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Roles of $\gamma\delta$ T Cells in Influenza Infections and Methods for TCR Expression and Characterization

Abstract

"Influenza virus is a significant pathogen in humans and animals with the ability to cause extensive morbidity and mortality. Exuberant immune responses associated with immune cell migration/activation and cytokine/chemokine release, can be induced after infections. Recent studies have painted a complex picture of viral clearance and tissue repair in adults. The immune responses in neonatal influenza infections and the potential methods to engineer antigen-specific T cells for immunotherapy against cancers and infections will be the focus of this dissertation." "Compared to adults, infants suffer higher rates of hospitalization, severe clinical complications, and mortality due to influenza infection. We found that $\gamma\delta$ T cells protected neonatal mice against mortality during influenza infection. $\gamma\delta$ T cell deficiency did not alter viral clearance or interferon- γ production. Instead, neonatal influenza infection induced the accumulation of interleukin-17A (IL-17A)-producing $\gamma\delta$ T cells, which was associated with IL-33 production by lung epithelial cells. Neonates lacking IL-17A-expressing $\gamma\delta$ T cells or IL33 had higher mortality upon influenza infection. $\gamma\delta$ T cells and IL-33 promoted lung infiltration of group 2 innate lymphoid cells and regulatory T cells, resulting in increased amphiregulin secretion and tissue repair. In influenza-infected children, IL-17A, IL-33, and amphiregulin expression were positively correlated, and increased IL-17A levels in nasal aspirates were associated with better clinical outcomes. Our results indicate that $\gamma\delta$ T cells are required in influenza-infected neonates to initiate protective immunity and mediate lung homeostasis." "In addition, transgenic expression of antigen-specific T cell receptor (TCR) genes is a promising approach for immunotherapy against infectious diseases and cancers. A key to the efficient application of this approach is the rapid and specific isolation and cloning of TCRs. Current methods are often labor-intensive, nonspecific, and/or relatively slow. Here, we describe an efficient system for antigen-specific TCR cloning and CDR3 substitution. We demonstrate the capability of cloning influenza-specific TCRs within 10 days using single-cell polymerase chain reaction (PCR) and Gibson Assembly techniques. This process can be accelerated to 5 days by generating receptor libraries, requiring only the exchange of the antigen-specific CDR3 region into an existing backbone. We describe the construction of this library for human $\gamma\delta$ TCRs and report the cloning and expression of a TRGV9/TRDV2 receptor that is activated by zoledronic acid. The functional activity of these $\alpha\beta$ and $\gamma\delta$ TCRs can be characterized in a novel reporter cell line (Nur77-GFP Jurkat 76 TCR-null) for screening of TCR specificity and avidity. In summary, we provide a rapid method for the cloning, expression, and functional characterization of human and mouse TCRs that can assist in the development of TCR-mediated therapeutics." "Overall, this dissertation addresses the protective roles of $\gamma\delta$ T cells in murine and human neonatal influenza infections, and establishes the rapid system for TCR characterization, cloning and expression, which may shed light on T cell adoptive transfer immunotherapy for viral infections."

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In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Xizhi Guo
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ABSTRACT

Influenza virus is a significant pathogen in humans and animals with the ability to cause extensive morbidity and mortality. Exuberant immune responses associated with immune cell migration/activation and cytokine/chemokine release, can be induced after infections. Recent studies have painted a complex picture of viral clearance and tissue repair in adults. The immune responses in neonatal influenza infections and the potential methods to engineer antigen-specific T cells for immunotherapy against cancers and infections will be the focus of this dissertation.

Compared to adults, infants suffer higher rates of hospitalization, severe clinical complications, and mortality due to influenza infection. We found that $\gamma\delta$ T cells protected neonatal mice against mortality during influenza infection. $\gamma\delta$ T cell deficiency did not alter viral clearance or interferon- γ production. Instead, neonatal influenza infection induced the accumulation of interleukin-17A (IL-17A)-producing $\gamma\delta$ T cells, which was associated with IL-33 production by lung epithelial cells. Neonates lacking IL-17A-expressing $\gamma\delta$ T cells or *Il33* had higher mortality upon influenza infection. $\gamma\delta$ T cells and IL-33 promoted lung infiltration of group 2 innate lymphoid cells and regulatory T cells, resulting in increased amphiregulin secretion and tissue repair. In influenza-infected children, IL-17A, IL-33, and amphiregulin expression were positively correlated, and increased IL-17A levels in nasal aspirates were associated with better clinical outcomes. Our results indicate that $\gamma\delta$ T cells are required in influenza-infected neonates to initiate protective immunity and mediate lung homeostasis.

In addition, transgenic expression of antigen-specific T cell receptor (TCR) genes is a promising approach for immunotherapy against infectious diseases and cancers. A key to the efficient application of this approach is the rapid and specific isolation and cloning of TCRs. Current methods are often labor-intensive, nonspecific, and/or relatively slow. Here, we describe an efficient system for antigen-specific TCR cloning and CDR3 substitution. We demonstrate the capability of cloning influenza-specific TCRs within 10 days using single-cell polymerase chain reaction (PCR) and Gibson Assembly techniques. This process can be accelerated to 5 days by generating receptor libraries, requiring only the exchange of the antigen-specific CDR3 region into an existing backbone. We describe the construction of this library for human $\gamma\delta$ TCRs and report the cloning and expression of a TRGV9/TRDV2 receptor that is activated by zoledronic acid. The functional activity of these $\alpha\beta$ and $\gamma\delta$ TCRs can be characterized in a novel reporter cell line (*Nur77*-GFP Jurkat 76 TCR-null) for screening of TCR specificity and avidity. In summary, we provide a rapid method for the cloning, expression, and functional characterization of human and mouse TCRs that can assist in the development of TCR-mediated therapeutics.

Overall, this dissertation addresses the protective roles of $\gamma\delta$ T cells in murine and human neonatal influenza infections, and establishes the rapid system for TCR characterization, cloning and expression, which may shed light on T cell adoptive transfer immunotherapy for viral infections.

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LIST OF ABBREVIATIONS

APC	Allophycocyanin
Areg	Amphiregulin
CAR	Chimeric Antigen Receptor
CD	Cluster of Differentiation
CDNA	Complementary Deoxyribonucleic Acid
CDR3	Complimentary Determining Region 3
DC	Dendritic Cells
EGFR	Epithelial Growth Factor Receptor
FITC	Fluorescein Isothiocyanate
HLA	Human Leukocyte Antigen
IFN	Interferon
IL	Interleukin
ILC	Innate Lymphoid Cells
KOMP	KnockOut Mouse Project
LET1	Lung Epithelial Cells 1
MDCK	Madin-Darby Canine Kidney Cells
MHC	Major Histocompatibility Complex
NF- κ B	Nuclear Factor – κ B
PAMP	Pathogen-Associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
pMHC	Peptide - Major Histocompatibility Complex

PRR	Pathogen Recognition Receptors
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RACE	Rapid Amplification of cDNA Ends
RBC	Red Blood Cells
RT-PCR	Reverse Transcription PCR
STAT	Signal Transducer and Activator of Transcription
TCR	T Cell Receptor
Th	T Helper Cells
TIL	Tumor Infiltrating Lymphocytes
Treg	Regulatory T Cells

CHAPTER 1. INTRODUCTION

Background

Immune Responses to Influenza Infections

Influenza virus is a pleomorphic enveloped virus of approximately 100nm in diameter (Dash and Thomas, 2014), belonging to the *Orthomyxoviridae*. Influenza virus contains a genome composed of eight segments of negative-sense, single-stranded RNA (ssRNA) tightly surrounded by nucleoprotein (NP). The surface haemagglutinin (HA) and neuraminidase (NA) are the major viral glycoproteins by which the subtype of the virus is defined. Influenza viruses can infect diverse host species, including pigs, birds, and humans. In the United States, it has been estimated that there were 49 million influenza illnesses, 960,000 hospitalizations and 79,000 influenza-associated deaths during 2017-2018 (Rolfes et al.). Influenza epidemics can seriously affect all populations, but children younger than 2 years of age and adults aged 65 years or older are the most vulnerable to influenza infection (Blanton, 2019).

Cellular Responses. Influenza virus causes acute respiratory infection and significant rates of hospitalization and mortality (Thompson et al., 2004). After infection, influenza virus is internalized into upper and lower respiratory epithelial cells via endocytosis. Viral RNAs can be recognized by the infected cell as pathogen-associated molecular patterns (PAMPs) by numerous pathogen recognition receptors (PRRs), which in turn can promote downstream cellular responses (Dash and Thomas, 2014).

Following the PRR recognition of influenza viral products by innate and epithelial cells, the immune system initiates the production and release of various inflammatory cytokines, including interferons, IL-1 β , IL-6, TNF- α , IL-33 and others, which further induce the activation of dendritic cells (DCs) and macrophages for antigen acquisition and presentation to trigger the maturation, migration, and stimulation of antigen-specific adaptive immune responses by CD8 T cells (Duan and Thomas, 2016).

Influenza-specific $\alpha\beta$ CD8 T cells play a critical role in eliminating virus-infected cells via two effector activities, antigen-specific cytotoxicity and cytokine/chemokine production. T cells use T cell receptors (TCRs) to recognize specific viral peptides presented by the major histocompatibility complex (MHC) molecules on antigen presenting cells (APCs). The recognition and engagement of the TCR to the peptide-MHC complex triggers downstream cytotoxicity and cytokine production by T cells. The cytotoxicity is mainly modulated by three routes, including perforin/granzyme-mediated cytotoxicity (Fan and Zhang, 2005), FasL/Fas-mediated apoptosis (Topham et al., 1997) and TRAIL/TRAIL-DR signaling (Brincks et al., 2008; Ishikawa et al., 2005). In addition, antigen-specific CD8 T cells can also produce various cytokines and chemokines, among which IFN- γ and TNF- α are the most prominent cytokines that regulate the functions of

other immune cells and induce the infected cell death (Gruta et al., 2004; Seder et al., 2008).

Cytokine Storm¹. The term "cytokine storm" to describe an immune response to influenza infection was first used in late 2003 in reference to influenza-associated encephalopathy (Clark, 2007; Yokota, 2003). Thus far, the influenza-induced cytokine storm has been linked to uncontrolled pro-inflammatory responses, which induce significant immunopathology and severe disease outcomes (D'Elia et al., 2013; Liu et al., 2016; Teijaro, 2014; Tisoncik et al., 2012). As we better understand the varied roles of individual cytokines, the concept of the cytokine storm has become more complicated. Beyond the direct effects of these cytokines on different cell types, their cross-regulatory functions within the cytokine network can have important effects on the outcome of an infection.

One useful framework for considering the role of cytokines is to divide them by those that are directly induced by virus infection (primary cytokines) and those that are induced downstream by other cytokines or features of the immune response (secondary cytokines). Influenza virus infection in epithelial cells, endothelial cells, and alveolar macrophages leads to the primary wave of cytokine production, especially type I interferons (IFNs), which upregulate the expression of numerous interferon-stimulated genes (ISGs) (Kato et al., 2005). Though originally not a primary focus of influenza virus biology, endothelial cells expressing the Sphingosine-1-phosphate (S1P₁) receptor have been demonstrated to be key orchestrators of the cytokine storm (Teijaro et al., 2011). Following the type I IFN release, higher expression of ISGs initiates downstream anti-viral responses and subsequent inflammatory cytokine production by innate immune cells, like dendritic cells (DCs), macrophages, neutrophils, and monocytes. In the adaptive phase of the immune response, different subsets of T cells and group 2 innate lymphoid cells (ILC2s) are activated and regulated to secrete the secondary cytokines that promote viral clearance, tissue homeostasis, and lung repair.

As we develop a comprehensive understanding of the multifaceted roles of innate and adaptive immune effectors during influenza infection, the traditional "cytokine storm" should be expanded to encompass the diversity of cytokines promoting pathogenic and protective immunity, including inducing local inflammation, eliminating infected cells, modulating cellular and molecular immune responses, and promoting tissue repair and homeostasis. Beyond the well-established functions of traditional antiviral cytokines like the IFNs, recent studies have implicated new cytokine subsets as being relevant to influenza infection. Most of the literature has focused on the strong type 1 immune response induced during infection, but type 2 cytokines have now been demonstrated to play important roles following infection, including IL-33, IL-5, and AREG. However, their functions remain, in many cases, ambiguous or controversial, with significant work needed to define their place in the immune network (**Figure 1-1**).

¹ This section adapted with permission from final submission. Guo, X.J. & Thomas, P.G. *Semin Immunopathol* (2017) 39: 541. DOI: <https://doi.org/10.1007/s00281-017-0636-y>.

Figure 1-1. Spatial and Temporal Segregation of Cytokine Production after Influenza Infection.

After internalization into epithelial cells, influenza virus can be detected by innate immune sensors and trigger downstream immune responses, including tremendous cytokine production, sometimes called the “cytokine storm”. Cytokines directly induced in a virally infected cell versus those downstream from other cytokine signaling can be segregated as primary cytokines and secondary cytokines, respectively. In the primary cytokine wave, virus-infected lung epithelial, endothelial, and other immune cells produce type I and III IFNs, IL-1 β , IL-18, TNF- α , IL-6, IL-33 and other cytokines, mainly to limit viral replication and spreading and to initiate downstream immune responses (bottom panel). Following their recruitment and activation by primary cytokines, CD8 T cells, NK cells, ILC2s, Tregs, and Th2 cells can secrete the secondary cytokines IFN- γ , IL-10, amphiregulin, and IL-5 to eliminate virus and virally infected cells, dampen inflammation, and restore lung function (top panel).

$\gamma\delta$ T Cells and Immunity

Development and Functions of $\gamma\delta$ T Cells. $\gamma\delta$ T cells are the first T cells to appear in the thymus during fetal thymic ontogeny. As a subset of T lymphocytes, $\gamma\delta$ T cells develop largely in the thymus, and generate $\gamma\delta$ TCR via recombination activating gene (RAG)-mediated V(D)J recombination. Compared to TCR α and TCR β chains, TCR γ and TCR δ chain loci have a small number of functional V gene segments. In mice, there are 7 V γ (TRGV1-7) and 10 V δ (TRDV1, 2, 4, 5, 6, 7, 8, 10, 11 and 12) functional segments, while the human V γ chain locus consists 8 segments (TRGV2, 3, 4, 5, 8, 9, 10, and 11) and 3 V δ segments (TRDV1-3) (Lefranc et al., 2015). Despite the limited diversity at the TCR γ and TCR δ chain loci, the potential diversity generated at the combined CDR3 regions (approximately 10^{18} combinations) is still higher than that of $\alpha\beta$ TCRs (about 10^{16}), resulting from the multiple D gene segments for recombination and the variety in CDR3 length of TCR δ chain (Chien et al., 2014).

After the TCR rearrangement, the functional expression of the $\gamma\delta$ TCR drives cells into the $\gamma\delta$ T cell lineage with the support of SRY-box13 (SOX13) expression at the stage of double-negative (DN3) or DN4 stage of thymocyte development. Unlike $\alpha\beta$ T cells, $\gamma\delta$ T cells undergo functional pre-programming in the thymus before entering the periphery, which is dependent on TCR signaling. It has been suggested that strong agonist-dependent signals, like SKINT1, leads to the loss of SOX13, the upregulation of the transcription factors, such as nuclear factor of activated T cells (NFAT), nuclear factor- κ B and T-bet, the surface expression of Cluster of Differentiation (CD)-27, and the capacity to produce interferon- γ (IFN- γ). In contrast, weaker signals tend to differentiate $\gamma\delta$ T cells with higher SOX13 and ROR γ t expression and the potential to produce IL-17A. Following development and differentiation, $\gamma\delta$ T cells exit the thymus and populate the periphery, especially in the mucosal barriers, to function in innate and adaptive immunity (Vantourout and Hayday, 2013).

Although $\gamma\delta$ T cells are considered a relatively small subset of T lymphocytes in the periphery of healthy humans and mice, numerous publications established the fact that $\gamma\delta$ T cells have vital roles in both innate and adaptive immunity against infectious diseases and cancers. Firstly, based on their diverse cytokine production, $\gamma\delta$ T cells can regulate immune responses, particularly orchestrating inflammation by IL-17A and IL-17F production (Crowe et al., 2009; Roark et al., 2008). Additionally, $\gamma\delta$ T cells can lyse the infected or stressed cells via the production of granzymes and perforins (Bade et al., 2005). Further, $\gamma\delta$ T cells can facilitate IgE production by B cells and promote the humoral response (Born et al., 2017; Huang et al., 2015). Also, it has been shown that $\gamma\delta$ T cells can produce epithelial growth factors to modulate stromal cell function and proliferation (Boismenu and Havran, 1994; Jameson and Havran, 2007; Nielsen et al., 2017; Sharp et al., 2005). Moreover, $\gamma\delta$ T cells can trigger dendritic cell maturation (Acker et al., 2015; Ismaili et al., 2002); lastly, $\gamma\delta$ T cells can present antigens for $\alpha\beta$ T cells functioning as antigen presenting cells (Brandes et al., 2005; Himoudi et al., 2012; Tyler et al., 2017).

$\gamma\delta$ T Cells and Neonatal Immunity. Among newborns and young infants, most of the global mortality is due to infections, because the hosts are particularly susceptible to microbes (Dowling and Levy, 2014). By studying the ontogeny of early life immunity, it has been suggested that the immune system of young hosts tends to have type 2 and type 17 polarized immune responses and limited type 1 immunity, compared to adults, possibly for the purpose of avoiding tissue damage from the overwhelming inflammation during development (Dowling and Levy, 2014; Langrish et al., 2002). Therefore, it is absolutely necessary to characterize the neonatal immune system for the purpose of therapeutics.

With this aim, we found the fact that $\gamma\delta$ T cells are the first T cells to appear in the immune system, and can respond to pathogen infections rapidly in different tissues because of their higher abundance in mucosal tissues and innate cell-like features. However, only limited research has been done to investigate the functions of $\gamma\delta$ T cells in neonatal immune responses. For examples, it has been indicated that impaired IL-17A production by $\gamma\delta$ T cells in neonatal respiratory syncytial virus (RSV) infections causes a lack of inflammasome activation and more severe disease outcomes, compared to adults (You et al., 2008). Additionally, a study found that large numbers of $\gamma\delta$ T cells in the brain of infants after injury, and the depletion of $\gamma\delta$ T cells provided protection to the hosts. However, the production of IL-17A and IFN- γ by $\gamma\delta$ T cells was not detected (Albertsson et al., 2018). Nonetheless, the responses of $\gamma\delta$ T cells in neonatal influenza infections still remain unclear.

$\gamma\delta$ T Cells and Influenza Infections. Influenza virus infection induces a typical Th1 immune response in adult mice, but the response is poorly characterized in neonates. Neonatal responses to other viral infections such as RSV and to bacterial challenge are characterized by Th2-type and anti-inflammatory responses (Dowling and Levy, 2014). The innate immune response to infection consists of diverse effectors, including cytokines, chemokines, and proteases with important antiviral functions that are generated rapidly in infected lungs. These effectors are produced by different types of innate immune cells, including innate lymphoid cells, macrophages, neutrophils, natural killer cells, and $\gamma\delta$ T cells. In acute infections, $\gamma\delta$ T cells are the major initial IL-17A producers (Roark et al., 2008). IL-17A produced by $\gamma\delta$ T cells can mediate the inflammatory response and recruit neutrophils to the inflammatory sites in lungs in response to pulmonary influenza infection. In one study, although IL-17 $r^{-/-}$ mice have higher body weight after influenza infection, their survival rate is significantly lower. Also, IL-17 $r^{-/-}$ mice have reduced neutrophil emigration and more severe lung damage (Crowe et al., 2009). Additionally, $\gamma\delta$ T cells can boost the adaptive immune response. The interaction between $\gamma\delta$ T cells and DCs leads to the maturation of DCs and the polarization of $\gamma\delta$ T cells (Devilder et al., 2006), which in turn modulates functional polarization of conventional T cells and promotes B cell responses and antibody class switching (Pao et al., 1996; Wen et al., 1996). Finally, $\gamma\delta$ T cells have the capacity to maintain tissue homeostasis and contribute to tissue repair. For example, Aryl hydrocarbon receptor (AhR) is highly expressed by $\gamma\delta$ T cells, and AhR activation promotes IL-22 production, but not IL-17A (Martin et al., 2009). IL-22 – expressing $\gamma\delta$ T cells are particularly important in pulmonary immune responses to prevent pulmonary

injury, repair damaged lung, and maintain lung homeostasis (Zenewicz and Flavell, 2011).

