y., . . . Urdahl, K. B. (2007). Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis. *J Exp Med, 204*(9), 2159-2169.

Sheth, A. N., Althoff, K. N., & Brooks, J. T. (2011). Influenza susceptibility, severity, and shedding in HIV-infected adults: a review of the literature. *Clin Infect Dis, 52*(2), 219-227.

Shin, D.-L., Hatesuer, B., Bergmann, S., Nedelko, T., & Schughart, K. (2015). Protection from Severe Influenza Virus Infections in Mice Carrying the Mx1 Influenza Virus Resistance Gene Strongly Depends on Genetic Background. *Journal of Virology, 89*(19), 9998-10009.

Shrestha, B., Pinto, A. K., Green, S., Bosch, I., & Diamond, M. S. (2012). CD8+ T cells use TRAIL to restrict West Nile virus pathogenesis by controlling infection in neurons. *J Virol, 86*(17), 8937-8948.

Skehel, J. J., & Wiley, D. C. (2000). Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu Rev Biochem, 69*, 531-569.

Slifka, M. K., & Whitton, J. L. (2001). Functional avidity maturation of CD8(+) T cells without selection of higher affinity TCR. *Nat Immunol, 2*(8), 711-717.

Smith, N. L., Wissink, E., Wang, J., Pinello, J. F., Davenport, M. P., Grimson, A., & Rudd, B. D. (2014). Rapid proliferation and differentiation impairs the development of memory CD8+ T cells in early life. *J Immunol, 193*(1), 177-184.

Smith, W., Andrewes, C. H., & Laidlaw, P. P. (1933). A VIRUS OBTAINED FROM INFLUENZA PATIENTS. *The Lancet, 222*(5732), 66-68.

Sridhar, S., Begom, S., Bermingham, A., Hoschler, K., Adamson, W., Carman, W., . . . Lalvani, A. (2013). Cellular immune correlates of protection against symptomatic pandemic influenza. *Nat Med, 19*(10), 1305-1312.

Sridhar, S., Brokstad, K. A., & Cox, R. J. (2015). Influenza Vaccination Strategies: Comparing Inactivated and Live Attenuated Influenza Vaccines. *Vaccines (Basel), 3*(2), 373-389.

Stellrecht, K. A., Nattanmai, S. M., Butt, J., Maceira, V. P., Espino, A. A., Castro, A. J., . . . Butt, S. A. (2017). Effect of genomic drift of influenza PCR tests. *J Clin Virol, 93*, 25-29.

Strickland, D., Kees, U. R., & Holt, P. G. (1996). Regulation of T-cell activation in the lung: isolated lung T cells exhibit surface phenotypic characteristics of recent activation including down-modulated T-cell receptors, but are locked into the G0/G1 phase of the cell cycle. *Immunology, 87*(2), 242-249.

Sun, C.-M., Fiette, L., Tanguy, M., Leclerc, C., & Lo-Man, R. (2003). Ontogeny and innate properties of neonatal dendritic cells. *Blood, 102*(2), 585-591.

Sun, F., Xiao, G., & Qu, Z. (2017). Murine Bronchoalveolar Lavage. *Bio-protocol, 7*(10), e2287.

Suvas, S., Azkur, A. K., Kim, B. S., Kumaraguru, U., & Rouse, B. T. (2004). CD4+CD25+ regulatory T cells control the severity of viral immunoinflammatory lesions. *J Immunol, 172*(7), 4123-4132.

Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G., & Glimcher, L. H. (2000). A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell, 100*(6), 655-669.

Takizawa, N., Momose, F., Morikawa, Y., & Nomoto, A. (2016). Influenza A Virus Hemagglutinin is Required for the Assembly of Viral Components Including Bundled vRNPs at the Lipid Raft. *Viruses, 8*(9).

Tang, M. L., Kemp, A. S., Thorburn, J., & Hill, D. J. (1994). Reduced interferon-gamma secretion in neonates and subsequent atopy. *Lancet, 344*(8928), 983-985.

Tarlinton, D., & Good-Jacobson, K. (2013). Diversity among memory B cells: origin, consequences, and utility. *Science, 341*(6151), 1205-1211.