Since $\gamma\delta$ T cells are the first T cells to develop in all the vertebrates and can protect the host against infections via different mechanisms, their striking ontogeny and functions suggest their primary contributions to neonatal protection, when conventional $\alpha\beta$ T cell responses are functionally less prominent and DCs are immature. Although the role of CD8 T cells in clearing influenza infection has been shown in both adults (Topham et al., 1997) and neonates (You et al., 2008), the response of neonatal CD8 T cells is less robust in cell number and IFN- γ production than that of adult CD8 T cells, which further leads to pathogenesis in the infected neonates. It has been reported that $\gamma\delta$ T cells make an important contribution to protect young mice from intestinal parasite infection (Randolph and Lewis, 2006). However, not much is known about the role of $\gamma\delta$ T cells in neonatal viral infection including influenza. Influenza infection still remains a significant cause of pulmonary morbidity and mortality, especially in neonates. Therefore, it is critical to investigate whether $\gamma\delta$ T cells confer protection against neonatal influenza infection.

T Cell Mediated Immunotherapy and Current Techniques

Adoptive T cell transfer (ACT) has emerged as a new therapeutic pillar for immunotherapy. Host T cells that have been *in vitro* expanded or TCR-engineered to generate antigen specific T cells, have been successfully used to control viruses and tumors in patients (Chapuis et al., 2012; Han et al., 2013; Hinrichs et al., 2009; Kershaw et al., 2006; Lamers et al., 2011). *In vitro* expansion of viral or tumor-specific T cells requires significant time to prepare, and the targets of the TCR are not usually fully characterized. On the other hand, T lymphocytes with genetically engineered TCRs or chimeric antigen receptors (CAR) have the advantages of defined high antigen-specificity and binding affinity. Despite the current techniques to profile and clone TCRs that have improved and shortened the process, these methods are still largely limited to the non-paired TCR bulk sequencing, require certain minimum amounts of cell number, and expansion of isolated cells prior to TCR isolation. Therefore, a new approach, which can overcome these obstacles, needs to be established to improve T cell mediated immunotherapy to infectious diseases and cancers.

In addition, current studies have shown increasing interest in determining ligands to certain TCRs (Di Marco Barros et al., 2016; Karunakaran and Herrmann, 2014; Vantourout et al., 2018; Vavassori et al., 2013; Willcox and Willcox, 2019), which can activate or inhibit T cell functions for use as potential drug candidates. Interestingly, numerous reports have attempted to identify $\gamma\delta$ TCR ligands, due to the non-MHC restricted recognition. However, the limited understanding and flexibility in the CDR3 of $\gamma\delta$ TCR makes the ligand identification more difficult (Willcox and Willcox, 2019). Hence, the establishment of a robust and efficient system with a reliable TCR activation readout is needed for the screening of specific TCRs.

Summary and Aims of Dissertation

Neonates are highly vulnerable to influenza infections with severe mortality. $\gamma\delta$ T cells are the first T cells to develop in ontogeny, and the immune responses are biased to type 2 immunity, which may render $\gamma\delta$ T cells responsive to infections via different mechanisms from adults. Therefore, we hypothesize that $\gamma\delta$ T cells have critical roles in protecting neonatal mice from influenza virus infection by modulating local inflammation, innate immune responses, and lung homeostasis. Our aim is to unravel the functions of $\gamma\delta$ T cells and related mechanisms following neonatal influenza infection.

As one of the promising methods for immunotherapy, T cell adoptive transfer has a great potential to protect hosts against infectious diseases and cancers. By improving the techniques of paired single-cell TCR characterization, cloning, and CDR3 substitution, our aim is to provide more opportunities for immunotherapy for infectious diseases and cancers.

CHAPTER 2. METHODS AND MATERIALS

Methods Related to Neonatal Influenza Infections²

Mice

C57BL/6J (wild-type) and C57BL/6J.129P2-*Tcrdtm1*Mom/J (*Tcrd*^{-/-}) mice were purchased from the Jackson Laboratory. *Il33*^{tm1(KOMP)Vlcg} (*Il33*^{-/-}) embryos were obtained from the KnockOut Mouse Project (KOMP; <https://www.komp.org/>) and rederived using C57BL/6 mice at St. Jude Children's Research Hospital's Animal Resource Center. *Tcrd*^{+/-}, *Il33*^{+/-}, and *Il17a*^{+/-} mice were bred to generate littermate control as well as homozygous knockout mice. *Il17a*^{-/-} mice were rederived from mice donated by Sarah Gaffen and Jay Kolls (University of Pittsburgh), with the agreement of Dr. Yoichiro Iwakura at the Tokyo University of Science. Both male and female neonates were infected at the age of 7-days-old, and female adult mice were infected at the age of 8-10 weeks. Infection methods are described below.

Human Samples

For this study, we used a subset of individuals from the FLU09 cohort which has been previously described (Oshansky et al., 2014). Nasal aspirates were collected at time of enrollment from 25 pediatric patients with laboratory confirmed influenza virus infection with no predisposing conditions (48% female, 88% African American, average age at enrollment = 1.5, age range 0.05 – 7.31, age mean $\pm$ STD 1.50 ± 1.47). Patients included in this study were stratified into mild and severe groups using treatment status, with 56% of patients having severe influenza illness outcome.

Ethical Compliance for Mouse and Human Study

Animal study protocols were approved by St. Jude Children's Research Hospital Committee on Use and Care of Animals. All the mice were bred and maintained at St. Jude Children's Research Hospital under specific-pathogen free conditions. For studies involving human samples, inclusion criteria required that participants meet the clinical case definition of influenza virus infection at the time of enrollment. This study was conducted in compliance with 45 CFR 46 and the Declaration of Helsinki. Institutional

² This section adapted with permission from final submission. Guo X.Z., Dash P., Crawford J.C., Allen E.K., Zamora A.E., Boyd D.F., Duan S., Bajracharya R., Awad W.A., Apiwattanakul N., Vogel P., Kanneganti T.D., Thomas P.G. (2018). Lung $\gamma\delta$ T cells mediate protective responses during neonatal influenza infection that are associated with type 2 immunity. *Immunity*. 49(3):531-544. DOI: <https://doi.org/10.1016/j.immuni.2018.07.011>.

Review Boards of St. Jude Children's Research Hospital and the University of Tennessee Health Science Center/Le Bonheur Children's Hospital approved the study.

Animal Model of Influenza A Infection

Neonatal mice were anesthetized by isoflurane inhalation and infected intranasally (i.n.) with A/HKx31 [H3N2, A/Aichi/2/68× A/Puerto Rico/8/34 (2 + 6)] in 10µl (0.6×10^2 EID₅₀/g body weight).

For neonatal EdU experiment, infected neonates were injected intraperitoneal (i.p.) with 50µl EdU (1µg/µl) diluted in PBS at 8 hrs before tissue harvest.

For neonatal influenza infection with rmIL-17A (low-dose: 100pg/mouse, high-dose: 1000pg/mouse) and rmIL-33 (10ng/mouse) administration experiment, *Tcrd*^{-/-} neonates were intranasally infected with A/HKx31 virus mixed with rmIL-17A (421-ML; R&D) or rmIL-33 (3626-ML; R&D) in 10µl.

For anti-Areg neutralizing experiments, wild-type neonates were infected with A/HKx31 virus in 10µl i.n. followed by intraperitoneal injection of 2µg of anti-Areg (AF989; R&D) or normal goat IgG control antibody (AB-108-C; R&D) in 50µl of PBS on day 4, 6 and 9 after infection.

For adult influenza infection, adult wild-type and *Tcrd*^{-/-} littermates (8-10 weeks old) were anesthetized by Tribromoethanol (Avertin) i.p. injection and infected i.n. with 1MLD₅₀ A/Puerto Rico/8/34 virus in 30µl.

Quantification of IAV in Infected Lung Tissue

Tissues were homogenized and centrifuged at 10,000 rpm for 15 mins. Supernatant from lung homogenates were measured by traditional 50% Tissue culture Infective Dose assay in Madin-Darby canine kidney (MDCK) cells.

Cytokine Measurement of Mouse Lung Homogenates

Cytokines in the supernatant of lung homogenates were measured by mouse DuoSet ELISA kits (R&D) or Milliplex assays (Millipore) according to manufacturer's instructions. The results were normalized by total protein determined by BCA assay (Thermo Fisher).

Flow Cytometry

Mice were euthanized at described timepoints and lungs were collected for analysis. For analysis of surface markers, cells were stained in PBS containing 2% (wt/vol) BSA, with TCR γ/δ (GL3; Biolegend), TCR β (H57-597; Biolegend), CD4 (RM4-5; Biolegend), CD8 α (53-6.7; Biolegend), CD3 (17A2; Biolegend), CD90.2 (30-H12; Biolegend), CD25 (PC61.5; eBioscience), ST2 (DIH9; Biolegend), CD27 (LG.3A10; Biolegend), CD44 (IM7; Biolegend), CCR6 (29-2L17; Biolegend), Sca-1 (D7; Biolegend), ST2 (DIH9; Biolegend), Lineage cocktail (lin-1; Biolegend), Gr-1 (RB6-8C5; Biolegend), CD11b (M1/70; Biolegend), CD11c (N418; Biolegend), CD31 (390; Biolegend) and CD326 (G8.8; Biolegend). Intracellular IL-17A (eBio17B7, eBioscience), IFN- γ (XMG1.2, Biolegend), Foxp3 (FJK-16s, eBioscience), Amphiregulin Biotinylated Antibody (R&D), and Brilliant Violet 421 Streptavidin (Biolegend) were analyzed by flow cytometry according to the manufacturer's instructions. For intracellular cytokine staining, T cells were stimulated for 4 hrs with Cell stimulation cocktail plus protein transport inhibitors (eBioscience) before intracellular staining, which is according to the manufacturer's instructions (eBioscience). Antibody validation profiles are available via 1DegreeBio (<http://1degreebio.org/>) or CiteAb (<http://www.citeab.com>). Flow cytometry data were acquired on Fortessa (BD Biosciences) and analyzed using FlowJo software (TreeStar).

RNA-Seq

Genomic DNA-free total RNA was extracted from whole, homogenized lungs using the RNeasy Mini kit (Qiagen). Individually indexed libraries were constructed for each mouse using the Illumina TruSeq Stranded Total RNA kit, and multiplexed libraries were sequenced across two Illumina HiSeq lanes, yielding over 580 million 100bp paired-end reads. Reads were mapped to the mm10 genome using STAR (version 2.5.2b; (Dobin et al., 2013) with default parameters, and gene counts were obtained with HTSeq (version 0.6.1p1; (Anders et al., 2015)) using Gencode comprehensive chromosomal annotations (vM13). Putative genes, pseudogenes, allosomal genes, and genes with fewer than 5 counts per million (CPM) were excluded from analyses. Gene counts were normalized to trimmed mean of M-values (TMM) using edgeR (Robinson and Oshlack, 2010) and then transformed to log₂-CPM and modeled as a function of genotype (*i.e.*, wild-type, *Tcrd*^{-/-}) using the limma package to determine differential expression across conditions (Ritchie et al., 2015). Results were corrected for multiple testing using the Benjamini and Hochberg method to produce FDR-adjusted p-values. For Gene Set Enrichment Analysis, each gene was assigned a rank by calculating the product of the log₂-fold change and the inverse of the FDR-adjusted p-value; this ranked set was then analyzed by GSEA (version 2.2.4; (Subramanian et al., 2005)) using the GseaPreranked function (1,000 permutations, uncollapsed dataset, classic enrichment statistic, 500/15 maximum/minimum set sizes) and the mouse NetPath gene set (Merico et al., 2010). RNA-Seq data were deposited at NCBI GEO under accession GSE99683.

Quantitative Real-time PCR

RNA of sorted $\gamma\delta$ T cells, ILC2s, ST2⁺CD4⁺ cells and ST2⁻CD4⁺ cells was isolated using RNeasy Mini kit (Qiagen) and reverse transcribed into cDNA with the SuperScript Vilo kit[®] (Invitrogen). Gene expression was assessed using Taqman[®] Gene Expression Master Mix according to the manufacturer's instructions (Applied Biosystems). All human and mouse primers and probe sets were purchased from Applied Biosystems. Data were analyzed with the method (Livak and Schmittgen, 2001) and results with Ct value higher than 35 were considered undetected.

In Vitro Stimulation

Murine lung epithelial cells (LET1s) and human lung epithelial cell line (A549) were maintained in our laboratory and cultured in DMEM supplemented with 10% (vol/vol) FBS, 1% (vol/vol) L-Glutamine, and 1% (vol/vol) penicillin-streptomycin. For *in vitro* stimulation, LET1 cells were plated overnight and treated with 3MOI of A/HKx31, and/or 50ng/ml recombinant mouse IL-17A (421-ML; R&D) for indicated time. For *in vitro* stimulation of human lung epithelial cells, A549 were plated overnight and treated with 3MOI of A/Brisbane/10/2007 (H3N2), and/or 50ng/ml recombinant human IL-17A (7955-IL-025/CF; R&D) for 48 hrs. For the pSTAT3 inhibition assay, LET1 and A549 cells were stimulated with virus and/or IL-17A with 100 μ M S3I-201 (SML0330; Sigma), resuspended in DMSO. All samples contained 0.05% DMSO in the culture medium.

Western Blotting

Proteins were extracted from stimulated cells using RIPA lysis buffer supplemented with proteinase inhibitors (cOmplete Protease Inhibitor Cocktail, Cat#11836170001, Sigma). Samples were loaded in 12%–15% SDS-PAGE and transferred onto PVDF membranes. Blocking was performed in 5% BSA for 1 hr, and membranes were incubated in primary antibodies overnight at 4°C. Membranes were incubated with HRP-conjugated secondary antibody for 1hr, and proteins were visualized using ECL substrate (Thermo Fisher). The primary antibodies were IL-33 (1:500 dilution, AF3626, R&D), pSTAT3 (1:1,000, 9145, Cell Signaling), STAT3 (1:1,000, 9139, Cell Signaling), and β -actin (1:2,000, 8457, Cell Signaling).

Neonatal $\gamma\delta$ T cell Transfer Experiment

Neonatal $\gamma\delta$ T cells were harvested and sorted from lungs of naive wild-type and *Il17a*^{-/-} neonatal littermates. Following sorting, wild-type or *Il17a*^{-/-} $\gamma\delta$ T cells were resuspended in a concentration of 2x10⁴ cells in 10 μ l of PBS and transferred to *Tcrd*^{-/-} littermate mice i.n., and a third transfer treatment of plain PBS was used as a control comparison. After 24 hrs, these *Tcrd*^{-/-} neonates were anesthetized by isoflurane and

infected i.n. with A/HKx31 in 10 μ l (0.6x10³ EID₅₀/g body weight). We used the log-rank test to assess survival differences as a function of treatment; after finding a significant effect, we then tested for pairwise differences between: 1) neonates receiving wild-type and *III7 α ^{-/-}* cells, and 2) between neonates receiving wild-type cells and PBS. We verified that there were no effects of experimental batch on survival by independently testing for effects of experiment number.

Single Cell Sorting and Multiplex PCR

Single cell suspension was made from wild-type lungs with mock- or influenza- (day 2 or 6) infections and stained with a live/dead exclusion dye (Live/dead aqua, Tonbo Biosciences), APC-conjugated anti-mouse CD3 (clone: 17A2) and PE-conjugated anti-mouse $\gamma\delta$ TCR (clone: GL3). The stained cells were resuspended in RNase inhibitor containing sort buffer (RNasin, Promega, 200U/ μ l) and sorted by gating on $\gamma\delta$ TCR⁺CD3⁺ cells directly into a 384-well PCR plate that had been preloaded with 1 μ l of reverse transcription reaction mix [1X SuperScript VILO Reaction Mix (Thermo Fisher), SuperScript VILO enzyme and 1X0.1% NP40 (Thermo Fisher)] with a sorter (Model SY3200, Sony Biotech Synergy sorter, Sony Biotech, San Jose, CA). The last column of the plate was left empty for use as PCR negative controls. After sorting, plates were sealed and kept on ice until end of the sort experiment, briefly centrifuged at 500g, and stored at -80°C until downstream processing.

Following single-cell sort into a 384 well PCR plate, direct lysis and reverse transcription were performed using SuperScript Vilo (Invitrogen) as described before (Guo et al., 2016). The resultant cDNA was subjected to a first round PCR reaction using a cocktail of TCRV γ region-specific forward and C region-specific reverse primers (**Table 2-1**). Using a corresponding internal primer pool, the first round PCR products were further subjected to a nested PCR (**Figure 2-1**). The PCR products were purified by Exonuclease I/Shrimp Alkaline Phosphatase treatment (Dash et al., 2015) and sequenced as described with a TRGC internal primer using an ABI Big Dye sequencer (Applied Biosystem) at the Hartwell Center, St Jude Children's Research Hospital. The sequence data were analyzed using a custom-built, macro-enabled Microsoft Excel sheet to derive CDR3 nucleotide and amino acid sequences (available upon request). To determine the corresponding TRGV-TRGJ usage, the sequence data derived from single cells were matched against the IMGT database (Lefranc et al., 2009).

Human Samples and Cytokine Measurement

To characterize human cytokines during influenza infection, we utilized nasal lavages obtained as part of the FLU09 study (Oshansky et al., 2014). FLU09 participants provided lavages at varying intervals from the day of enrollment through 12 days after enrollment. For this study, we focused on samples from a subset of infants and children below the age of 8 years (n = 25) who were verifiably infected with influenza A (pH1 or

Table 2-1. Primers Targeting Mouse T Cell Receptor Gamma (TRGV) Genes.

Primer Name	External Primer Sequence	Internal Primer Sequence
TRGV1-3 For	5'GCAGCTGGAGCAAAC TG3'	5'CTGAATTATCGGTCACCAG3'
TRGV4 For	5'CAAATATCCTGTAAAGTTTTCATC3'	5'GTTTAGAGTTTCTATTATATGTCCT TGCAAC3'
TRGV5 For	5'GATATCTCAGGATCAGCTCTCC3'	5'TACCCGAAGACCAAACAAGAC3'
TRGV6 For	5'TCACCTCTGGGGTCATATG3'	5'AGAGGAAAGGAAATACGGC3'
TRGV7 For	5'CAACTTGGAAGAAAGAATAATGTC3'	5'CACCAAGCTAGAGGGGTC3'
TRGC Rev	5'CTTTTCTTTCCAATACACCC3'	5'TCDGGAAAGAAGCTTTTCAAGG3'

Figure 2-1. Schematic of Reverse Transcription, Multiplex Nested Single-Cell PCR and Sequencing.

Complementary DNA (cDNA) from TCR $\gamma\delta$ and TCR $\alpha\beta$ mRNA was reverse transcribed directly from the sorted and stored single cells in the PCR plate without any RNA extraction step. The TCR $\alpha\beta/\gamma\delta$ transcripts from each cell were amplified by a multiplex nested PCR strategy, following which the products were purified by a modified Exonuclease I - rShrimp alkaline phosphatase. The purified PCR products were sequenced using the relevant TRAC, TRBC, TRGC or TRDC primer.

H3). Cells were separated from cell-free/mucus-free supernatants, and samples were stored at -80°C until analysis. Cytokines were measured in supernatants as follows: IL-17A, IL-33, and amphiregulin were assayed by Human IL-17A, IL-33, and amphiregulin DuoSet ELISA kits (R&D), and the level of IFN- γ was detected by Human Milliplex assays (Millipore) according to manufacturer instructions. To determine severity of influenza disease outcome, patients who were admitted to an emergency room or hospital were considered “severe” cases, whereas all other participants were considered “mild” ca

Quantification and Statistical Analysis

Data were analyzed with Prism 7.0 software (GraphPad) and presented as mean $\pm$ S.E.M. unless otherwise indicated. Experiments were repeated at least twice, as indicated. Statistical significance was determined by Mann-Whitney test between two groups. For comparison across three or more groups, non-parametric one-way ANOVA (Kruskal-Wallis test) was used, and differences between individual groups were estimated using Dunn’s multiple comparisons test. For the comparison of two groups at different time points, two-way ANOVA was used, and differences between individual groups were estimated using Sidak’s multiple comparisons test. To compare relative weight gain between wild-type and *Tcrd*^{-/-} neonates from 1-15 days after infection, we used a linear mixed-effects model via the “lmer” function in the R package lme4 (v1.1-13) (Bates et al., 2015) and assessed the significance of effects with Wald tests; specifically, genotype was modeled as a function of relative weight gain, with mixed effects allowing intercepts to vary across subjects (in order to control for individual differences among the repeatedly measured mice) and allowing the effect of genotype to vary across time points. The difference of survival rate was determined using the log-rank test. $p < 0.05$ was considered statistically significant where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and n.s., non significant.

For the repertoire analysis, because chi-square tests were not applicable due to the presence of zero-values in contingency table cells, statistical association across treatment conditions was assessed using Fisher’s exact test as implemented in R (Clarkson et al., 1993). Overall comparisons across days-post-infection for a given cell type (i.e., CD27⁺ or CD27⁻) were constructed as 3x7 contingency tables representing 3 treatment conditions and 7 possible gene segments. In the case that this overall comparison was significant, the simple effects were resolved by constructing 2x7 contingency tables for each pairwise comparison. P-values were adjusted to control family-wise error rate using Holm’s method (Holm, 1979).

For human cytokine analyses, we initially focused on the putative correlations underlying the proposed IL-17A/IL-33/Areg axis, using generalized linear mixed models with a log-link of the Gaussian distribution, as implemented by the R package MASS (v7.3-47) (Venables and Ripley, 2002), to investigate correlations among the adjacent components of the axis (i.e., IL-33 as a function of IL-17A, and Areg as a function of IL-33). In each case, we also included age, study day, and IFN- γ expression

as fixed effects, and patient-ID was included as a random effect in order to account for repeated sampling within patients over the course of the study. We also analyzed IFN- γ expression as a function of axis cytokines, using both the Gamma and Gaussian distributions. To investigate potential correlations between cytokine levels and disease severity, we focused on samples collected on the day at which disease severity was assessed (i.e., study day 0); for these analyses, we used the natural logarithm transformation to obtain normally distributed cytokine measures and ages, and we employed multifactor Analysis of Variance models (R aov function), with age included as a covariate. For significant effects in aov models, we confirmed equal variances between groups using an F-test (R var.test function).

Data and Software Availability

The RNA-seq data from whole lung RNA of wild-type and *Tcrd*^{-/-} neonates (day 8 after infection) has been deposited in the Gene Expression Omnibus (GEO, NCBI) under accession code GSE99683.

Methods Related to TCR Characterization and Cloning³

Subjects and Peripheral Blood Mononuclear Cells (PBMCs) Samples

Samples were obtained on research protocols approved by St. Jude Children's Research Hospital's institutional review boards (IRB) (Memphis, TN). Peripheral whole blood was collected from heparinized apheresis rings from healthy immunocompetent individuals not taking immunomodulatory pharmaceutical agents. PBMCs were isolated via density gradient centrifugation (GE Healthcare Ficoll-Paque PLUS, Marlborough, MA), and red blood cells (RBCs) were removed using RBC lysis buffer (8.3g NH₄Cl, 1g KHCO₃, and 1ml 0.1% Phenol Red in 1L distilled water). Isolated PBMCs were frozen in -80°C for future use. All PBMCs used in the paper were stored frozen. Compared to fresh PBMC data from healthy apheresis rings, our frozen PBMCs did not have a significantly lower success rate for single-cell amplification (data not shown).

Single Cell Sorting and Staining

PBMCs were treated with human FcR blocking reagent (Miltenyi Biotec, Auburn, CA) on ice for 20 minutes. Human TCR $\gamma\delta$ cells were isolated by staining with PE-conjugated anti-human TCR γ/δ (Biolegend, San Diego, CA, clone: B1), FITC-

³ This section adapted with open access permission from final submission. Guo X.Z., Dash P., Calverley M., Tomchuck S., Dallas M.H., Thomas P.G. (2016). Rapid cloning, expression, and functional characterization of paired $\alpha\beta$ and $\gamma\delta$ T-cell receptor chains from single-cell analysis. *Mol Ther Methods Clin Dev.* 3:15054. DOI: <https://doi.org/10.1038/mtm.2015.54>.

conjugated anti-human CD3 (Biolegend, San Diego, CA, clone: OKT3), a dump gate consisting of APC-conjugated anti-human CD11b/CD14/CD19 (Biolegend, San Diego, CA, CD11b clone: ICRF44; Biolegend, San Diego, CA, CD14 clone: HCD14; Biolegend, San Diego, CA, CD19 clone: HIB19) and Live/Dead Violet exclusion dye (Invitrogen, Carlsbad, CA, L34955) on ice for 30 minutes. After staining, TCR $\gamma\delta$ + CD3+ cells were sorted directly into a 96-well PCR plate (Biorad, Hercules, CA) with a sorter (Model sy3200, Sony Biotech Synergy sorter, San Jose, CA) by the following gating strategy: Single cells gate - Lymphocytes gate - Singlets gate – Live/dead gate – Dump gate (CD11b/14/19) – TCR $\gamma\delta$ /CD3 gate (**Figure 2-2**). The last 2 columns of the plate were left empty for use as PCR negative controls. After sorting, plates were stored at -80°C until downstream processing. Human $\alpha\beta$ T cells were also isolated using a similar method by using the staining and gating strategy described in this paper (Wang et al., 2012).

Reverse Transcription, Multiplex, Nested Single Cell PCR and Sequencing

Complementary DNA (cDNA) from TCR $\gamma\delta$ and TCR $\alpha\beta$ mRNA was reverse transcribed directly from the sorted and stored single cells in the PCR plate without any RNA extraction step using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) in a 2.5 μl reaction mix as per the method described previously (Dash et al., 2011). The cDNA synthesis was carried out by incubating at 25°C for 5min, 42°C for 30min, and 80°C for 5min. Alternatively, we used the SuperScript® VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA) which produces a higher success rate for single cell PCR by incubating the reaction mixture at 25°C for 10min, 42°C for 60min, and 80°C for 5min. The TCR $\alpha\beta$ transcripts from each cell were amplified by a multiplex nested PCR strategy as described previously (Dash et al., 2011; Wang et al., 2012). For amplification of TCR $\gamma\delta$ transcripts, the overall strategy was similar to the published TCR $\alpha\beta$ amplification, except for the primers described in **Tables 2-2** and **2-3**. We designed 9 TRGV external sense primers, 9 TRGV internal sense primers, 8 TRDV external sense primers and 8 TRDV internal sense primers targeted for individual TRGV and TRDV families based on the sequences derived from the IMGT database (<http://www.imgt.org/genedb/>; (Lefranc et al., 2009)). For the antisense primer, we designed single TRGC external, TRGC internal, TRDC external, and TRDC internal primers complementary to the published TRGC and TRDC sequences in IMGT. Human TRAV14/DV4, TRAV23/DV6, TRAV29/DV5, TRAV36/DV7, and TRAV38-2/DV8 are shared primers in TRAV and TRDV primer sets. The primers were synthesized by IDT and stored at -20°C at a stock concentration of 100 $\mu\text{mol/l}$ in TE buffer (pH8.0). The primers for each category (sense external, sense internal of TRGV and TRDV) were combined so that the final concentration of each primer in the mixture was 10 $\mu\text{mol/l}$. The antisense primers were diluted to 10 $\mu\text{mol/l}$. The PCR conditions for the TCR $\gamma\delta$ nested PCR were 95°C for 2min, followed by 35 cycles of 95°C for 20s, 53°C for 20s, and 72°C for 45s, followed by final extension of 72°C for 7min. The PCR products were run on a 2% agarose gel to check for the success rate of the PCR as well as contamination following which the products were purified by a modified Exonuclease I - rShrimp

Figure 2-2. Gating Strategy of Human TCR γ/δ + CD3+ Cells Single Cell Sorting.

Human TCR γ/δ + CD3+ cells from PBMC samples were single-cell sorted into 96-well plate by applying the gating strategy above.

“Single cells gate – Lymphocytes gate – Singlets gate – Live/dead gate – Dump gate (CD11b/14/19) – TCR γ/δ + /CD3+ gate”.

Table 2-2. Primers Targeting Human T Cell Receptor Gamma (TRGV) Genes.

TRGV Primer Name	External Primer Sequence	Internal Primer Sequence
HuTRGV3.5 For	5'TCTTCCAACCTTGAAGGG3'	5'GGTCATCTGCTGAAATCAC3'
HuTRGV7 For	5'TCTTCCAACCTTGAAGGG3'	5'GGTCATCTGCTGTAATCACTTG3'
HuTRGVA For	5'GGGTCATCCTGTTTCCAG3'	5'TACCTAAGGACCTGTGTAGAGG3'
HuTRGVB For	5'TGGCCTCCCAAAGTACTG3'	5'TCCTCTTTCTATGTCCCAGG3'
HuTRGV8 For	5'CCAACCTTGAAGGGAGAAC3'	5'AAAATGCCGTCTACACCC3'
HuTRGV9 For	5'CCAGGTCACCTAGAGCAAC3'	5'TGTCCATTTTCATATGACGG3'
HuTRGV10 For	5'TTATCAAAAAGTGGAGCAGTTC3'	5'CAGCTATCCATTTCCACGG3'
HuTRGV11 For	5'GAACAACCTGAAATATCTATTTCC3'	5'CATATCTTGGAAGGCATCC3'
HuTRGV1.2.4.6 For	5'GGGTCATCTGCTGAAATCAC3'	5'CCAGGAGGGGAAGGC3'
HuTRGC Rev	5'GGTGTTCCTCCTGG3'	5'CCCAGAATCGTGTGCT3'

Table 2-3. Primers Targeting Human T Cell Receptor Delta (TRDV) Genes.

TRDV Primer Name	External Primer Sequence	Internal Primer Sequence
HuTRDV1 For	5'GCCCAGAAGGTTACTCAAG3'	5'AGCAAAGAGATGATTTTCCTTA3'
HuTRDV2 For	5'ATTGAGTTGGTGCCTGAAC3'	5'TATATCAACTGGTACAGGAAGACC3'
HuTRDV3 For	5'TGTGACAAAGTAACCCAGAGTTC3'	5'GGTACTGCTCTGCACTTACGAC3'
HuTRDV4/TRAV14 For	5'CAAACCCAACCAGGAATG3'	5'AGGAAAAGGAGGCTGTGAC3'
HuTRDV5/TRAV29 For	5'GCAAGTTAAGCAAAATTCACC3'	5'CTGCTGAAGGTCCTACATTC3'
HuTRDV6/TRAV23 For	5'TTGATAGTCCAGAAAGGAGG3'	5'CGTTTGACTACTTTCCATGG3'
HuTRDV7/TRAV36 For	5'GACAAGGTGGTACAAAGCC3'	5'ATCTCTGGTTGTCCACGAG3'
HuTRDV8/TRAV38-2 For	5'CAGTCACTCAGTCTCAACCAG3'	5'TCTGGTACAAGCAGCCTC3'
HuTRDC Rev	5'CTTCATATTTACCAAGCTTGACAG3'	5'GATGACAATAGCAGGATCAAAC3'

alkaline phosphatase (ExoSAP-IT®, BioRad, Hercules, CA) method (Bell, 2008) to eliminate unincorporated primers and dNTPs for high quality DNA sequencing. 1µl of the single cell PCR product was added into the mixture of 4.6µl of Tris-Cl (50mmol/l, pH8.0), 0.2µl of Exonuclease I and 0.2µl of rShimp alkaline phosphatase and was incubated at 37°C for 15min and 80°C for 15min. The purified PCR products were sequenced using the relevant TRAC, TRBC, TRGC or TRDC primer. A schematic of the PCR strategy is shown in Figure 2-1.

gBlock® Gene Fragments, Gibson Assembly® and Transformation

The gBlock® gene fragments encoding the library of TRGVs and TRDVs were obtained from Integrated DNA Technologies (IDT, Coralville, IA). The expression vector pMCherry (10µg), which was modified from the parental pMIGII (Workman et al., 2002) by changing GFP to an mCherry reporter, was double digested by EcoR I (20units) and Xho I (20units) restriction enzymes (New England Biolabs, Ipswich, MA) at 37°C for 3h as per manufacturer's instruction. Agarose gel purified-linearized pMCherry vector (100ng) and 2x TCR gBlock inserts were ligated in a three-way ligation, including the TCRγ gene, TCRδ gene, and linearized vector by using the Gibson Assembly® Cloning kit (New England Biolabs, Ipswich, MA) per manufacturer's instructions. Two microliters of the ligation mixture was transformed into DH5α Competent E. coli (New England Biolabs, Ipswich, MA) per manufacturer's instructions.

Generation of Human CD3 Construct

Human CD3 δ, γ, ε and ζ genes were amplified from human PBMC cDNA using the primers in **Table 2-4**. All the genes were linked together by overlap PCR with species-specific 2A regions inserted (Szymczak et al., 2004). The types and amino acid sequences of the 2As used are shown in **Table 2-5**. The CD3 gene complex was then cloned into an MSCV-based retroviral vector that contains an IRES (Bettini et al., 2013; Holst et al., 2006; Persons et al., 1997) and ametrine as a reporter gene.

DNA Isolation, Cell Culture and Transfection

Recombinant pMCherry plasmids with full length TCRαβ or TCRγδ inserts were isolated in small scale by using a NucleoSpin® Plasmid kit (Clontech, Mountain View, CA) and in large scale for transfection using a Plasmid Midi kit (Qiagen, Hilden, Germany) per the manufacturers' instructions. The Neon® Transfection System was used to transfect 10µg TCRαβ or γδ DNA in the pMCherry vector into the human Jurkat 76 TCRαβ⁻ cell line (2×10^7 cells/mL, 100ul) (Heemskerk et al., 2003), followed by three pulses with a voltage of 1,350V and a width of 10ms. The transfected cells were cultured for 48h before being assayed for TCRαβ or TCRγδ expression on the surface by FACS

Table 2-4. 2A Primers Targeting Human CD3 δ , γ , ϵ and ζ Genes.

Primer Name	Primer Sequence
CD3δ sense	5'CCCTCACTCCTTCTCTAGGCGCCGGAATTCGCCAGGATGGAACATAGCACG3'
CD3δ antisense	5'CCACGTCTCCCGCCAACTTGAGAAGGTCAAAATTCAAAGTCGTGTTTCACCGGTCCCTTGTTCCGAGCC3'
CD3γ sense	5'GAATTTTGACCTTCTCAAGTTGGCGGGAGACGTGGAGTCCAACCCAGGGCCCATGGAACAGGGGAAG3'
CD3γ antisense	5'CCTCGACGTCACCGCATGTTAGCAGACTTCCTCTGCCCTCAGATCTTCTATTCTCTCTCAAC3'
CD3ϵ sense	5'CAGAGGAAGTCTGCTAACATGCGGTGACGTCGAGGAGAATCCTGGCCCAATGCAGTCGGGCACTC3'
CD3ϵ antisense	5'GTTTTCTTCCACGTCTCCTGCTTGCTTTAACAGAGAGAAGTTCGTGGCGGATCCTCCGATGCGTCTCTG3'
CD3ζ sense	5'CTCTCTGTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGGTCCCATGAAGTGGAAGTG3'
CD3ζ antisense	5'GAGGGAGAGGGGCGGAATTGATCCTCGAGCAATTGTTAGCGAGGGGCCAG3'

Table 2-5. Types and Sequences of 2A Regions.

2A Type	2A Amino Acid Sequence	Separation
F2A (foot-and-mouth disease virus)	VKQTLNFDLLKLAGDVESNPGP	CD3 δ and CD3 γ
T2A (Thosea asigna virus)	EGRGSLTTCGDVEENPGP	CD3 γ and CD3 ϵ
P2A (porcine teschovirus-1)	ATNFSLLKQAGDVEENPGP	CD3 ϵ and CD3 ζ

analysis. The human Jurkat 76 cells TCR $\alpha\beta$ ⁻ cell line was cultured in complete-RPMI 1640 medium, which is RPMI 1640 with 10% of fetal bovine serum, 1% Penicillin Streptomycin, and 1% L-glutamine at 37°C and 5% CO₂.

Immunofluorescent and Flow Cytometric Analyses

For surface staining, cells ($1-5 \times 10^5$) were harvested from culture and washed with FACS buffer (PBS with 1% of BSA and 0.1% sodium azide) prior to staining. The cells were treated with human FcR blocking reagent (Miltenyi Biotec, Auburn, CA) on ice for 20 min, and cells were then treated with various fluorescent conjugated antibodies against cell surface markers in FACS buffer. Human $\gamma\delta$ T cells were stained with APC-conjugated anti-human TCR γ/δ (Biolegend, San Diego, CA, clone: B1) or APC-conjugated anti-human TCR α/β (Biolegend, San Diego, CA, clone: IP26) and Pacific Blue-conjugated anti-human CD3 (Biolegend, San Diego, CA, clone: OKT3). For influenza-specific tetramer staining, cells ($1-5 \times 10^5$) were stained with APC-conjugated Influenza-M1 tetramer (Beckman Coulter, Brea, CA, HLA-A*0201, GILGFVFTL) in FACS buffer at room temperature for 1h prior to surface staining with the same staining antibodies described above.

Modification of the CDR3 Region by Two-Step Overlap Extension PCR Cloning

The substitution of the CDR3 was carried out by an overlap extension PCR cloning protocol (Bryksin and Matsumura, 2010). Briefly, we generated a library of linker DNA by gBlock synthesis at IDT (**Table 2-6**). The linker DNA consists of TRGC-2A-TRDV_x (X represents the TRDV family) sequence. Using the single cell PCR products of γ and δ chains of the desired clonotype and the relevant linker gBlock DNA we carried out an overlap PCR. The PCR reaction was set up and carried out as follows: 12.5 μ l 2 $\times$ Phusion[®] high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA), 0.25 μ l of 100 $\times$ DMSO, 1 μ l of 10 μ mol/l TRGV internal sense primer, 1 μ l of TRDV internal antisense primer (**Tables 2-2 and 2-3**), 1ng of linker DNA, and deionized H₂O up to 25 μ l. The PCR program was 98°C for 30s; 34 cycles of each at 98°C for 10s, 58°C for 30s, 72°C for 1min; then finally 72°C for 10min. The PCR products were visualized on a 1% agarose gel, and purified from the gel to use for cloning into the existing construct with the same TRGV and TRDV family usage. The reaction conditions used were as follows: 20ng of a TCR construct in pMCherry vector with identical TRGV and TRDV but an irrelevant CDR3 γ and δ , with 50ng of the first step PCR products, 12.5 μ l of 2 $\times$ Phusion[®] high-fidelity DNA polymerase, 0.25 μ l of 100 $\times$ DMSO, and deionized H₂O up to 25 μ l. The PCR conditions used were 98°C for 30s; 17 cycles of each at 98°C for 10s, 65°C for 30s, 72°C for 4min; then finally 72°C for 10min. The PCR products were incubated with 1 μ l DpnI enzyme (New England Biolabs, Ipswich, MA) at 37°C for 1h, and 2-3 μ l of the digested products transformed into NovaBlue Singles[®] competent cells (EMD Millipore, Darmstadt, Germany).