Teijeira, A., Hunter, M. C., Russo, E., Proulx, S. T., Frei, T., Debes, G. F., . . . Halin, C. (2017). T Cell Migration from Inflamed Skin to Draining Lymph Nodes Requires Intralymphatic Crawling Supported by ICAM-1/LFA-1 Interactions. *Cell Rep, 18*(4), 857-865.

Thangavel, R. R., & Bouvier, N. M. (2014). Animal models for influenza virus pathogenesis, transmission, and immunology. *J Immunol Methods, 410*, 60-79.

Thommes, E. W., Kruse, M., Kohli, M., Sharma, R., & Noorduyn, S. G. (2017). Review of seasonal influenza in Canada: Burden of disease and the cost-effectiveness of quadrivalent inactivated influenza vaccines. *Human Vaccines & Immunotherapeutics, 13*(4), 867-876.

Tucker, R. M., Feldman, A. G., Fenner, E. K., & Mack, C. L. (2013). Regulatory T cells inhibit Th1 cell-mediated bile duct injury in murine biliary atresia. *J Hepatol, 59*(4), 790-796.

Uddback, I. E., Steffensen, M. A., Pedersen, S. R., Nazerai, L., Thomsen, A. R., & Christensen, J. P. (2016). PB1 as a potential target for increasing the breadth of T-cell mediated immunity to Influenza A. *Sci Rep, 6*, 35033.

Utay, N. S., & Hunt, P. W. (2016). Role of immune activation in progression to AIDS. *Curr Opin HIV AIDS, 11*(2), 131-137.

van der Waart, A. B., van der Velden, W. J., Blijlevens, N. M., & Dolstra, H. (2014). Targeting the IL17 pathway for the prevention of graft-versus-host disease. *Biol Blood Marrow Transplant, 20*(6), 752-759.

van Doorn, E., Pleguezuelos, O., Liu, H., Fernandez, A., Bannister, R., Stoloff, G., . . . Hak, E. (2017). Evaluation of the immunogenicity and safety of different doses and formulations of a broad spectrum influenza vaccine (FLU-v) developed by SEEK: study protocol for a single-center, randomized, double-blind and placebo-controlled clinical phase IIb trial. *BMC Infect Dis, 17*(1), 241.

van Haren, S. D., Dowling, D. J., Foppen, W., Christensen, D., Andersen, P., Reed, S. G., . . . Levy, O. (2016). Age-Specific Adjuvant Synergy: Dual TLR7/8 and Mincle Activation of Human Newborn Dendritic Cells Enables Th1 Polarization. *J Immunol, 197*(11), 4413-4424.

Veiga-Fernandes, H., Walter, U., Bourgeois, C., McLean, A., & Rocha, B. (2000). Response of naive and memory CD8+ T cells to antigen stimulation in vivo. *Nat Immunol, 1*(1), 47-53.

Veldhoen, M. (2017). Interleukin 17 is a chief orchestrator of immunity. *Nat Immunol, 18*(6), 612-621.

Verhoeven, D. (2019). Influence of Immunological Maturity on Respiratory Syncytial Virus-Induced Morbidity in Young Children. *Viral Immunol, 32*(2), 76-83.

Verhoeven, D., Perry, S., & Pryharski, K. (2016). Control of influenza infection is impaired by diminished interferon-gamma secretion by CD4 T cells in the lungs of toddler mice. *J Leukoc Biol, 100*(1), 203-212.

Vignali, D. A., Collison, L. W., & Workman, C. J. (2008). How regulatory T cells work. *Nat Rev Immunol, 8*(7), 523-532.

Waaijenborg, S., Hahne, S. J., Mollema, L., Smits, G. P., Berbers, G. A., van der Klis, F. R., . . . Wallinga, J. (2013). Waning of maternal antibodies against measles, mumps, rubella, and varicella in communities with contrasting vaccination coverage. *J Infect Dis, 208*(1), 10-16.

Wan, Y. Y. (2014). GATA3: a master of many trades in immune regulation. *Trends Immunol, 35*(6), 233-242.

Wang, G., Miyahara, Y., Guo, Z., Khattar, M., Stepkowski, S. M., & Chen, W. (2010). "Default" generation of neonatal regulatory T cells. *J Immunol, 185*(1), 71-78.