Table 2-6. Human TCR $\gamma\delta$ Linker DNA Library.

Linker DNA	Sequence
HuLinker DV1	5'cataccttgtctcttgagaaatcttccagatattattaagatacattggcaagaaaagaagagcaacacgattctgggatcc caggagggggaacaccatgaagactaacgacacatacatgaaatttagctggtaacgggtgccagaagagtcactggacaaag aacacagatgtatcgtcagacatgagaataataaaaacggaattgatcaagaaattatcttctccaataaagacagatgtcatc acaatggatcccaagacaattgggtcaaaagatgcaaatgatacactactgctgcagctcacaacacacctctgcatattacatgta cctcctcctgctcctcaagagtgtggtctatcttggcatcatcacctgctgtctgcttggagaacggctttctgctgcaatggagag aatcaGCCACGAACTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAAC CCCGGTCCCATGCTGTTCTCCAGCCTGCTGTGTGTATTTGTGGCCTTCAGCTA CTCTGGATCAAGTGTGGCCCAAGGTTACTCAAGCCCAGTCATCAGTATCC ATGCCAGTGAGGAAAGCAGTCACCCTGAACTGCCTGTATGAAACAAGTTGGT GGTCATATTATATTTTTTGGTACAAGCAACTTCCCAGCAAAGAGATGATTTTC CTTATTCGCC3'
HuLinker DV2	5'cataccttgtctcttgagaaatcttccagatattattaagatacattggcaagaaaagaagagcaacacgattctgggatcc caggagggggaacaccatgaagactaacgacacatacatgaaatttagctggtaacgggtgccagaagagtcactggacaaag aacacagatgtatcgtcagacatgagaataataaaaacggaattgatcaagaaattatcttctccaataaagacagatgtcatc acaatggatcccaagacaattgggtcaaaagatgcaaatgatacactactgctgcagctcacaacacacctctgcatattacatgta cctcctcctgctcctcaagagtgtggtctatcttggcatcatcacctgctgtctgcttggagaacggctttctgctgcaatggagag aatcaGCCACGAACTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAAC CCCGGTCCCATGCAGAGGATCTCCTCCCTCATCCATCTCTCTCTCTTCTGGGC AGGAGTCATGTCAGCCATTGAGTTGGTGCCTGAACACCAAACAGTGCCTGTG TCAATAGGGGTCCCTGCCACCCTCAGGTGCTCCATGAAAGGAGAAGCGATCG GTAAGTACTATATCAACTGGTACAGGAAGACCCAAGG3'
HuLinker DV3	5'cataccttgtctcttgagaaatcttccagatattattaagatacattggcaagaaaagaagagcaacacgattctgggatcc caggagggggaacaccatgaagactaacgacacatacatgaaatttagctggtaacgggtgccagaagagtcactggacaaag aacacagatgtatcgtcagacatgagaataataaaaacggaattgatcaagaaattatcttctccaataaagacagatgtcatc acaatggatcccaagacaattgggtcaaaagatgcaaatgatacactactgctgcagctcacaacacacctctgcatattacatgta cctcctcctgctcctcaagagtgtggtctatcttggcatcatcacctgctgtctgcttggagaacggctttctgctgcaatggagag aatcaGCCACGAACTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAAC CCCGGTCCCATGATTCTTACTGTGGGCTTTAGCTTTTTGTTTTTCTACAG GGGCACGCTGTGTGACAAAGTAACCCAGAGTTCCCCGGACCAGACGG TGGCGAGTGGCAGTGAGGTGGTACTGCTCTGCACTTACGACACTG3'
HuLinker DV4	5'cataccttgtctcttgagaaatcttccagatattattaagatacattggcaagaaaagaagagcaacacgattctgggatcc caggagggggaacaccatgaagactaacgacacatacatgaaatttagctggtaacgggtgccagaagagtcactggacaaag aacacagatgtatcgtcagacatgagaataataaaaacggaattgatcaagaaattatcttctccaataaagacagatgtcatc acaatggatcccaagacaattgggtcaaaagatgcaaatgatacactactgctgcagctcacaacacacctctgcatattacatgta cctcctcctgctcctcaagagtgtggtctatcttggcatcatcacctgctgtctgcttggagaacggctttctgctgcaatggagag aatcaGCCACGAACTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAAC CCCGGTCCCATGTCACTTTCTAGCCTGCTGAAGGTGGTCACAGCTTCACTGTG GCTAGGACCTGGCATTGCCCAAGATAACTCAAACCCAACCAGGAATGTTC GTGCAGGAAAAGGAGGCTGTGACTCTGG3'

Table 2-6. Continued.

Linker DNA	Sequence
HuLinker DV5	5'cataccttgtctcttgagaaatcttccagatattattaagatacattggcaagaaaagaagagcaacacgattctgggatcc caggagggaacaccatgaagactaacgacacatacatgaaatttagctggtaacgggtgccagaagagtcactggacaaag aacacagatgtatcgtagacatgagaataataaaaacggaattgatcaagaaattatcttctccaataaagacagatgcatc acaatggatcccaagacaattggtaaaaagatgcaaatgatactactgctgcagctcacaaacacctctgcatattacatgta cctcctcctgctcctcaagagtgtggtctatcttggcatcatcacctgctgtctgcttgggaagaacggcttctgctgcaatggagag aatcaGCCACGAACTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAAC CCCGGTCCCATGGCCATGCTCCTGGGGGCATCAGTGCTGATTCTGTGGCTTCA GCCAGACTGGGTAAACAGTCAACAGAAGAATGATGACCAGCAAGTTAAGCA AAATTCACCATCCCTGAGCGTCCAGGAAGGAAGAATTTCTATTCTGAACTGT GACTATACTAACAGCATGTTTGATTATTTCTATGGTACAAAAAATACCCTGC TGAAGGTCCTACATTCCTGATATC3'
HuLinker DV6	5'cataccttgtctcttgagaaatcttccagatattattaagatacattggcaagaaaagaagagcaacacgattctgggatcc caggagggaacaccatgaagactaacgacacatacatgaaatttagctggtaacgggtgccagaagagtcactggacaaag aacacagatgtatcgtagacatgagaataataaaaacggaattgatcaagaaattatcttctccaataaagacagatgcatc acaatggatcccaagacaattggtaaaaagatgcaaatgatactactgctgcagctcacaaacacctctgcatattacatgta cctcctcctgctcctcaagagtgtggtctatcttggcatcatcacctgctgtctgcttgggaagaacggcttctgctgcaatggagag aatcaGCCACGAACTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAAC CCCGGTCCCATGGACAAGATCTTAGGAGCATCATTTTTAGTTCTGTGGCTTCA ACTATGCTGGGTGAGTGGCCAACAGAAGGAGAAAAGTGACCAGCAGCAGGT GAAACAAAGTCCTCAATCTTTGATAGTCCAGAAAGGAGGGATTTC AATTATA AACTGTGCTTATGAGAACACTGCGTTTGACTACTTTCCATGGTACC3'
HuLinker DV7	5'cataccttgtctcttgagaaatcttccagatattattaagatacattggcaagaaaagaagagcaacacgattctgggatcc caggagggaacaccatgaagactaacgacacatacatgaaatttagctggtaacgggtgccagaagagtcactggacaaag aacacagatgtatcgtagacatgagaataataaaaacggaattgatcaagaaattatcttctccaataaagacagatgcatc acaatggatcccaagacaattggtaaaaagatgcaaatgatactactgctgcagctcacaaacacctctgcatattacatgta cctcctcctgctcctcaagagtgtggtctatcttggcatcatcacctgctgtctgcttgggaagaacggcttctgctgcaatggagag aatcaGCCACGAACTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAAC CCCGGTCCCATGATGAAGTGTCCACAGGCTTTACTAGCTATCTTTTGGCTTCT ACTGAGCTGGGTGAGCAGTGAAGACAAGGTGGTACAAAGCCCTCTATCTCTG GTTGTCCACGAGGGAG3'
HuLinker DV8	5'cataccttgtctcttgagaaatcttccagatattattaagatacattggcaagaaaagaagagcaacacgattctgggatcc caggagggaacaccatgaagactaacgacacatacatgaaatttagctggtaacgggtgccagaagagtcactggacaaag aacacagatgtatcgtagacatgagaataataaaaacggaattgatcaagaaattatcttctccaataaagacagatgcatc acaatggatcccaagacaattggtaaaaagatgcaaatgatactactgctgcagctcacaaacacctctgcatattacatgta cctcctcctgctcctcaagagtgtggtctatcttggcatcatcacctgctgtctgcttgggaagaacggcttctgctgcaatggagag aatcaGCCACGAACTTCTCTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAAC CCCGGTCCCATGGCATGCCCTGGCTTCCTGTGGGCACTTGTGATCTCCACCTG TCTTGAATTTAGCATGGCTCAGACAGTCACTCAGTCTCAACCAGAGATGTCT GTGCAGGAGGCAGAGACCGTGACCCTGAGCTGCACATATGACACCAGTGAG AGTGATTATTATTTATTCTGGTACAAGCAGCCTCCCAG3'

Yellow – TRGC region; Purple – 2A sequence; Gray – TRDV region

***Nur77*-GFP Jurkat 76 TCR $\alpha\beta$ ⁻ Cell Line**

To characterize the functionality of TCR $\alpha\beta$ or $\gamma\delta$ clones, we established the *Nur77*-GFP Jurkat 76 TCR $\alpha\beta$ ⁻ cell line (NJ76 cells). After linearization of a *Nur77*-GFP BAC clone (constructed based on pTARBAC)(Moran et al., 2011) by mixing 10 μ g BAC DNA, 2 μ l 10 $\times$ reaction buffer, 10units of PI-SceI restriction enzyme (New England Biolabs, Ipswich, MA), and nuclease-free water to make the volume up to 20 μ l with incubation at 37°C for 3h and inactivation at 65°C for 20min, we added 80 μ l of nuclease-free water, 15 μ l of sterile sodium acetate (3mol/l, pH7.0), and 300 μ l of ethanol to the reaction mixture, and centrifuged at 12,000g for 30min at 4°C. The resulting DNA pellet was washed with 75% ethanol, air dried, and resuspended by Tris-EDTA buffer (pH 8.0). We used the Neon® Transfection System (Invitrogen, Carlsbad, CA) following the manufacturer's instruction to transfect the linearized BAC DNA into the human Jurkat 76 TCR $\alpha\beta$ ⁻ cell line (2×10^7 cells/mL, 100 μ l), with three pulses with a voltage of 1,350V and a width of 10ms. Cells were then cultured in complete-RPMI 1640 medium containing 500 μ g/ml Geneticin (Invitrogen, Carlsbad, CA) for selection.

Stimulation of K^bPB1₇₀₃+TCR $\alpha\beta$ ⁺ NJ76 Cells (PB1-NJ76) by Flu Peptide PB1

NJ76 cell transfected with a murine K^bPB1₇₀₃-specific TCR $\alpha\beta$ derived from influenza-infected mice and transfected cells were incubated with mouse splenocytes (cell number ratio of PB1-NJ76/splenocytes is 2:1), the influenza PB1₇₀₃₋₇₁₁ peptide (1 μ mol/ml), mouse splenocytes and peptide, and mouse α -CD3 (Biolegend, San Diego, CA, 2C11; 10 μ g/ml) and human α -CD28 (Biolegend, San Diego, CA, CD28.2; 10 μ g/ml) in c-RPMI 1640 medium at 37°C for 4h. The GFP expression in the mouse TCR $\alpha\beta$ + CD3⁺ cell population was quantified by flow cytometry.

Stimulation of TRGV9/TRDV2-NJ76 Cells by Zoledronic Acid

NJ76 cells transfected with a TRGV9/TRDV2 clone were incubated with 50 μ g/ml zoledronic acid (Zometa, Novartis, Basel, Switzerland) in c-RPMI 1640 medium at 37°C for 3h, washed three times and incubated for 12h. The GFP expression in the TCR $\gamma\delta$ + CD3⁺ cell population was quantified by flow cytometry.

CHAPTER 3. LUNG $\gamma\delta$ T CELLS MEDIATE PROTECTIVE RESPONSES DURING NEONATAL INFLUENZA INFECTION THAT ARE ASSOCIATED WITH TYPE 2 IMMUNITY⁴

Introduction

Influenza viruses are among the most common and significant pathogens that underlie human respiratory diseases, with high morbidity and mortality (Taubenberger and Kash, 2010; Taubenberger and Morens, 2008). These viruses primarily infect respiratory epithelial cells where, after detection by immune sensors, the host will initiate innate and adaptive immune responses that guide viral clearance and tissue repair (Dash and Thomas, 2014; Iwasaki and Pillai, 2014). Whereas viral clearance is primarily promoted by type 1 immune responses through effector CD8⁺ T cells (Cerwenka et al., 1999; Duan and Thomas, 2016; Duan et al., 2015), group 2 innate lymphoid cells (ILC2s) regulated by interleukin (IL)-33-IL-33R signaling promote type 2 immune responses and are important for lung homeostasis and repair following influenza infection (Chang et al., 2011; Gorski et al., 2013; Guo and Thomas, 2017; Le Goffic et al., 2011; Monticelli et al., 2011). Similarly, regulatory T (Treg) cells and T helper (Th) cells have been implicated in beneficial influenza immune responses mediated by IL-33-driven tissue restoration via production of amphiregulin (Areg) (Arpaia et al., 2015). Although the downstream immune responses and tissue repair process induced by IL-33 have been well defined, the production and upstream regulation of IL-33 is still unclear.

Compared to adults, infants suffer relatively higher rates of hospitalization, severe clinical complications, and mortality as a result of influenza infection (Bhat et al., 2005; Munoz, 2003). The proximate mechanisms guiding these age-dependent differences may also be related to exaggerated type 2 responses that are characteristic of the infant immune system (Adkins et al., 2004; Dowling and Levy, 2014; Garcia et al., 2000; Kleer et al., 2016), mediated by the IL-33 pathway (Kleer et al., 2016; Saluzzo et al., 2017), and may function to prevent tissue damage owed to excessive inflammation. Thus, the pathologies concomitant with influenza infection, and their respective therapeutic targets, likely differ between infants and adults. In adult influenza infection, CD8⁺ T cells predominate the antiviral response (Cerwenka et al., 1999; Duan and Thomas, 2016; Duan et al., 2015), but in infant influenza infection, CD8⁺ T cells exhibit relatively reduced functionality (You et al., 2008).

In contrast to CD8⁺ T cells, $\gamma\delta$ T cells are the first T cells to appear in the thymus during fetal development and have the capacity to recognize a wide range of antigens and respond rapidly to infections (Chien et al., 2014; Vantourout and Hayday, 2013) even

⁴ Adapted with permission from final submission. Guo X.Z., Dash P., Crawford J.C., Allen E.K., Zamora A.E., Boyd D.F., Duan S., Bajracharya R., Awad W.A., Apiwattanakul N., Vogel P., Kanneganti T.D., Thomas P.G. (2018). Lung $\gamma\delta$ T cells mediate protective responses during neonatal influenza infection that are associated with type 2 immunity. *Immunity*. 49(3):531-544. DOI: <https://doi.org/10.1016/j.immuni.2018.07.011>.

during infancy; for instance, these cells are known to play important roles in the infant immune response to RSV infection (Huang et al., 2015) and enterocolitis (Weitkamp et al., 2014). In mice, $\gamma\delta$ T cells are generally categorized according to their secretion of IFN- γ (Gao et al., 2003), IL-17A (Coffelt et al., 2015; Papotto et al., 2017), or M-CSF (Mamedov et al., 2018). The IL-17A producers, in particular, are known to play a prominent role in lung immunity to bacterial infections, where rapid IL-17A production is critical for the recruitment of protective neutrophil responses (Romagnoli et al., 2016). For the most part, $\gamma\delta$ T cells have not been associated with inducing type 2 immune effectors. Although $\gamma\delta$ T cells may likewise act as immune sentinels during neonatal influenza infection, the functions of $\gamma\delta$ T cells in influenza-infected infants remain to be elucidated.

In this study, utilizing a neonatal mouse model of influenza infection, we report that $\gamma\delta$ T cells played an important role in protecting suckling mice against viral infection. This protection was mediated by augmentation of IL-33 production from the mucosal compartment that was induced by IL-17A secreted from the responding $\gamma\delta$ T cells. We further showed that this IL-17A-dependent IL-33 production subsequently generates a local type 2 immune response with increased accumulation of Areg-producing ILC2s and Treg cells in the lung, thus promoting tissue repair and lung integrity following infection. We also observed correlations between IL-17A, IL-33, and Areg in nasal washes of human influenza-infected infants. Thus, our findings reveal important roles for $\gamma\delta$ T cells in directing an axis of IL-17A and IL-33 production, promoting tissue recovery after infection and providing a potential therapeutic target.

Results

$\gamma\delta$ T Cells Protect Neonatal Mice Against Influenza Infection by Promoting Tissue Restoration Independent of Viral Clearance

In influenza-infected adult mice, cytotoxic CD8⁺ T cells can express death receptors and produce cytokines and lytic mediators that promote viral clearance (Brincks et al., 2008; Gruta and Turner, 2014; Price et al., 2000). However, during infancy the immune system remains developmentally immature, with relatively fewer and less diverse CD8⁺ T cells compared to adults (You et al., 2008). In contrast, many $\gamma\delta$ T cell subsets develop fetally and thus are available to respond in neonates. To investigate if $\gamma\delta$ T cells can respond to neonatal influenza infection, we infected 7-day-old wild-type neonates with A/HKx31 (H3N2) influenza virus. After infection, we observed a significant accumulation of $\gamma\delta$ T cells, characterized by increases in both frequency and cell number (**Figure 3-1A**) although there was no change in mock-infected lungs of the same age (**Figure 3-2A, B**). EdU (5-ethynyl-2'-deoxyuridine) cell incorporation assays indicated that the increases in $\gamma\delta$ T cells in infected lungs were at least partially due to $\gamma\delta$ T cell proliferation (**Figure 3-1B**). In order to understand the function of $\gamma\delta$ T cells during influenza infection, we next infected a neonatal cohort of wild-type and $\gamma\delta$ T cell

Figure 3-1. $\gamma\delta$ T Cells Protect Neonatal Mice Against Influenza Infection via Promotion of Lung Homeostasis and Repair, Independent of Viral Clearance.

(A) Representative flow cytometric plots (left), and frequency and number (right) of $\gamma\delta$ T cells in mock- (open circle, n=14) or virus-infected (solid) lungs of wild-type neonates at 1 (n=11) and 2 (n=10) days following intranasal influenza A/x31 virus infection. (B) Representative flow cytometric plots (left) and summary frequency plot (right) of EdU⁺ $\gamma\delta$ T cells in mock-infected (n=7) and influenza virus-infected (n=7) lungs of wild-type neonates 2 days after infection. (C and D) Body weight profile (% of original weight) (C) and survival rate (D) of wild-type (black, n=28) and *Tcrd*^{-/-} (red, n=26) neonates following influenza infection. (E) Viral titer (Log₁₀TCID₅₀/ml) of wild-type (black) and *Tcrd*^{-/-} (red) neonates assessed by plaque assay at days 0, 3, 5, 7 and 10 after influenza infection. (F) Measurement of IFN- γ in the total lung homogenates by ELISA of wild-type (black, n=5) and *Tcrd*^{-/-} (red, n=5) neonates at 7 days after influenza infection. (G) Gene Set Enrichment Analysis of whole-lung gene expression, ranked by significance (-Log₁₀[FDR q-value]), from wild-type (black, n=3) and *Tcrd*^{-/-} (red, n=3) neonates at 8 days after influenza infection. (H) Representative images of H&E staining of influenza infected wild-type and *Tcrd*^{-/-} lungs at 15 days after infection. (I) Summary of histological analysis from influenza-infected wild-type (black, n=8) and *Tcrd*^{-/-} (red, n=6) lungs at 15 days after infection. Data are combined from at least two independent experiments and shown as mean $\pm$ SEM. *p<0.05, **p<0.01, ****p<0.0001, n.s., not significant.

Figure 3-2. Gating Strategy and $\gamma\delta$ T Cell Prevalence in Mock-Infected Lungs.

(A) Schematic flow cytometric plots of the gating strategies employed during experiments. (B) Frequency (left) and number (right) of $\gamma\delta$ T cells in the lungs of wild-type neonates at days 7, 8, and 9 after birth (equivalent to d0, d1 and d2 of mock infection). Data are combined from two independent experiments and shown as mean $\pm$ SEM. n.s., not significant.

receptor-deficient (*Tcrd*^{-/-}) animals with influenza virus. Compared to *Tcrd*^{-/-} mice, relative weight gains were overall significantly larger in wild-type neonates (**Figure 3-1C**). Wild-type neonates also had a significantly increased survival rate compared to *Tcrd*^{-/-} mice (**Figure 3-1D**), yet the two groups demonstrated no detectable difference in tissue viral clearance at any time point after infection (**Figure 3-1E**). Consistent with the viral titer, loss of $\gamma\delta$ T cells did not alter the levels of IFN- γ at Day 7 after infection (**Figure 3-1F**). To determine if $\gamma\delta$ T cells also play a role in adult influenza infection, we infected wild-type and *Tcrd*^{-/-} littermates (8-10 weeks old); no significant differences were observed in the weight loss profile or survival rate (**Figure 3-3A, B**) between wild-type and *Tcrd*^{-/-} adults. These data indicate that in neonatal mice $\gamma\delta$ T cells provide protection against influenza infection without influencing antiviral responses.

To determine if $\gamma\delta$ T cells function as immune regulators during infection, we performed RNA-Seq using total RNA obtained from whole lungs of 3 wild-type and 3 *Tcrd*^{-/-} neonates 8 days after infection. Gene Set Enrichment Analysis demonstrated distinct immune pathways in lungs obtained from wild-type and *Tcrd*^{-/-} mice (**Figure 3-1G**). Several pathways relevant to tissue development and regeneration were enriched in wild-type lungs, including the epithelial growth factor receptor (EGFR) pathway (Hall et al., 2016; Monticelli et al., 2011; Zaiss et al., 2015) and the hedgehog pathway (Hogan et al., 2014; Sriperumbudur et al., 2017). The absence of $\gamma\delta$ T cells was associated with increased inflammatory pathways, including TNF- α , IL-6, and IL-5. In order to investigate the downstream effects of these $\gamma\delta$ T cell-mediated immune responses on infected lungs, we performed H&E staining of lung sections. At 15 days after infection, *Tcrd*^{-/-} mice were characterized by increased perivascular & interstitial inflammation and bronchiolar hyperplasia & metaplasia (**Figure 3-1H, I**), suggesting that $\gamma\delta$ T cell deficiency disrupted lung homeostasis and tissue repair. Collectively, these data indicate that $\gamma\delta$ T cells expand after influenza infection and provide protection to infants by promoting tissue restoration rather than by enhancing the antiviral response.

$\gamma\delta$ T Cells Rapidly Produce IL-17A After Neonatal Influenza Infection

To determine the mechanism by which $\gamma\delta$ T cells promoted protection in neonatal animals, we examined their phenotypes after infection by using CD27 and CD44 surface marker expression, which distinguish IL-17A- and IFN- γ - producing $\gamma\delta$ T cells (Ribot et al., 2009). We found that $\gamma\delta$ T cells were predominantly CD27⁻CD44^{hi} in virus-infected neonatal lungs, corresponding to IL-17A production, while they were mainly CD27⁺CD44^{lo} (corresponding to IFN- γ production) after mock infection (**Figure 3-4A**). After influenza infection, lung $\gamma\delta$ T cells also had higher cell surface expression of CCR6 and Sca-1 (**Figure 3-4B**), consistent with the phenotype of IL-17A-producing $\gamma\delta$ T cells. To confirm the IL-17A-producing phenotype of lung $\gamma\delta$ T cells after influenza infection, we assayed *Il17a* gene expression using quantitative real-time PCR (qRT-PCR) in sorted $\gamma\delta$ T cells at different points. This analysis indicated that *Il17a* was upregulated transiently 1 day after infection. (**Figure 3-4C**). In concordance with these qRT-PCR

Figure 3-3 The Role of $\gamma\delta$ T Cells in Adult Influenza Infection.

(A and B) Body weight profile (A) and survival rate (B) of wild-type (black, n=15) and *Tcrd*^{-/-} (red, n=23) adults after virus infection, normalized to the original weight. Data are combined from four individual experiments and weight change data are shown as mean $\pm$ SEM. (C) Cell number of adult lung $\gamma\delta$ T cells at indicated time point after infection. (D) Cell number of adult lung IL-17A-producing $\gamma\delta$ T cells at indicated time point after infection. (E) Cell number of adult lung IFN- γ -producing $\gamma\delta$ T cells at indicated time point after infection. (C-E) Data are combined from two individual experiments with mock infection (n=7), or day 3 (n=10), day 5 (n=5), and day 8 (n=5) after influenza infection. **p<0.01.

Figure 3-4. IL-17A-Producing $\gamma\delta$ T Cells Rapidly Accumulate and Respond to Influenza Infection in Neonatal Mice.

(A) Representative flow cytometric plots (left, with CD27 and CD44 expression on x- and y-axis, respectively) and summary frequency plot (right) of $\gamma\delta$ T cells from mock- (open, n=12) and influenza virus- (solid, n=11) infected mice at 2 days following infection. (B) Representative flow cytometric histogram showing expression of CCR6 (left) and Sca-1 (right) gated on $\gamma\delta$ T cells from mock- (open) and virus- (shaded) infected neonates (2 days after infection). Mean fluorescence intensity are shown in the upper right corners. (C) Relative expression of *Il17a* by quantitative real-time PCR in sort-purified $\gamma\delta$ T cells from mock (open, n=6) and virus-infected neonates at 1 (n=6) and 2 (n=6) days following infection. (D) Representative flow cytometric plots of IL-17A (top) and IFN- γ (bottom) expression in $\gamma\delta$ T cells from mock- (left) and virus-infected (right) neonates at 1 day after infection. (E) Scatter plot showing frequency and number of IL-17A- (top) and IFN- γ - (bottom) producing $\gamma\delta$ T cells from (D). Samples from mock-infected (n=14) neonates were pooled from animals 1 (n=11) and 2 (n=10) days after mock infection. (F) Representative flow cytometric plots (left) and summary frequency plots (right) of $\gamma\delta$ TCR expression gated on total IL-17A-producing cells from mock- (n=14) and virus- (n=11) infected mice at 1 day following infection. Data are combined from at least 2 independent experiments and presented as mean $\pm$ SEM.

p<0.01, *p<0.001, ****p<0.0001, n.s., not significant.

data, the intracellular staining of IL-17A in $\gamma\delta$ T cells showed an enhanced frequency and number of IL-17A-producing $\gamma\delta$ T cells in virus-infected lungs, whereas IFN- γ -producing $\gamma\delta$ T cells were not significantly different (**Figure 3-4D, E**; kinetics shown in **Figure 3-5A, B**). This transient IL-17A production by $\gamma\delta$ T cells did not significantly alter neutrophil infiltration or accumulation (**Figure 3-5C**).

$\gamma\delta$ T cells can be highly clonotypic in different tissues, especially with respect to V γ chain usage (Carding and Egan, 2002). Given the enrichment in CD27⁻ $\gamma\delta$ T cells secreting IL-17A in influenza-infected neonates, we decided to investigate if the T cell receptor repertoires were altered in cells exhibiting this phenotypes. By utilizing single-cell PCR technology (Dash et al., 2011; Guo et al., 2016; Wang et al., 2012), we characterized the TRGV family usage by CD27⁺ or CD27⁻ neonatal $\gamma\delta$ T cells (Kashani et al., 2015) that were mock- or influenza-infected. TRGV family usage varied significantly between CD27⁺ and CD27⁻ $\gamma\delta$ T cells at different time points after infection (**Figure 3-5D and Table 2-1**). Furthermore, we observed shifts in the representation of individual TRGV families in CD27⁻, but not CD27⁺, populations. Specifically, TRGV2⁺ receptors, which were associated with CD27⁺ cells at baseline, became enriched in the CD27⁻ population.

Upon gating on IL-17A-positive cells, we also found that neonates contained elevated frequencies of $\gamma\delta$ TCR⁺ cells among total IL-17A-producing cells in the early stages of influenza infection. Furthermore, $\gamma\delta$ T cells became the dominant producers of IL-17A after infection (**Figure 3-4F**). However, the expression of IL-17A and IFN- γ by $\gamma\delta$ T cells in adult mice showed a distinct pattern from that in neonates: consistent with a previous study (Crowe et al., 2009), adult $\gamma\delta$ T cells produced additional IL-17A at a later stage of infection (**Figure 3-3C, through E**). In conjunction, these data from neonates and adults suggest distinct functionality of $\gamma\delta$ T cells across ontogeny, such that neonatal $\gamma\delta$ T cells rapidly secrete and are the primary source of IL-17A in response to influenza infection.

IL-17A, Primarily Secreted by $\gamma\delta$ T Cells, Improves the Survival of Influenza-Infected Neonates by Enhancing IL-33 Production

IL-17A is the canonical cytokine that regulates type 3 immunity, and its roles in adult influenza infection are still controversial. Some have suggested that IL-17A can be beneficial by modulating B cell responses (Wang et al., 2011), whereas others have proposed that IL-17A signaling may increase immune pathology by promoting neutrophil infiltration in adults (Crowe et al., 2009). Our data suggest that IL-17A might play a protective role in neonates because, in comparison to the lungs of virus-infected *Tcrd*^{-/-} neonates, IL-17A (mainly produced by $\gamma\delta$ T cells) is increased in wild-type lungs one day after influenza infection (**Figure 3-6A**). To test this formally in our experimental model, we infected *Tcrd*^{-/-} neonates concomitant with administration of a low-level of recombinant mouse IL-17A, the dose for which was calculated based on the difference of lung total IL-17A between wild-type and *Tcrd*^{-/-} neonates, and monitored the survival

Figure 3-5. Characterization of $\gamma\delta$ T Cells and Neutrophils in Influenza-Infected Neonatal Lungs.

(A) Frequency (top panel) and cell number (bottom panel) of lung $\gamma\delta$ T cells (left), IL-17A-producing $\gamma\delta$ T cells (middle) and IFN- γ -producing $\gamma\delta$ T cells (right), with mock infection (n=14), or day 1 (n=11), 2 (n=10), 5 (n=13), and 8 (n=7) after infection. (B) Frequency of IL-17A-producing $\gamma\delta$ T cells in mock- (n=10) or influenza- (n=15) infected neonatal lungs at 1 day after infection, without PMA/Ionomycin stimulation. (C) Percent (left) and number (right) of neutrophils of wild-type or *Tcrd*^{-/-} neonates with influenza-infected neonatal lungs at Day 0, 3, 5 and 8 following infections. (D) TRGV family usage of CD27⁺ (left) or CD27⁻ (right) $\gamma\delta$ T cells estimated by single-cell RT-PCR at mock-infection, 2 days after influenza infection, and 6 days after influenza infection (top to bottom). TRGV sequence data in the pie chart present Mock-CD27⁺ (n=11), Mock-CD27⁻ (n=19), Day2-CD27⁺ (n=65), Day2-CD27⁻ (n=59), Day6-CD27⁺ (n=45), and Day6-CD27⁻ (n=32). Data are combined from at least two independent experiments and presented as mean $\pm$ SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n.s., not significant.

Figure 3-6. IL-17A, Predominantly Secreted by $\gamma\delta$ T Cells, Improves the Survival of Influenza-Infected Neonates by Promoting IL-33 Production.

(A) Estimation of IL-17A in whole lung homogenates from mock- (open) or influenza virus-(solid)- infected wild-type (black) and *Tcrd*^{-/-} (red) neonates by ELISA at 1 day after infection. (B) Survival rate of influenza virus-infected *Tcrd*^{-/-} neonates administered with low levels of recombinant mouse IL-17A (rmIL-17A, green, n=37, 100pg/mouse) or PBS control (red, n=19) at the time of infection. (C) Schema outlining wild-type and *Il17a*^{-/-} $\gamma\delta$ T cell transfers to *Tcrd*^{-/-} neonates and subsequent infection. (D and E) Body weight profile (D) and survival rate (E) of influenza-infected *Tcrd*^{-/-} neonates receiving wild-type (black, n=15) or *Il17a*^{-/-} (red, n=13) $\gamma\delta$ T cells intranasally or no cell transfer (grey, n=10). (F) Protein levels of IL-33 assessed by ELISA in influenza virus-infected wild-type (black, n=15) and *Tcrd*^{-/-} lungs (red, n=9) at 1 day following infection. (G) Protein levels of IL-33 detected by ELISA in the lungs of *Tcrd*^{-/-} neonates that had been infected with influenza virus and simultaneously treated with either a low level of rmIL-17A (green, n=7, 100pg/mouse) or PBS control (red, n=6). Data were collected 1 day after infection/treatment. (H) Survival rate of influenza virus-infected wild-type (black, n=20) and *Il33*^{-/-} neonates (blue, n=17) with intranasal influenza virus infection. (I) Survival rate of influenza virus-infected *Tcrd*^{-/-} neonates administered with recombinant mouse IL-33 (rmIL-33, brown, n=18, 10ng/mouse) or PBS control (red, n=18) at the time of infection. Data are combined from at least 2 individual experiments. *p<0.05, **p<0.01, n.s., not significant.

rate. Low dose IL-17A significantly improved the survival of $\gamma\delta$ T cell-deficient mice, indicating that the IL-17A pathway is protective in the neonatal animals (**Figure 3-6B**). In contrast, a high dose of IL-17A administered to infected *Tcrd*^{-/-} mice was detrimental to the host (**Figure 3-7A**), as was a low dose of IL-17A administered to infected wild-type neonates (**Figure 3-7B**), suggesting that IL-17A fine-tunes the balance between pathogenicity and protection. In order to determine if IL-17A specifically produced by $\gamma\delta$ T cells is critical for protection against neonatal influenza infection, we isolated $\gamma\delta$ T cells from wild-type or *Il17a*^{-/-} neonatal lungs, transferred the cells to littermate *Tcrd*^{-/-} neonates intranasally and infected the recipients with influenza virus (**Figure 3-6C**). The weight change (**Figure 3-6D**) and survival rate (**Figure 3-6E**) profiles suggested that IL-17A production by $\gamma\delta$ T cells contributed to the protection $\gamma\delta$ T cells provide to infected neonates.

To explore which immune processes may have been disrupted by the loss of IL-17A in *Tcrd*^{-/-} neonates, we next assessed the concentration of a panel of cytokines in homogenized lungs at day 2 after infection. The majority of cytokines generally associated with responses to influenza infection, including IFN- γ , IL-2, IL-1 β , KC, GM-CSF, and IP-10, exhibited no differences between wild-type and *Tcrd*^{-/-} neonatal lungs at day 2 after infection, suggesting that traditional innate and Type 1 responses are not dependent on $\gamma\delta$ T cells (**Figure 3-7C**). In contrast, we found that loss of $\gamma\delta$ T cells led to diminished levels of IL-33 (**Figures 3-6F and 3-7D**). To confirm this observation, we again assayed IL-33 to determine if $\gamma\delta$ T cell-derived IL-17A was sufficient for inducing IL-33 in $\gamma\delta$ T cell-deficient mice treated with IL-17A. Compared to virus-only controls, IL-33 levels in whole lung homogenates were elevated in response to low-dose IL-17A (**Figure 3-6G**). These data suggest that IL-33 production is upregulated downstream of IL-17A, which is secreted by $\gamma\delta$ T cells in influenza virus-infected mice. In order to determine if IL-33 production functions as a component of the protection provided by $\gamma\delta$ T cells and IL-17A in influenza-infected neonates, we next infected wild-type and *Il33*^{-/-} deficient neonates with influenza virus. *Il33*^{-/-} neonates exhibited increased mortality compared to wild-type littermates (**Figure 3-6H**). In addition, and consistent with our IL-17A results, the administration of IL-33 also improved the survival of infected *Tcrd*^{-/-} neonate littermates (**Figure 3-6I**). Therefore, in neonatal mice, $\gamma\delta$ T cells are the primary source of IL-17A, which in turn is necessary for IL-33 production, which itself is protective in these animals.

IL-17A Promotes IL-33 Production in Mouse Lung Epithelial Cells in Synergy with Influenza Virus by Elevating STAT3 Phosphorylation

One study recently demonstrated that IL-17A promotes the exacerbation of IL-33-induced airway hyperresponsiveness in an allergic asthma model in BALB/c mice (Mizutani et al., 2014). However, the precise relationship between IL-17A and IL-33 production remains unclear. Although IL-33 can be produced by multiple cell types, epithelial cells are thought to be the primary source in mouse lungs (Pichery et al., 2012), and we found sorted lung epithelial cells from wild-type neonates to have the highest *Il33*

Figure 3-7. Cytokine Expression and Infected Neonates' Survival with Cytokine Administration.

(A) Survival rate of influenza-infected *Tcrd*^{-/-} neonates administered with of high-dose of recombinant mouse IL-17A (rmIL-17A, navy, 1000pg/mouse, n=12) or PBS control (red, n=21) at the time of infection. (B) Survival rate of influenza-infected wild-type neonates administered with recombinant mouse IL-17A (rmIL-17A, orange, 100pg/mouse, n=10) or PBS control (black, n=8) at the time of infection. (C) Protein levels of IFN- γ , IL-2, IL-1 β , KC, GM-CSF and IP-10 assessed by Milliplex assay and normalized to the total protein in the lungs of infected wild-type (black, n=5) and *Tcrd*^{-/-} (red, n=5) neonates at day 2 after infection. (D) Protein levels of IL-33 assessed by ELISA and normalized to the total protein in the lungs of infected wild-type (black) and *Tcrd*^{-/-} (red) neonates at indicated time points after infection. Samples are pooled from at least two independent trials at each time point, and data are presented as mean $\pm$ SEM. € Relative expression results of *Il33* gene in endothelial cells, epithelial cells, CD11c⁺ cells, and lung fibroblasts isolated from neonatal lungs 2 days after infection (n=3). Data are combined from at least two independent experiments and presented as mean $\pm$ SEM. *p<0.05, **p<0.01, n.s., not significant.

gene expression among other lung stromal cell types at day 2 after influenza infection (**Figure 3-7E**). We consequently set out to determine if IL-17A could directly induce IL-33 production in lung epithelial cells. To test this, we stimulated a mouse lung type 1 epithelial cell line (LET1s) (Rosenberger et al., 2014) *in vitro* using influenza virus and recombinant mouse IL-17A (either individually or in combination). After 24 or 48 hours (hrs) of treatment, the cells in each group were collected to assay IL-33 transcripts and protein with qRT-PCR and immunoblot, respectively. Quantification of the relative expression of *Il33* mRNA showed that administration of either virus or IL-17A could enhance IL-33 production in LET1 cells, but the effect of the virus and IL-17A combination was especially striking (**Figure 3-8A**). Consistent with these findings, substantial IL-33 protein was also detected after treatment with both stimuli by immunoblot (**Figure 3-8B**). Interestingly, IL-17A alone was more potent than virus alone in elevating IL-33 mRNA and protein expression.