Wang, J., Siffert, M., Spiliotis, M., & Gottstein, B. (2016). Repeated Long-Term DT Application in the DEREG Mouse Induces a Neutralizing Anti-DT Antibody Response. *J Immunol Res, 2016*, 1450398-1450398.

Wang, Z., Friedrich, C., Hagemann, S. C., Korte, W. H., Goharani, N., Cording, S., . . . Lochner, M. (2014). Regulatory T cells promote a protective Th17-associated immune response to intestinal bacterial infection with C. rodentium. *Mucosal Immunol, 7*(6), 1290-1301.

Welliver, T. P., Garofalo, R. P., Hosakote, Y., Hintz, K. H., Avendano, L., Sanchez, K., . . . Welliver, R. C., Sr. (2007). Severe human lower respiratory tract illness caused by respiratory syncytial virus and influenza virus is characterized by the absence of pulmonary cytotoxic lymphocyte responses. *J Infect Dis, 195*(8), 1126-1136.

Willart, M. A., Deswarte, K., Pouliot, P., Braun, H., Beyaert, R., Lambrecht, B. N., & Hammad, H. (2012). Interleukin-1alpha controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. *J Exp Med, 209*(8), 1505-1517.

Willis, R. A., Kappler, J. W., & Marrack, P. C. (2006). CD8 T cell competition for dendritic cells in vivo is an early event in activation. *Proc Natl Acad Sci U S A, 103*(32), 12063-12068.

Wohlfert, E. A., Grainger, J. R., Bouladoux, N., Konkel, J. E., Oldenhove, G., Ribeiro, C. H., . . . Belkaid, Y. (2011). GATA3 controls Foxp3(+) regulatory T cell fate during inflammation in mice. *J Clin Invest, 121*(11), 4503-4515.

Wu, T., Hu, Y., Lee, Y.-T., Bouchard, K. R., Benechet, A., Khanna, K., & Cauley, L. S. (2014). Lung-resident memory CD8 T cells (T(RM)) are indispensable for optimal cross-protection against pulmonary virus infection. *Journal of Leukocyte Biology, 95*(2), 215-224.

Xiang, K., Ying, G., Yan, Z., Shanshan, Y., Lei, Z., Hongjun, L., & Maosheng, S. (2015). Progress on adenovirus-vectored universal influenza vaccines. *Hum Vaccin Immunother, 11*(5), 1209-1222.

Xing, Z., & Cardona, C. J. (2009). Preexisting immunity to pandemic (H1N1) 2009. *Emerg Infect Dis, 15*(11), 1847-1849.

Xu, H., Ziani, W., Shao, J., Doyle-Meyers, L. A., Russell-Lodrigue, K. E., Ratterree, M. S., . . . Wang, X. (2018). Impaired Development and Expansion of Germinal Center Follicular Th Cells in Simian Immunodeficiency Virus-Infected Neonatal Macaques. *J Immunol, 201*(7), 1994-2003.

Xu, K., Yang, W. Y., Nanayakkara, G. K., Shao, Y., Yang, F., Hu, W., . . . Yang, X. (2018). GATA3, HDAC6, and BCL6 Regulate FOXP3+ Treg Plasticity and Determine Treg Conversion into Either Novel Antigen-Presenting Cell-Like Treg or Th1-Treg. *Front Immunol, 9*, 45.

Yang, X. O., Panopoulos, A. D., Nurieva, R., Chang, S. H., Wang, D., Watowich, S. S., & Dong, C. (2007). STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J Biol Chem, 282*(13), 9358-9363.

Yoo, J. K., Kim, T. S., Hufford, M. M., & Braciale, T. J. (2013). Viral infection of the lung: host response and sequelae. *J Allergy Clin Immunol, 132*(6), 1263-1276; quiz 1277.

You, D., Ripple, M., Balakrishna, S., Troxclair, D., Sandquist, D., Ding, L., . . . Cormier, S. A. (2008). Inchoate CD8+ T Cell Responses in Neonatal Mice Permit Influenza Induced Persistent Pulmonary Dysfunction. *Journal of immunology (Baltimore, Md. : 1950), 181*(5), 3486-3494.

Yu, F., Sharma, S., Edwards, J., Feigenbaum, L., & Zhu, J. (2015). Dynamic expression of T-bet and GATA3 by regulatory T cells maintains immune tolerance. *Nat Immunol, 16*(2), 197-206.