We next examined the signaling pathways induced by influenza virus and IL-17A treatment in LET1 cells. Examination of the transcriptional binding sites in the human IL-33 promoter (ENCODE data in UCSC genome browser, Build hg19) identified Signal Transducer and Activator of Transcription 3 (STAT3) as a potential regulator of *Il33* transcription, so we performed a Western blot assay to test whether STAT3 was activated downstream of treatment with either influenza virus or IL-17A. We found that phosphorylated-STAT3 (pSTAT3) was coordinately upregulated with IL-33 (data not shown). To confirm the role of pSTAT3 in IL-33 production, we inhibited the phosphorylation of STAT3 by co-incubating with S3I-201, a well-established small molecule inhibitor that prevents the dimerization and phosphorylation of STAT3 (Siddiquee et al., 2007). Under the same stimulation conditions as above, we compared the relative expression levels of *Il33* among IL-17A- and/or virus-stimulated LET1 cells that were co-stimulated with either DMSO or S3I-201 for 48 hrs. The pSTAT3 inhibitor robustly blocked the upregulation of IL-33 induced by IL-17A, even in the presence of influenza infection and IL-17A co-treatment (**Figure 3-8C**). Consistent with these transcript assays, IL-33 protein abundance was also attenuated after treatment with S3I-201 (**Figure 3-8D**). Thus, our results indicate that IL-17A can directly increase the production of IL-33 in lung epithelial cells downstream of STAT3 phosphorylation.

Deficiency in $\gamma\delta$ T Cells Compromises the Accumulation and Functions of ILC2s and Treg Cells

ILC2s (Monticelli et al., 2011), Treg cells, and Th cells (Arpaia et al., 2015) have been reported to play roles in maintaining tissue homeostasis for influenza-infected adults. Each of these cell types can express the IL-33 receptor (ST2) on their cell surface and can be recruited to the lungs by IL-33. Compared to *Tcrd*^{-/-} lungs, we observed more ILC2s and total Treg cells in wild-type lungs, but comparable amounts of Th cells were present in both mouse strains at day 5 after infection (**Figures 3-9A, B and 3-10A**). ILC2s and Treg cells have both been implicated in the production of Areg, which is important for tissue repair after influenza infection in adult mice (Arpaia et al., 2015;

Figure 3-8. IL-17A Elevates IL-33 Production in Murine Lung Epithelial Cells via STAT3 Phosphorylation.

(A) Relative expression of *I/33* mRNA, as assessed by quantitative real-time PCR, in mouse lung epithelial cells (LET1s) treated with medium (white), A/x31 influenza virus (blue, 3 MOI), rmIL-17A (green, 50ng/ml), or virus+rmIL-17A (red) at 24hrs and 48hrs after treatment. Data are shown as mean $\pm$ SEM and combined from three separate experiments that independently showed the same trend. (B) Immunoblot assay of IL-33 protein in stimulated LET1s for the same conditions as in (A) at 48 hrs after stimulation. (C) Quantitative real-time PCR analysis of *I/33* mRNA in LET1 cells treated as in A and B with and without STAT3 phosphorylation inhibitor S3I-201 (100 μ M in 0.05% DMSO) at 48hrs after treatment. Data are shown as mean $\pm$ SEM and combined from two separate experiments that independently showed the same trend. (D) Immunoblot analysis of IL-33 and phosphorylated STAT3 (pSTAT3) in the LET1 lysates treated as before (A, B, and C). Total STAT3 (tSTAT3) and β -actin are also shown. Immunoblots were repeated twice showing similar results. * $p < 0.05$, **** $p < 0.0001$

Figure 3-9. Deficiency in $\gamma\delta$ T Cells Results in Decreased Accumulation and Functionality of ILC2s and Treg Cells.

(A) Representative flow cytometric plots (left) and cell number (right) of ILC2s (CD4⁻ CD90.2⁺ Lin⁻ ST2⁺) in the lungs of infected wild-type (black, n=14) and *Tcrd*^{-/-} (red, n=10) neonates at day 5 following infection. (B) Representative flow cytometric plots (left) and cell number (right) of Treg cells (CD4⁺ Foxp3⁺) and Th cells (CD4⁺ Foxp3⁻) in the lungs of infected wild-type (black, n=14) and *Tcrd*^{-/-} (red, n=10) neonates at day 5 following infection. (C) Representative flow cytometric plots of Areg-producing cells gated on ILC2s and Treg cells in the lungs of infected wild-type (black, n=14) and *Tcrd*^{-/-} (red, n=10) neonates at 5 days following infection. (D) Frequency (left) and cell number (right) of Areg-producing ILC2s, Treg cells, and Th cells in the lungs of infected wild-type (black, n=14) and *Tcrd*^{-/-} (red, n=10) neonates at 5 days after infection. (E) Representative flow cytometric plots of Areg-producing cells gated on ILC2s and Treg cells in the lungs of infected wild-type (black, n=8) and *Il33*^{-/-} (blue, n=6) neonates at 5 days following infection. (F) Frequency (left) and cell number (right) of Areg-producing ILC2s, Treg cells, and Th cells in the lungs of infected wild-type (black, n=8) and *Il33*^{-/-} (blue, n=6) neonates at 5 days after infection. (G) Relative expression levels of *Areg* of ILC2 cells, ST2⁺CD4⁺ cells, and ST2⁻CD4⁺ cells sorted from wild-type (black, n=5) and *Tcrd*^{-/-} (red, n=5) neonatal lungs at 5 days after infection measured by quantitative real-time PCR. (H) Protein levels of Areg in whole-lung lysate from infected wild-type (black, n=6) and *Tcrd*^{-/-} (red, n=6) neonates at 5 days after infection. (I) Survival rate of infected wild-type neonates that were injected intraperitoneally at days 4, 6, and 9 (shown by arrows) after influenza virus infection with Areg neutralizing antibody (purple, α -Areg antibody, 2ug/mouse/timepoint, n=21) or isotype control antibody (black, normal goat IgG control, 2ug/mouse/time point, n=20). Data are combined from at least 2 independent experiments. *p<0.05, **p<0.01, ***p<0.001, n.s., not significant.

Figure 3-10. Areg Expression Dynamics During Neonatal Influenza Infection.

(A) Cell number of ILC2s, Treg cells, and Th cells in the lungs of wild-type (black) and *Tcrd*^{-/-} (red) influenza-infected neonates at various time point after infection. Data are combined from at least two individual experiments at each time point and shown as mean $\pm$ SEM. (B) Abundance of Areg protein was assessed by ELISA and normalized to the total protein in the lungs of wild-type (black) and *Tcrd*^{-/-} (red) influenza-infected neonates at different time points. Samples are pooled from at least two individual experiments at each time point, and data are shown as mean $\pm$ SEM. (C) Representative flow cytometric plots of Areg-producing Th cells in wild-type and *Tcrd*^{-/-} lungs at day 5 following infection. (D) Frequency of Areg-producing neutrophils in influenza-infected wild-type (n=7) and *Tcrd*^{-/-} (n=6) lungs at day 5 after infection. Data are combined from two individual experiments and shown as mean $\pm$ SEM. € Relative gene expression of *Il5*, *Il10*, *Gata3*, *Rorc*, *Stat3*, *Tbx21* and *Foxp3* in ILC2s, ST2⁺CD4⁺ cells and ST2⁻CD4⁺ cells sorted from wild-type (n=5) and *Tcrd*^{-/-} (n=5) lungs at day 5 after infection. Samples are pooled from two independent experiments and data are presented as mean $\pm$ SEM.

*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n.s., not significant.

Monticelli et al., 2011; Zaiss et al., 2015). The assessment of Areg levels in total wild-type lung homogenates showed that the peak of Areg production was at day 5 after infection (**Figure 3-10B**). Thus, we measured the amount of Areg-producing ILC2s, Treg cells, and Th cells in infected wild-type and *Tcrd*^{-/-} neonates at this time point. We found higher frequencies and numbers of Areg-producing ILC2s and Treg cells in wild-type, infected lungs compared to those from *TCRδ*-deficient mice (**Figure 3-9C, D**). In contrast, Th cell responses were equivalent between the two groups at day 5 after infection (**Figures 3-9D and 3-10C**). In addition, neutrophils accumulating in the infected lungs rarely produced Areg (**Figure 3-10D**). Consistent with the importance of IL-33 in regulating type 2 responses, the frequency and number of Areg-producing ILC2s were also higher in wild-type neonates compared to *Il33*^{-/-} animals (**Figure 3-9E, F**). In order to characterize the Areg-producing cells more thoroughly, we isolated ILC2s, ST2⁺CD4⁺ cells, and ST2⁻CD4⁺ cells from wild-type and *Tcrd*^{-/-} neonatal lungs at day 5 after infection and performed qRT-PCR assays to measure gene expression of multiple signature genes. Although many factors remained unchanged, cells from wild-type mice exhibited higher levels of *Areg* and *Il10*, while cells from *Tcrd*^{-/-} had elevations in type 2 response genes, including *Il5* and *Gata3* (**Figures 3-9G and 3-10E, F**). The consistently lower *Areg* expression in cells from *Tcrd*^{-/-} mice strongly suggests that the deficiency in $\gamma\delta$ T cells compromises tissue repair after infection (**Figure 3-9G**).

In order to investigate the importance of Areg in the neonatal influenza model, we next assessed the levels of Areg by ELISA. The overall abundance of Areg protein was lower in *Tcrd*^{-/-} whole-lungs relative to wild-type lungs (**Figure 3-9H**), and this deficiency is likely implicated in the reduced survival rate observed in *Tcrd*^{-/-} mice after infection (**Figure 3-1D**). Based on the kinetics of Areg production (**Figure 3-10B**), we also delivered Areg neutralizing antibody or isotype control antibody intraperitoneally to wild-type mice on Days 4, 6, and 9 after infection. After monitoring the survival of these treated mice, we found that Areg is protective during neonatal influenza infection (**Figure 3-9I**) in wild-type animals. Taken together, these results suggest that neonatal $\gamma\delta$ T cells benefit neonatal mice during influenza infection specifically by mediating Areg production via ILC2s and Treg cells.

IL-17A, IL-33, and Areg Are Correlated with Each Other and with Improved Outcomes in Influenza-Infected Human Children

To determine whether the findings in our neonatal influenza mouse model were translatable to humans, we assessed the abundance of IL-17A, IL-33, and Areg in nasal aspirates obtained from children and infants with community-acquired influenza infection. We found that IL-33 levels were significantly and positively correlated with IL-17A levels in the airways of influenza-infected subjects (**Figure 3-11A**). However, IL-33 was not correlated with IFN- γ (**Figure 3-11B**), suggesting that IL-33 production is not controlled by IFN- γ in this context and that all cytokine levels were not generally correlated. We also found a positive correlation between IL-33 and Areg (**Figure 3-11C**).

Figure 3-11. The IL-17A/IL-33/Areg Cascade in Influenza-Infected Children Is Associated with Robust Disease Outcome.

(A-C) Correlation between concentrations of human IL-17A and IL-33 (A), IFN- γ and IL-33 (B) and IL-33 and Areg protein levels (C) in the nasal aspirates of influenza-infected children (< 8 years old, n=51). (D) Concentration of IL-17A at Day 0 after enrollment in the nasal aspirates of children with mild (black, n=11) and severe (red, n=12) influenza disease outcomes. (E) Concentration of IFN- γ at Day 0 after enrollment in the nasal aspirates of children with mild (black, n=11) and severe (red, n=12) disease outcomes. Data are presented as mean $\pm$ SEM, and in each case cytokine values (pg/ml) were log₁₀ transformed for visualization only. *p<0.05.

However, IFN- γ and Areg (**Figure 3-12A**) were also correlated, possibly indicating the existence of independent pathways associated with Areg-production in human infection, or a non-mechanistic correlation between IFN- γ and Areg kinetics.

In searching for correlates of disease outcome, we found that IL-17A concentrations at diagnosis (within 96 hours of symptom onset) in nasal aspirates predicted the severity of influenza illness in infants and children, as those with mild disease outcomes had significantly higher levels of IL-17A than those with severe outcomes (**Figure 3-11D**). In contrast, IFN- γ was neither correlated with IL-17A (**Figure 3-12B**) nor associated with disease severity (**Figure 3-11E**).

To confirm our observation in mice that IL-17A can directly induce IL-33 production by lung epithelial cells, we stimulated cells from a human lung epithelial cell line (A549) with virus and/or recombinant human IL-17A together with either DMSO or the STAT3 inhibitor S3I-201 (as above). qRT-PCR analysis of these treatments indicated that, as in mice, human IL-17A can enhance the gene expression of *IL33* in lung epithelial cells in a STAT3 phosphorylation-dependent manner during influenza infection (**Figure 3-12C**). In summary, IL-17A (rather than IFN- γ) is positively associated with the IL-33-Areg axis and potentially influences the disease outcomes in children with influenza infections.

Discussion

In comparison to older children and young adults, infant influenza infections are associated with higher rates of hospitalization and mortality. In the human FLU09 cohort, we previously reported that viral clearance was not impaired in infants compared to other demographic groups, and yet disease severity was increased (Oshansky et al., 2014). Intriguingly, previous reports have also shown that neonates cannot mount robust type 1 immune responses, as revealed by the insufficient magnitude and function of neonatal CD8⁺ T cells (You et al., 2008). Instead, type 2 immune responses are more dominant in the neonatal immune system (Adkins et al., 2004; Dowling and Levy, 2014; Garcia et al., 2000), perhaps as a mechanism limiting potential inflammatory damage. As a result of the variations in the immune system evident throughout ontogeny, the parameters that determine mild or severe outcomes after influenza infection likely differ between infants and adults.

During the development of the immune system, $\gamma\delta$ T cells develop and migrate to mucosal organs prior to $\alpha\beta$ T cells, suggesting that the former may play a critical role in immune competence in neonates (Chien et al., 2014; Hayday, 2000; Vantourout and Hayday, 2013). Here, we confirmed that notion by defining a significant role for $\gamma\delta$ T cells in maintaining lung homeostasis and tissue integrity during neonatal influenza infection.

The primary function of $\gamma\delta$ T cells in this model of influenza infection appears to be the rapid production of IL-17A to initiate a cascade of type 2 and tissue repair F 3-12

Figure 3-12. Correlation of IFN- γ - Areg and IFN- γ - IL-17A in Children, and *IL33* Gene Expression in Stimulated A549 Cells.

(A) Correlation between concentration of human IFN- γ (x-axis) and Areg (y-axis) in influenza-infected children (n=51). Cytokine values (pg/ml) were log₁₀ transformed for visualization. (B) Correlation between concentration of human IFN- γ (x-axis) and IL-17A (y-axis) in influenza-infected children (n=51). Cytokine values (pg/ml) were log₁₀ transformed for visualization. (C) Relative expression of *IL33* transcripts in human lung epithelial cells (A549) treated with DMSO or the pSTAT3 inhibitor and stimulated for 48 hours with medium, influenza virus, rmIL-17A, or the virus-rmIL-17A combination. Data are shown as mean $\pm$ SEM and combined from three separate experiments that independently showed the same trend. * $p < 0.05$, ** $p < 0.01$.

responses, which were characterized by the induction of IL-33, the recruitment of ILC2s and Treg cells, and the production of amphiregulin. However, the ligands for $\gamma\delta$ T cell activation in this system are undetermined. This unconventional role for IL-17A diverges significantly from its better characterized contributions to type 3 immunity, for instance in responses to lung infections by bacteria such as *Staphylococcus aureus* (Cheng et al., 2012) and *Mycobacterium tuberculosis* (Lockhart et al., 2006). Thus far, little has been discovered about the potential role of IL-17A in type 2 immunity, which predominates during allergic reactions and helminth infections rather than bacterial infections (Spellberg and Edwards, 2001). One study has suggested IL-17A-producing $\gamma\delta$ T cells can modulate regulatory T cell homeostasis and thermogenesis in adipose tissues by inducing IL-33 production (Kohlgruber et al., 2018). Another study has reported an exacerbation of IL-33 production after IL-17A administration, but this was in a model of allergic asthma, where the effects of IL-17A were primarily pathological (Mizutani et al., 2014). Although IL-17A and influenza virus both induce robust IL-33 production, IL-17A is more potent than virus in upregulating IL-33 expression in lung epithelial cells through STAT3 phosphorylation. Recent studies also found that IL-17A can promote tumor growth (Wang et al., 2009) and angiogenesis (Pan et al., 2015) via STAT3 phosphorylation, suggesting that this may be a common potential target for IL-17A signaling, with effects on tissue repair and remodeling. In contrast, canonical IL-17A signaling proceeds through the nuclear factor- κ B (NF- κ B) pathway mediated by ACT1 (Gaffen, 2009), which results in the activation of the type 3 immune response traditionally associated with IL-17A. In LET1 cells we found that NF- κ B activation (data not shown) was dispensable for IL-33 induction.

To confirm the specific role of IL-17A produced by $\gamma\delta$ T cells in protection, we performed a $\gamma\delta$ T cell-transfer experiment from IL-17A wild-type or deficient mice. Our results showed a significant difference in the survival between infected *Tcrd*^{-/-} neonates receiving wild-type $\gamma\delta$ T cells and those receiving *Il17a*^{-/-} $\gamma\delta$ T cells. However, we also observed a delayed, but not significantly different, survival among *Tcrd*^{-/-} neonates with *Il17a*^{-/-} $\gamma\delta$ T cells, compared to *Tcrd*^{-/-} neonates receiving a PBS control. Thus, we speculate there may be some small additional potential IL-17A-independent effects of neonatal $\gamma\delta$ T cells. Transfer of $\gamma\delta$ T cells into mice lacking both IL-33 and $\gamma\delta$ T cells would confirm the requirement for an intact IL-33 pathway downstream of $\gamma\delta$ T cell activity and will be the subject of future experiments in the lab.

Given the important role of type 2 immunity in mediating allergy and asthma, as well as the epidemiological linkages between infant infections and future development of these diseases, it seems possible that this IL-17A-IL-33 pathway may be a key link between early infection and atopy. One hypothesis for why this pathway may be more active or essential in neonates than in adults, where the effects of IL-17A appear minimal, is the significantly smaller lung volumes in the very young. Lung damage resulting from infection can, as a proportion of total lung volume, generate a greater impact on lung efficiency. Thus, robust induction of type 2 immunity and its associated tissue repair function may be necessary to maintain the capacity of the infant lung, albeit at the cost of an increased risk of subsequent pathologies.

Of particular interest, we observed that IL-17A, instead of IFN- γ , is positively correlated with IL-33 in nasal aspirate samples from children with naturally-acquired influenza infection. This correlation also extends to IL-33 and Areg in these samples, consistent with the findings in our mouse model demonstrating an early regulatory role of IL-17A for the subsequent immune response to neonatal influenza infection.

Interestingly, this is thus far the only protective association with an increased cytokine concentration that we have observed in this cohort. In contrast, type 1 immune responses were not associated with IL-33. These data indicate the novel IL-17A-IL-33-Areg axis may serve as a potential therapeutic target during infant influenza infection; further study of these markers across additional human cohorts will be important not only for verifying the importance of this axis in infant influenza infection, but also for fully elucidating the particular conditions underlying this aspect of $\gamma\delta$ T cell function in humans in general.

In conclusion, our findings provide evidence that IL-17A, secreted by $\gamma\delta$ T cells, has a critical function in protecting infants during influenza infection. IL-17A-producing $\gamma\delta$ T cells may provide this protection by inducing IL-33 secretion and type 2 immune responses which, in turn promote tissue homeostasis and integrity.

CHAPTER 4. RAPID CLONING, EXPRESSION, AND FUNCTIONAL CHARACTERIZATION OF PAIRED $\alpha\beta$ AND $\gamma\delta$ T-CELL RECEPTOR CHAINS FROM SINGLE-CELL ANALYSIS⁵

Introduction

T cells play a vital role in the control of viral infections and tumors. T cells are activated by antigen presenting cells via interactions between peptide-major histocompatibility complex (pMHC) and TCRs. This interaction can induce proliferation and the development of effector functions, including cytokine production and cytotoxic activity. T cells can also infiltrate infected or transformed tissues, e.g. as tumor-infiltrating lymphocytes (TILs), to perform these effector functions (Restifo et al., 2012; Rosenberg and Restifo, 2015). However, in some chronic viral infections and tumors, responding effector T cells progressively get exhausted and become dysfunctional (Baitsch et al., 2011; Wherry and Kurachi, 2015; Wherry et al., 2007). In addition, control of tumors and/or infection may require large numbers of highly reactive lymphocytes that cannot be achieved due to normal tolerance mechanisms. One effective method to overcome this barrier is the use of therapeutic adoptive transfer of lymphocytes (Besser et al., 2013; Klaver et al., 2015; Rosenberg and Restifo, 2015).

Adoptive transfer of lymphocytes such as in vitro expanded or TCR-engineered antigen specific T cells has been successfully used to control viruses and tumors in patients (Besser et al., 2013; Chapuis et al., 2012; Hinrichs et al., 2009; Kershaw et al., 2006; Lamers et al., 2011). In vitro expansion of viral or tumor-specific T cells require significant time to prepare and the targets are not usually fully characterized. Lymphocytes expressing engineered TCRs and chimeric antigen receptors (CAR) target specific antigens, with CARs recognizing surface antigens through immunoglobulin-type interactions (Han et al., 2013; Ramos and Dotti, 2011) and TCRs recognizing tumor-associated pMHC complexes. CAR therapy directed against surface antigens requires a tumor-associated antigen that can be universally targeted (even on healthy, non-tumor tissue) without significant toxicity. Tumor-specific antigens that are targeted by TCRs represent an attractive alternative that can provide greater specificity and reduce non-tumor associated toxicities (Schmitt et al., 2009; Stauss et al., 2015; Varela-Rohena et al., 2008). Additionally, engineered T cells expressing high-affinity antigen receptors can be conditioned to overcome immune tolerance, which has been a major limitation for immunotherapy (Schmitt et al., 2009, 2015; Varela-Rohena et al., 2008). Apart from the clinical applications, a robust system for the cloning and expression of TCRs is a valuable tool for the investigation of TCR structure and functions (Birnbbaum et al., 2014; Luoma et al., 2013; Vavassori et al., 2013).

⁵ Adapted with open access permission from final submission. Guo X.Z., Dash P., Calverley M., Tomchuck S., Dallas M.H., Thomas P.G. (2016). Rapid cloning, expression, and functional characterization of paired $\alpha\beta$ and $\gamma\delta$ T-cell receptor chains from single-cell analysis. *Mol Ther Methods Clin Dev.* 3:15054. DOI: <https://doi.org/10.1038/mtm.2015.54>.

Techniques to rapidly profile and clone antigen-specific TCRs have improved and shortened the process of TCR-engineered immunotherapy (Kobayashi et al., 2013; Linnemann et al., 2013). These approaches are useful contributions to the field and are able to handle large cell inputs very effectively. However, for certain applications, the reported methods still have some limitations. First, approaches that rely on deep sequencing and cloning of bulk sorted cells can still be limited by target cell numbers. In contrast, single cell approaches can utilize input sizes starting with a single cell but are less efficient at dealing with high cell number inputs (greater than 10,000 cells). As a result, single cell methods are best directed at defined samples such as antigen-specific responses or tissue-associated infiltrating cells. Second, for bulk sorting, pairing of TCR chains requires algorithmic imputation, which can have difficulty dealing with cells expressing two distinct TCR chains of one type (e.g. two TCR α chains), which are quite common. A recently reported algorithm has addressed this concern and efficiently pairs bulk processed TCRs, using barcoded pools of cells (Howie et al., 2015). This method, though, requires relatively large inputs to successfully pair and would likely not be appropriate for very small sample sizes as might be obtained from tissue biopsies or tetramer sorting of small populations.

Third, while currently described methods are able to generate full length receptors either by synthesis or by 5' RACE-associated approaches at the single cell level, these methods often are reported to require expansion of the isolated cells prior to TCR isolation. Lastly, the majority of antiviral and antitumor adoptive therapy has focused on $\alpha\beta$ T cell clones due to their exquisite antigen specificity. However, $\gamma\delta$ T cells have also been shown to mediate antiviral and antitumor effects and are novel candidates for therapeutic development (Perko et al., 2015; Silva-Santos et al., 2015). To date, there is little research about profiling and utilizing the TCR $\gamma\delta$ repertoire for therapeutic purposes, and as such applying $\gamma\delta$ T cells for immunotherapeutic applications may be a promising future approach in conjunction with traditional TCR $\alpha\beta$ techniques. Therefore, it is important to establish a system to define the repertoire and functional activity for $\gamma\delta$ T cells. Additionally, improving efficiencies for cloning $\alpha\beta$ TCRs from single cells may have complementary uses in the lab and in the clinic.

To overcome these limitations, we developed a rapid cloning and expression system for specific TCRs. In conjunction with single cell multiplex PCR techniques for TCR $\alpha\beta$ or TCR $\gamma\delta$ profiling (Dash et al., 2011; Wang et al., 2012), and Gibson Assembly® cloning of synthesized DNA, we were able to rapidly sequence and clone specific TCRs in a retroviral expression vector. Moreover, by generating V region-specific libraries, this protocol can be significantly accelerated by only requiring the substitution of the CDR3 region (Bryksin and Matsumura, 2010), resulting in cloned TCRs in appropriate expression vectors in as little as five days after cell isolation with highly robust, inexpensive methods. Thus, this protocol provides an efficient and relatively high-throughput means for TCR engineering for therapeutic or research applications.

Results

Paired TCR $\gamma\delta$ Analysis of Human PBMC Samples at the Single Cell Level

Following on our previously reported single cell PCR protocols for TCR $\alpha\beta$ (Dash et al., 2011; Wang et al., 2012), we developed a similar strategy to characterize the paired TCR $\gamma\delta$ repertoire in humans (**Figure 4-1A**). The primers were designed for all non-pseudogene TRGV and TRDV regions along with antisense primers for their respective constant regions. Two sets of primers (external and internal) were designed in order to perform a nested PCR (**Tables 2-2 and 2-3**). The PCR products were examined by agarose gel electrophoresis before sequencing (**Figure 4-1B**). The average success rate for obtaining paired CDR3 γ and CDR3 δ sequences at the single cell level from human PBMC samples is $71.25 \pm 18.75\%$. The TRGV/TRDV family usage was determined from the multiplex PCR products (**Figure 4-1C**). On average, 20% of the sequences from our analysis of 14 human PBMCs were TRGV9/TRDV2. This technique along with the established mouse and human $\alpha\beta$ single cell multiplex PCR offers a rapid method (turnaround time ~ 3 days per 160 cells) for characterizing paired TCR $\gamma\delta$ chains at the single cell level. The data of paired TRGV/TRDV family usage percentage in each human sample are shown in **Table 4-1**.

Establishment of Human TCR $\alpha\beta$ and TCR $\gamma\delta$ Retroviral Expression Clones

Many of the downstream applications of paired TCR $\alpha\beta$ or TCR $\gamma\delta$ sequence analysis require cloning and expression of the antigen specific receptors for immunological studies such as structural and functional characterization, biochemical characterization, epitope identification, and gene therapy (Uldrich et al., 2013; Vavassori et al., 2013; Yoshio Ogawa, 2013). Thus, we sought to develop a rapid cloning method to improve on conventional restriction enzyme-mediated ligation techniques, which can be cumbersome and time consuming. In addition, use of restriction enzymes for cloning becomes problematic because of the potential for restriction sites to appear in some variable regions and the non-germline CDR3 sequences of the TCR chains. The vector that we chose for TCR expression is pMCherry, which has been successfully used to construct TCR clones for the generation of retrogenic mice (Bettini et al., 2013; Holst et al., 2006). A schematic diagram of the cloned TCR chains in the pMCherry vector is shown in **Figure 4-2A**.

To clone full length TCR chains, as a proof of principle, we chose a TRGV9/TRDV2 clone to demonstrate the feasibility of our cloning system, since the TRGV9/TRDV2 clonotype is dominant in the TCR $\gamma\delta$ repertoire analysis from human PBMCs (**Figure 4-1C**). Similarly, a human TCR $\alpha\beta$ pair was chosen derived from an influenza-specific CD8 T cell from an infected individual (unpublished data). Using the IMGT-reported human TRGV, TRDV, TRGC, and TRDC sequences for TCR $\gamma\delta$ or human TRAV, TRBV, TRAC and TRBC sequences for TCR $\alpha\beta$ and our single cell

Figure 4-1. Unbiased Single-Cell Amplification of Paired TCR CDR3 Regions.

(A) Overview of the multiplex PCR protocol to amplify and sequence paired TCR CDR3 α/γ and CDR3 β/δ . After sorting single human $\alpha\beta$ or $\gamma\delta$ T cells into a 96-well plate, reverse transcription is performed to obtain single-cell cDNA. Taking human $\gamma\delta$ T cells as an example, a first round of PCR is performed by using an external primer mixture of 9 TRGV and 8 TRDV sense and single TRGC and TRDC antisense primers following RT-PCR. The first-round PCR products are subjected to two separate second-round PCRs using a corresponding internal primers mix (9 sense TRGV, single antisense TRGC, and 8 sense TRDV, single antisense TRDC, respectively). The timeline of this process is shown on the left. (B) An agarose gel electrophoresis image of TCR segments containing CDR3 γ and CDR3 δ is shown. Paired CDR3 γ and CDR3 δ products from the same cell were loaded in adjacent lanes. Negative control PCR reactions are shown in the boxed region and in the ladder lane, a 500bp label is shown. (C) Paired TRGV/TRDV usage is determined by multiplex PCR and sequencing (n=14 human apheresis rings). The percentage of different TRGV/TRDV usage in each sample was assessed (mean $\pm$ SEM).

A

Characterizing the TCR $\alpha\beta/\gamma\delta$ repertoire

B

C

Table 4-1. TRGV/TRDV Repertoire Among 14 Human Samples.

TRGV/TRDV Usage	Mean	Standard Deviation	Standard Error
TRGV9/TRDV2	20.06	16.11	4.306
TRGV4/TRDV1	12.71	15	4.008
TRGV8/TRDV1	8.714	10.63	2.842
TRGV9/TRDV3	8.357	21.25	5.68
TRGV2/TRDV1	7.836	14.61	3.906
TRGV1/TRDV2	4.643	13.51	3.61
TRGV3/TRDV3	4.35	13.09	3.498
TRGV9/TRDV1	4.279	8.702	2.326
TRGV4/TRDV3	4.071	9.059	2.421
TRGV3/TRDV1	4.029	5.646	1.509
TRGV2/TRDV3	3.071	8.801	2.352
TRGV5/TRDV3	2.643	6.744	1.802
TRGV8/TRDV2	1.971	5.388	1.44
TRGV5/TRDV1	1.871	3.633	0.9711
TRGV2/TRDV2	1.871	3.633	0.9711
TRGV8/TRDV3	1.643	4.557	1.218
TRGV2/TRDV5/TRAV29	1.492	4.706	1.305
TRGV3/TRDV2	1.279	2.922	0.7808
TRGV10/TRDV1	1.071	2.31	0.6175
TRGV9/TRDV8/TRAV38	0.3571	1.336	0.3571
TRGV4/TRDV2	0.1857	0.6949	0.1857
TRGV9/TRDV5/TRAV29	0.1714	0.6414	0.1714

Figure 4-2. Rapid Cloning and Expression of Human TCR $\alpha\beta$ or TCR $\gamma\delta$ in a Retroviral Vector.

(A) Schematic diagram of TCR cloning using gBlock[®] synthesized DNA fragments and a linearized retroviral vector (pMCherry) is shown. Family specific TRGV and TRDV full length TCR chains were synthesized with a 15-20bp overlap sequence (purple) in the 2A region (pink). Together with a linearized pMCherry expression vector, a three-way ligation is performed by using Gibson Assembly[®] Cloning. The timeline of this process is presented on the left. (B) Expression of TCR constructs in the Jurkat 76 TCR $\alpha\beta$ cell line. The vectors with human TRGV9/TRDV2 TCR genes (*top panel*) and human influenza-specific TCR $\alpha\beta$ genes (*bottom panel*) were co-transfected with the human CD3 construct into the Jurkat 76 TCR $\alpha\beta$ cell line. The flow cytometry results of transfected cells are shown.

CDR3 γ and δ or CDR3 α and β sequence data we constructed full length TCR γ and δ chains and TCR α and β chains joined by the 2A “self-cleaving” site in silico. 2A oligopeptides can interact with the ribosomal exit tunnel to terminate sequence translation at the final codon (Pro) of the 2A sequence, and reinitiate translation of the following sequence (Sharma et al., 2012). Recently, multi-cistronic 2A based retroviral vectors have been widely used for TCR:CD3 structural and functional studies (Bartok et al., 2010; Bettini et al., 2012, 2013; Day et al., 2011; Holst et al., 2006; Szymczak et al., 2004). The entire sequence of TCR γ -2A-TCR δ along with an 25bp overhang complementary to the ends of the linearized pMCherry vector were synthesized in two fragments of approximately 1kb each as gBlock® DNA fragments (IDT) with an internal 25bp overlap in the 2A segment. By using Gibson Assembly® Master Mix, we ligated the two gBlocks spanning the TCR γ -2A-TCR δ with the linearized vector in a three-way ligation. The process of cloning is shown in **Figure 4-2A**. After this cloning procedure, an average of 70.9% of the colonies picked after transformation were entirely matched with target sequences. The others contained either point mutations resulting from the cloning process or no inserts. To date, we have cloned more than 30 different TCR constructs by using this system, including, mouse and human TCR $\alpha\beta$ and TCR $\gamma\delta$. The cloning system is highly reproducible and we have succeeded in generating clones in all attempts.

To test the functionality of the TCR clones that were made following the method described in **Figure 4-2A**, we transfected the human TRGV9/TRDV2 construct into the Jurkat 76 TCR $\alpha\beta$ ⁻ cell line and checked for the cell surface expression by anti-TCR $\gamma\delta$ and anti-CD3 antibody staining and flow cytometry. Although Jurkat cells have endogenous CD3, the expression of TCR $\gamma\delta$ was not robust. Since $\gamma\delta$ T cells do not develop in CD3-deficient mice and patients (Siegers et al., 2007), we cloned the human CD3 complex into an MSCV vector (pMIAMetrine) and co-transfected it with our human TCR constructs. mCherry and ametrine are the reporter genes in the pMCherry vector with human TCR genes and the pMIAMetrine vector with human CD3 genes, respectively. We demonstrated that co-transfection of the human CD3 construct with the human TCR $\gamma\delta$ and $\alpha\beta$ chains can improve the surface expression level of TCR in a Jurkat cell line (**Figure 4-3A through C**). 3.76% of cells were double positive for mCherry and ametrine, and 19.5% of double-positive cells were TCR $\gamma\delta$ and CD3 positive, which proved the functionality of our cloning and expression platform (top panel, **Figure 4-2B**). We analyzed the expression of the influenza virus-specific TCR $\alpha\beta$ by staining the transfected cells with APC-conjugated influenza-M1 tetramer (HLA-A*0201, GILGFVFTL) and CD3 antibody. The FACS plot shows 5.03% of transfected cells were double positive for mCherry and ametrine, 16.1% of which were positive for tetramer staining (bottom panel, **Figure 4-2B**).

Effective TCR Activation Reporting by Nur77-GFP Jurkat 76 TCR $\alpha\beta$ ⁻ Cells

An important application of TCR cloning and expression is to screen molecules that can activate or inhibit TCR signaling. Thus far, the common methods to detect TCR

Figure 4-3. Co-Transfection of Human CD3 Can Improve the Expression of Human TCR Constructs.

(A-B) Comparison of single transfection of human TCR constructs and co-transfection of human TCR constructs and human CD3. (C) Quantification of mCherry/ameitrine and TCR/CD3 expression is shown. Statistical differences were determined by One-way ANOVA; $p < 0.05$ was considered statistically significant. Data are mean $\pm$ SEM of two independent experiments *** $p < 0.001$, **** $p < 0.0001$.

activation are using ELISA to detect cytokines (e.g. IFN- γ) in the cell culture medium (Kobayashi et al., 2013; Linnemann et al., 2013), intracellular staining to report cytokine production by flow cytometry, or qRT-PCR to quantify the mRNA expression of cytokines, which are time-consuming, labor-intensive, and expensive. Hence, we established a TCR activation reporter cell line, *Nur77*-GFP Jurkat 76 TCR $\alpha\beta^-$ (NJ76 cells). The *Nur77*-GFP reporting system has been demonstrated to reflect specific TCR signal strength by GFP expression (Au-Yeung et al., 2014; Gao et al., 2014; Moran et al., 2011). Here, we established the NJ76 cell line by stably transducing *Nur77*-GFP BAC DNA into Jurkat 76 TCR $\alpha\beta^-$ cells.

To test the functionality of NJ76 cells in reporting TCR activation, we transfected NJ76 cells with a murine K^bPB1₇₀₃-specific TCR $\alpha\beta$ derived from influenza-infected mice along with mouse CD3. The K^bPB1₇₀₃+TCR $\alpha\beta$ + NJ76 cells (PB1-NJ76) were incubated with mouse splenocytes, the influenza PB1₇₀₃ peptide, splenocytes and peptide, or mouse α -CD3/human α -CD28 as a positive control for 4 hours and GFP expression in transfected NJ76 cells was detected by flow cytometry (**Figure 4-4A**). The quantification of GFP levels is shown in **Figure 4-4B**. The results show that PB1-NJ76 cells can robustly express GFP after specific peptide stimulation (PB1) with antigen-presenting cells. The gating strategy for GFP detection is shown in **Figure 4-5**.

This TCR-activation reporting system has also been tested for TCR $\gamma\delta$ signaling. Zoledronic acid (Zometa, Novartis, Basel, Switzerland) is an aminobisphosphonate that has demonstrated antitumor effects via inhibition of tumor growth and angiogenesis and induction of malignant cell apoptosis in humans (Gnant and Clézardin, 2012; Peng et al., 2007). In addition, zoledronic acid can specifically stimulate and expand human TRGV9/TRDV2 cells (Di Carlo et al., 2013; Goto et al., 2013; Sprini et al., 2014; Wada et al., 2014). Since it can result in the accumulation of upstream metabolites in the mevalonate pathway, e.g. IPP, which induce the expansion of $\gamma\delta$ T cells in vitro and in vivo, zoledronic acid pre-treatment can increase the cytotoxicity of some cancer cell lines by $\gamma\delta$ T cells (Suzuki et al., 2010). After transfection of the human TRGV9/TRDV2 vector and human CD3 vector into NJ76 cells, we pulsed the transfected TRGV9/TRDV2-NJ76 cells with 50 μ g/ml of zoledronic acid for 3 hours, and washed and incubated the cells at 37°C for 12h. We quantified the GFP expression level in transfected TRGV9/TRDV2-NJ76 cells and control cells by flow cytometry. The *Nur77*-GFP expression level is shown in **Figure 4-4C, top**. The transfected TRGV9/TRDV2-NJ76 cells showed a significantly higher level of GFP expression, which demonstrates that zoledronic acid can trigger $\gamma\delta$ TCR signaling for the stimulation and expansion of $\gamma\delta$ T cells. The fold change of GFP expression over time in stimulated TRGV9/TRDV2-NJ76 cells (Red line) and non-stimulated TRGV9/TRDV2-NJ76 (Black line) is shown in **Figure 4-4C, bottom**.

Rapid TCR Cloning by CDR3 Substitution Using Overlap Extension PCR and TCR Library

For TCRs, the only hypervariable regions are the CDR3 regions. Thus, cloning full length TCRs de novo for each application may expend unnecessary resources. To

Figure 4-4. *Nur77*-GFP Jurkat 76 TCRαβ⁺ Cells Can Report the TCR Signaling Activation.