Yu, H. R., Tsai, C. C., Chang, L. S., Huang, H. C., Cheng, H. H., Wang, J. Y., . . . Hsu, T. Y. (2017). l-Arginine-Dependent Epigenetic Regulation of Interleukin-10, but Not Transforming Growth Factor-beta, Production by Neonatal Regulatory T Lymphocytes. *Front Immunol, 8*, 487.

Yu, X., Tsibane, T., McGraw, P. A., House, F. S., Keefer, C. J., Hicar, M. D., . . . Crowe, J. E., Jr. (2008). Neutralizing antibodies derived from the B cells of 1918 influenza pandemic survivors. *Nature, 455*(7212), 532-536.

Zens, K. D., Chen, J. K., Guyer, R. S., Wu, F. L., Cvetkovski, F., Miron, M., & Farber, D. L. (2017). Reduced generation of lung tissue–resident memory T cells during infancy. *The Journal of Experimental Medicine, 214*(10), 2915-2932.

Zhan, Y., Carrington, E. M., van Nieuwenhuijze, A., Bedoui, S., Seah, S., Xu, Y., . . . Lew, A. M. (2011). GM-CSF increases cross-presentation and CD103 expression by mouse CD8(+) spleen dendritic cells. *Eur J Immunol, 41*(9), 2585-2595.

Zhao, D., Feng, F., Zhao, C., Wu, F., Ma, C., Bai, Y., . . . Li, H. (2018). Role of perforin secretion from CD8+ T-cells in neuronal cytotoxicity in multiple sclerosis. *Neurol Res, 40*(1), 62-67.

Zheng, W., & Tao, Y. J. (2013). Structure and assembly of the influenza A virus ribonucleoprotein complex. *FEBS Letters, 587*(8), 1206-1214.

Zhou, X., Bailey-Bucktrout, S., Jeker, L. T., Penaranda, C., Martínez-Llordella, M., Ashby, M., . . . Bluestone, J. A. (2009). Foxp3 instability leads to the generation of pathogenic memory T cells in vivo. *Nat Immunol, 10*(9), 1000-1007.

VITA

Luke Harrison Heil

Education:

Davidson College, Bachelor of Science: Biology- 2012
Christ Presbyterian Academy, Nashville TN, High School Diploma- 2008

Professional Positions:
Vanderbilt University- Research Technician

Publications:

Miller EK, Gebretsadik T, Carroll KN, Dupont WD, Mohamed YA, Morin LL, Heil L, Minton PA, Woodward K, Liu Z, Hartert TV, Williams JV. Viral etiologies of infant bronchiolitis, croup and upper respiratory illness during 4 consecutive years. Pediatr Infect Dis J. 2013 Sep;32(9):950-5.

Linder JE, Kraft DC, Mohamed Y, Lu Z, Heil L, Tollefson S, Saville BR, Wright PF, Williams JV, Miller EK. Human rhinovirus C: Age, season, and lower respiratory illness over the past 3 decades. J Allergy Clin Immunol. 2013 Jan;131(1):69-77.e1-6.

Miller EK, Williams JV, Gebretsadik T, Carroll KN, Dupont WD, Mohamed YA, Morin LL, Heil L, Minton PA, Woodward K, Liu Z, Hartert TV. Host and viral factors associated with severity of human rhinovirus-associated infant respiratory tract illness. J Allergy Clin Immunol. 2011 Apr;127(4):883-91.

Miller EK, Khuri-Bulos N, Williams JV, Shehabi AA, Faouri S, Al Jundi I, Chen Q, Heil L, Mohamed Y, Morin LL, Ali A, Halasa NB. Human rhinovirus C associated with wheezing in hospitalised children in the Middle East. J Clin Virol. 2009 Sep;46(1):85-9.

Miller EK, Edwards KM, Weinberg GA, Iwane MK, Griffin MR, Hall CB, Zhu Y, Szilagyi PG, Morin LL, Heil LH, Lu X, Williams JV; New Vaccine Surveillance Network. A novel group of rhinoviruses is associated with asthma hospitalizations. J Allergy Clin Immunol. 2009 Jan;123(1):98-104.e1.