(A) Following co-transfection of a murine K^bPB1₇₀₃-specific TCR αβ derived from influenza-infected mice and a mouse CD3 construct, the K^bPB1₇₀₃⁺TCRαβ⁺ NJ76 cells (PB1-NJ76) were stimulated either with influenza PB1₇₀₃ peptide alone or PB1₇₀₃ peptide-pulsed splenocytes for 4 hrs. Anti-mouse CD3/α-human CD28 stimulation was also done as a positive control. The GFP expression was assessed by flow cytometry. (B) The quantification of GFP expression in PB1-NJ76 cells is shown. Statistical differences were determined by One-way ANOVA. (C) The transfected human TRGV9/TRDV2-NJ76 cells were pulsed with zoledronic acid (50ug/ml) for 3h at 37°C, and washed and incubated at 37°C for 12h. The GFP expression of stimulated NJ76 cells (Grey line), non-stimulated TRGV9/TRDV2-NJ76 (Black line) and stimulated TRGV9/TRDV2-NJ76 cells (Red line) is shown (*top panel*). Fold change of GFP expression in stimulated TRGV9/TRDV2-NJ76 cells (Red line) compared to non-stimulated TRGV9/TRDV2-NJ76 (Black line) is shown as a time course (*bottom panel*). Statistical differences were determined by Two-way ANOVA; $p < 0.05$ was considered statistically significant. Data are mean $\pm$ SEM of two independent experiments. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s. non-significant.

Figure 4-5. Gating Strategy of TCR-Transfected-NJ76 Cells in Flow Cytometry. The data of TCR-transfected-NJ76 cells after stimulation in Figure 3 were analyzed by applying the gating strategy to all the samples. The gating strategy is “Single cell gate – Lymphocytes gate – Singlets gate – mCherry+Ametrine+ gate – TCR+CD3+ gate – GFP+ gate”.

improve on this, we have generated a library of potential TRGV and TRDV “backbone” combinations that only require the swapping of individual CDR3 regions directly from PCR products. For example, in TRGV9/TRDV2 cells from PBMCs of healthy donors, the CDR3s of both γ and δ chains were found to be highly diverse (**Table 4-2**). To rapidly generate a library of diverse TRGV/TRDV clones, we designed DNA linkers, whose ends overlap with the TRGC and TRDV in our single cell PCR products. These DNA linkers contain the TRGC region, 2A and one of the TRDV regions, as is shown in **Table 2-6**. By overlap PCR with the single cell PCR products, DNA linkers, TRGV sense primers, and TRDC antisense primers, we can connect any pair of TCR $\gamma\delta$ together. Next, we use these first-step PCR products as a mega primer with the appropriate clone from our library as a template for the second-step overlap extension PCR. By using this substitution method, we have successfully cloned different $\gamma\delta$ TCRs with matched CDR3s from our human single cell PCR products. In principle, the same approach could be used with $\alpha\beta$ TCRs, although the clone library would be larger. As an estimate, this CDR3 substitution approach can shorten the cloning process to within 5 days (**Figure 4-6**).

Discussion

Here we report several useful techniques for the analysis of TCR biology, including a single-cell based protocol for $\gamma\delta$ TCR amplification, a rapid protocol for TCR cloning and expression, and a novel platform for functional characterization of TCR clones. Our system provides an accurate and efficient method to approach rapid cloning at the single cell level, which can improve the development of multiple applications, including TCR-mediated immunotherapy.

The most prominent recent immunotherapy approaches involve T cell checkpoint blockade inhibitors (Pardoll, 2012). However, these therapies depend on the presence of significant numbers of anti-tumor T cells. The ex vivo expansion of tumor infiltrating lymphocytes has also been successful, but is time consuming (Dudley et al., 2003). Our protocol could significantly accelerate the amount of time needed to generate large numbers of anti-tumor T cells, by allowing the efficient transduction of identified anti-tumor receptors. The key to the application of this form of directed T cell immunotherapy is the rapid and accurate isolation and cloning of paired TCRs. Thus far, various methods have been developed for the cloning of TCR genes by traditional PCR, but the acquisition and expression of TCRs is often labor-intensive, time-consuming, expensive, and non-specific. The system we have described provides efficient acquisition of TCR gene products for cloning based on single cell isolation, with an amplification success rate of isolated paired single cell TCR γ and TCR δ CDR3 products of $71.25 \pm 18.75\%$ based on total sorted single cells in each sample. We have also developed a platform for screening TCR activation after cloning. By inserting the *Nur77* reporter into a Jurkat 76 TCR $\alpha\beta$ cell line, we have generated a useful system for monitoring specific TCR activation, as demonstrated by stimulation of PB1-NJ76 by its cognate influenza-derived peptide and stimulation of TRGV9/TRDV2 T cells with zoledronic acid (Zometa).

Table 4-2. CDR3 Amino Acid Sequences of Paired Human TRGV9/TRDV2 Cells Isolated from PBMCs (n=14).

Paired Amino Acid Sequence in TRGV9-CDR3 Region	Paired Amino Acid Sequence in TRDV2-CDR3 Region	Frequency
ALFIQELGKKIKV	ACDVLGDTEGRLI	2
ALWDGPYYKKL	ACDTVFTGGYSSWDTRQMF	2
ALWDIPPGQELGKKIKV	ACDTLGETSSWDTRQMF	2
ALWEAQELGKKIKV	ACDSGGYSSWDTRQMF	2
ALWEARQELGKKIKV	ACDTLFPGGSATDKLI	2
ALWEGTRGQELGKKIKV	ACDTVGAHTDKLI	2
ALWEVGDQELGKKIKV	ACDPLNTGGSFSLYTDKLI	2
ALWEVHSELGKKIKV	ACDTGGFRSSWDTRQMF	2
ALWEVHSELGKKIKV	ACDTGGFRSSWDTRQMF	2
ALWEVLELGKKIKV	ACDTVGMGIRLGDKLI	2
ALWEVLVGELGKKIKV	ACDILGINTDKLI	2
ALWEVPELGKKIKV	ACERLGDYVPDKLI	2
ALWEVQELGKKIKV	ACDRLLGDTDKLI	2
ALWEVQELGKKIKV	ACDTVAPRIGGLKYTDKLI	2
ALWEVQELGKKIKV	ACDTVGGPYTDKLI	2
ALWEVQELGKKIKV	ACDTVGGTAQ	2
ALWEVQELGKKIKV	ACDTVSGGSTPTWYTDKLI	2
ALWEVQELGKKIKV	ACDTVSIFTGDTTDKLI	2
ALWEVRELGKKIKV	ACDTILIFSPTGGDTDKLI	2
ALWEVRELGKKIKV	ACVPLGDWTDKLI	2

Table 4-2. Continued.

Paired Amino Acid Sequence in TRGV9-CDR3 Region	Paired Amino Acid Sequence in TRDV2-CDR3 Region	Frequency
ALWEVRKQELGKKIKV	ACDTLGDDFDKLI	2
ALWEVTHNRQELGKKIKV	ACDTLLGTEAWDTRQMF	2
ALWGGAAGAYYKKL	ACDGKTTDTDKLI	2
ALWGGEELGKKIKV	ACDLLGDTRYTDKLI	2
ALWVQELGKKIKV	ACVGITGDTDKLI	2
ALWEAHQELGKKIKV	ACDSLGDSDVKLI	1
ALWEANKKL	ACDLLRGAGGQIDKLI	1
ALWEAQELGKKIKV	ACDTVGGAFDTDKLI	1
ALWEATGLGKKIKV	ACDMGDTRSWDTRQMF	1
ALWEDLELGKKIKV	ACDTVSWGKNTDKLI	1
ALWEKEELGKKIKV	ACDTGDWGSSWDTRQMF	1
ALWEKELGKKIKV	ACDILDSTGGTDLTAQLF	1
ALWEMTQELGKKIKV	ACDTVRNTGGYAFAGIDKLI	1
ALWEPQELGKKIKV	ACDKVLGDSSWDTRQMF	1
ALWESKELGKKIKV	ACEGLGATQSSWDTRQMF	1
ALWEVGELGKKIKV	ACDKLLGDNELI	1
ALWEVHKLGGKIKV	ACDSLLGKGTDKLI	1
ALWEVKELGKKIKV	ACDTLRGSADKLI	1
ALWEVLQQELGKKIKV	ACDTPARHTDKLI	1
ALWEVPVLGGKIKV	ACDTADRSSYTDKLI	1
ALWEVQELGKKIKV	ACDTLLGDPSSSWDTRQMF	1

Table 4-2. Continued.

Paired Amino Acid Sequence in TRGV9-CDR3 Region	Paired Amino Acid Sequence in TRDV2-CDR3 Region	Frequency
ALWEVQELGKKIKV	ACDTLSGGYARTDKLI	1
ALWEVQELGKKIKV	ACDTVGLGDTGLGLI	1
ALWEVRELGKKIKV	ACDTIVSGYDGYDKLI	1
ALWEVRELGKKIKV	ACSILGDKTSDKLI	1
ALWEVRQELGKKIKV	ACDTVSRGGYSDKLI	1
ALWEVRVQELGKKIKV	ACDPLERVGGPANTDKLI	1
ALWEVTELGKKIKV	ACDVLGDTGDDKLI	1
ALWGRELGKKIKV	ACDTVGSNTDKLI	1
ALWVQELGKKIKV	ACDVLGDTEADKLI	1
ALYGSPSGEELGKKNQG	ACDPLEGAGGHNTDKLI	1

Figure 4-6. Schematic Strategy of CDR3 Substitution by Overlap Extension PCR. Based on the library of TCR $\alpha\beta$ and TCR $\gamma\delta$ established by the described cloning platform, the strategy for CDR3 substitution using multiplex single cell PCR products and linker DNA is shown. After the sequence analysis of single-cell PCR, the target pairs of TCRs are chosen from the respective second round paired PCR plates, which include TCR α/γ_m and TCR β/δ_n (m represents a particular TRAV or TRGV subfamily; n represents a particular TRBV or TRDV subfamily). Beforehand, we generated a library of linker DNA by gBlock synthesis (IDT) (Table 2-6). The linker DNA consists of TRAC/TRGC-2A-TRBV n /TRDV n (n represents the TRB/DV subfamily) sequence. Using the single cell PCR products of α/γ and β/δ chains of the desired clonotypes and the relevant linker gBlock DNA, we carried out an overlap PCR with TRAm/GVm internal sense primer and TRB/DC internal antisense primer. The PCR products were visualized on an agarose gel, and subsequently purified to use as “mega-primer” for cloning into the existing construct from our TCR cloning library (pMIC-TCR α/γ_m -TCR β/δ_n) with the same TRGV and TRDV family usage but different CDR3s by overlap extension PCR. The timeline of the whole process is on the left.

Considering the variability of CDR3 sequences and TCR variable regions (approximately 10^{18} combinations in human TCR $\gamma\delta$ cells and 10^{16} combinations for TCR $\alpha\beta$ cells (Chien et al., 2014)) and the complexity of cloning all the different clones de novo, we established a method using overlap extension PCR of a linker molecule with amplified single cell CDR3 products and a constructed $\gamma\delta$ TCR library to rapidly (less than 5 days) generate diverse TCR clones. The applications of this rapid approach for immunotherapy are obvious. Tumor-specific T cells have been characterized by broad non-specific surface phenotypes that can be used to isolate, clone, and express potential tumor-targeted clones (Gros et al., 2014). The recent advancement of tumor sequencing has allowed for identification of tumor neoantigens and overexpressed self-antigens (Downing et al., 2012; Robinson et al., 2012; Zhang et al., 2012a, 2012b). Combining these technologies will allow for promptly characterized and tailored anti-tumor therapy.

T cell transfers have also been used for the treatment of opportunistic infections in immunosuppressed patients, particularly after hematopoietic stem cell transplant. The reactivation of herpes viruses like human cytomegalovirus and Epstein-Barr virus is a clinical dilemma that cannot always be addressed with antivirals (Barrett and Bollard, 2015; Perko et al., 2015; Saglio et al., 2014). Analogous to TIL therapies, ex vivo expansion of antiviral T cell specificities can be clinically useful, but suffers from similar workflow limitations. By generating a library of specific TCR constructs reactive against a range of viruses and HLA types, TCR-directed therapies could be used prophylactically or immediately at the earliest signs of reactivation.

In addition to these therapeutic applications, our protocol significantly improves the workflow for cloning and expressing TCRs for study in vitro. This can include the characterization of biochemical features of the TCR-peptide-MHC interaction, or, in the case where ligands have not been identified, transduced cell lines can be used for the screening of novel antigens. This is particularly useful in the context of $\gamma\delta$ T cells, where very few ligands have been identified and confirmed (Vantourout and Hayday, 2013; Vavassori et al., 2013). The GFP reporter line we have engineered can be used directly in high-throughput screening platforms; alternative reporters (such as luciferase) can be easily substituted as well.

In conclusion, we introduce a novel method to rapidly clone, express and characterize the function of paired $\alpha\beta$ and $\gamma\delta$ TCR chains from single cells. Our platform addresses the non-specific, labor-intensive, and time-consuming issues of traditional PCR-based cloning and it provides a relatively high-throughput, accurate, and efficient method of TCR engineering for therapeutic or research applications.

CHAPTER 5. DISCUSSION

Infants suffer higher rates of hospitalization and mortality after influenza virus infection, compared to young adults. It has been reported that infants cannot mount robust type 1 immune responses, due to the insufficient magnitude and function of neonatal CD8 T cells (You et al., 2008). Therefore, the unique type 2-biased immune system potentially makes the treatment to neonates different from that to adults, perhaps as a mechanism limiting potential inflammatory damage. To further characterize the neonatal immune responses to influenza infection, we found $\gamma\delta$ T cells develop and migrate to mucosal organs prior to $\alpha\beta$ T cells during the immune response, suggesting that the former may play a critical role in immune competence in neonates (Chien et al., 2014; Hayday, 2000; Vantourout and Hayday, 2013). Here, we demonstrated the critical role for $\gamma\delta$ T cells in maintaining lung homeostasis and tissue integrity during neonatal influenza infection.

As reported previously, adult $\gamma\delta$ T cells respond to influenza infections by producing IL-17A and IL-17F to initiate lung inflammation, which is immuno-pathogenic to the infected hosts (Crowe et al., 2009; Li et al., 2012). In contrast, other groups have shown that $\gamma\delta$ T cells can induce robust humoral responses and protect adults from influenza infections (Dong et al., 2018; Wang et al., 2011, 2016). However, in neonates, we found the primary function of $\gamma\delta$ T cells in this model of influenza infection appears to be the rapid production of IL-17A to initiate a cascade of type 2 and tissue repair responses, which were characterized by the induction of IL-33, the recruitment of ILC2s and Treg cells, and the production of amphiregulin for the tissue repair and homeostasis.

To determine if the $\gamma\delta$ T cells can also protect adults, we infected wild-type and *Tcrd*^{-/-} adult mice with H1N1 influenza virus, but we did not observe a significant difference between these two groups in the weight loss and survival. We found that adult $\gamma\delta$ T cells can also produce IL-17A following influenza infections, while the peak of the response is at the relative late stage of the infections (Day 5-8), which may be the reason why it leads to a different disease outcome in adults, compared to the infected neonates. We speculate that the lung environment, biased to type 2 immunity, and the compacted lung structure can cause the different responses from $\gamma\delta$ T cells.

Interestingly, one recent study has also suggested IL-17A-producing $\gamma\delta$ T cells can modulate regulatory T cell homeostasis and thermogenesis in adipose tissues by inducing IL-33 production from fibroblasts, which further confirms the novel IL-17A-IL-33 axis that we identified in our study (Kohlgruber et al., 2018). In addition, another study has reported an exacerbation of IL-33 production after IL-17A administration, but this was in a model of allergic asthma, where the effects of IL-17A were primarily pathological (Mizutani et al., 2014). This unconventional function of IL-17A to induce type 2 immunity, which is commonly considered to regulate type 3 immunity in bacterial infections (Cheng et al., 2012; Lockhart et al., 2006), is perhaps a fine-tuning mechanism from the innate immune cells for balancing pro-inflammatory type 1/17 immunity and anti-inflammatory type 2 immunity for tissue repair and homeostasis.

An important factor previously identified involved in neonatal immunity is the potential effect of environmental variation, such as microbiota (Cicin-Sain et al., 2012). Indeed, in our own experiments we have observed differences in neonates reared by different wild-type and *Tcrd*^{-/-} dams. These observations suggest that $\gamma\delta$ T cells may regulate additional features of immunity via breast milk or some other maternal factor. To avoid such confounding factors in the experiments reported here, all $\gamma\delta$ T cell- and IL-33-deficient mouse studies were performed with heterozygous parents and utilized comparisons of littermate controls.

Although $\gamma\delta$ T cells respond to neonatal influenza infections rapidly, we have not identified any ligands that $\gamma\delta$ T cells can recognize upon the infections. Similar to $\alpha\beta$ TCR, $\gamma\delta$ TCR can be stimulated by the peptides presented by MHC I. Interestingly, it has also been reported that V γ 9V δ 2 cells can recognize phospholipids mediated by butyrophilin 3A1 (BTN3A1), which is a ubiquitously expressed cell surface protein (Willcox and Willcox, 2019). Therefore, identifying the ligands for $\gamma\delta$ T cells can be different from the current methods for $\alpha\beta$ T cells.

In addition, as innate-like lymphocytes, it is potentially intriguing to determine if $\gamma\delta$ T cells have immunological memory. One study has indicated that $\gamma\delta$ T cells can generate a bacteria-specific memory response following *Listeria monocytogenes* (*Lm*) primary and secondary infections (Sheridan et al., 2013). The intestinal $\gamma\delta$ T cells, which are mainly IL-17A and IFN- γ producing cells, exhibit more robust cell expansion and cytokine production after secondary challenge. Therefore, studying if influenza can also induce the generation and functions of memory $\gamma\delta$ T cells and how we can manipulate this population has a great potential in the treatment to infections.

In conclusion, our findings provide evidence that IL-17A, secreted by $\gamma\delta$ T cells, has a critical function in protecting infants during influenza infection. IL-17A-producing $\gamma\delta$ T cells may provide this protection by inducing IL-33 secretion and type 2 immune responses which, in turn promote tissue homeostasis and integrity.

Nevertheless, the ligands for $\gamma\delta$ T cell activation in this system are undetermined. Therefore, we developed techniques for studying TCRs and their ligands, including single-cell based protocol for $\gamma\delta$ TCR amplification, a rapid protocol for TCR cloning and expression, and a novel platform for functional characterization of TCR clones. Our system provides an accurate and efficient method to approach rapid cloning at the single cell level, which can improve the development of multiple applications, including TCR-mediated immunotherapy and high-throughput ligand screening for antigen-specific TCR.

Adoptive cell transfer therapy has been considered as one of the most prominent immunotherapy approaches recently. However, the current protocols for defining paired TCR repertoire and cloning the antigen-specific TCRs are still less efficient, labor-intensive and time-consuming, while the key to the application of T cell immunotherapy is the rapid and accurate isolation and cloning of paired TCRs. Our methods provide

efficient acquisition of TCR gene products for cloning based on single cell isolation (approximately 70-80% of total sorted single cells in each sample).

Following TCR analysis and characterization, the discovery of ligands to the antigen-specific T cells is another important application of our methods. By stably expressing *Nur77*-GFP or *Nur77*-Luciferase in Jurkat 76 TCR $\alpha\beta$ ⁺ cell line, we can identify molecules that specifically trigger TCR signaling based on different TCRs expressed on the surface of the Jurkat cells, as demonstrated by stimulation of PB1-NJ76 by its cognate influenza-derived peptide and stimulation of TRGV9/TRDV2 T cells with zoledronic acid (Zometa). Thus, our methods of determining TCR ligands will provide a powerful tool for therapeutic applications.

In conclusion, this dissertation has suggested a protective role of IL-17A-producing $\gamma\delta$ T cells in neonatal influenza infections via initiating downstream effects on IL-33 induction by lung epithelial cells and further amphiregulin secretion by lung regulatory T cells and group 2 innate lymphoid cells for tissue repair and homeostasis. Additionally, this dissertation introduces a novel platform to rapidly clone, express and characterize the functions of paired $\alpha\beta$ and $\gamma\delta$ TCR chains from single cells. Altogether, our findings shed more lights on the potential treatment of infant respiratory infections and research applications.

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