ORCHESTRATION OF AN IMMUNE RESPONSE TO RESPIRATORY PATHOGENS

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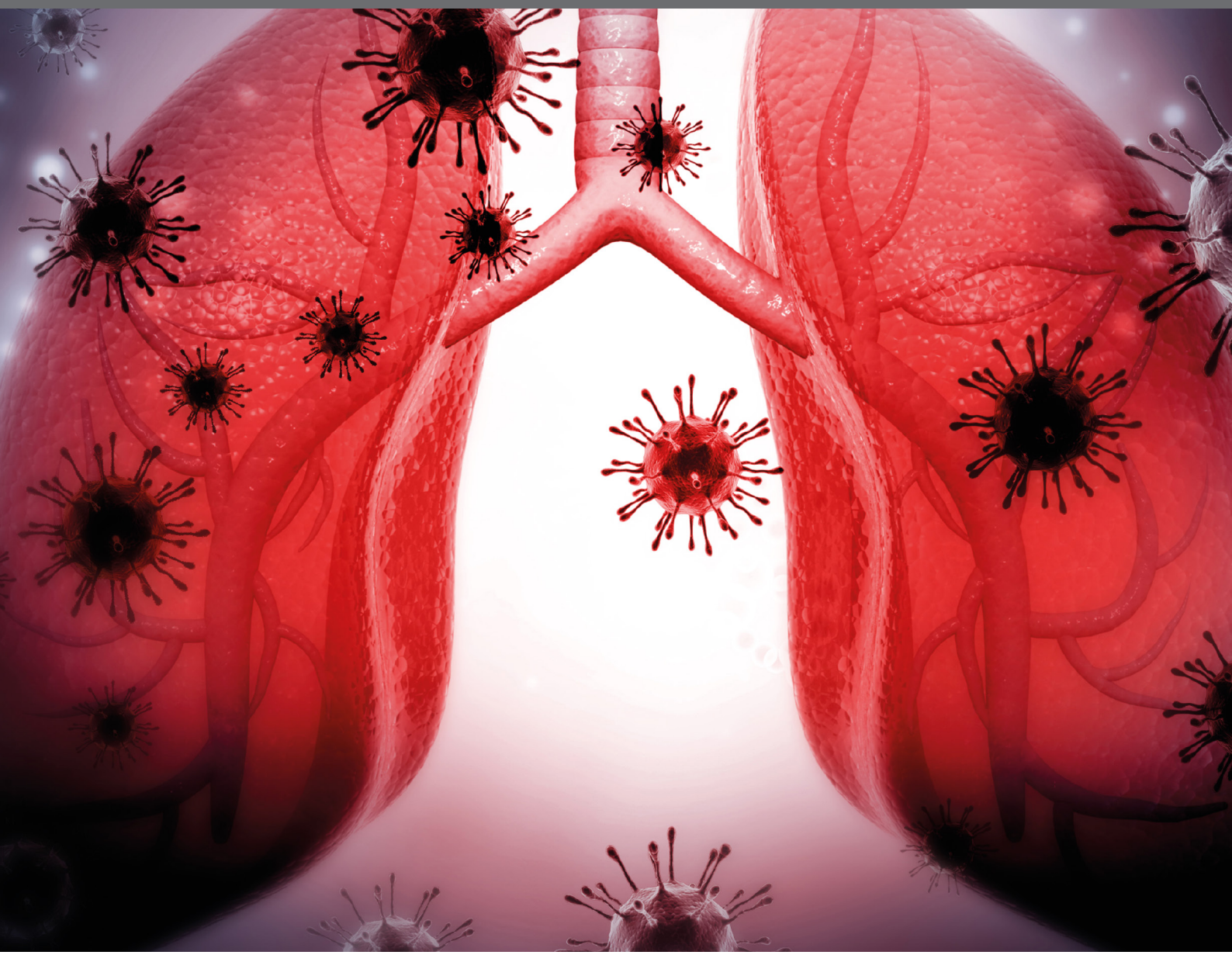

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ORCHESTRATION OF AN IMMUNE RESPONSE TO RESPIRATORY PATHOGENS

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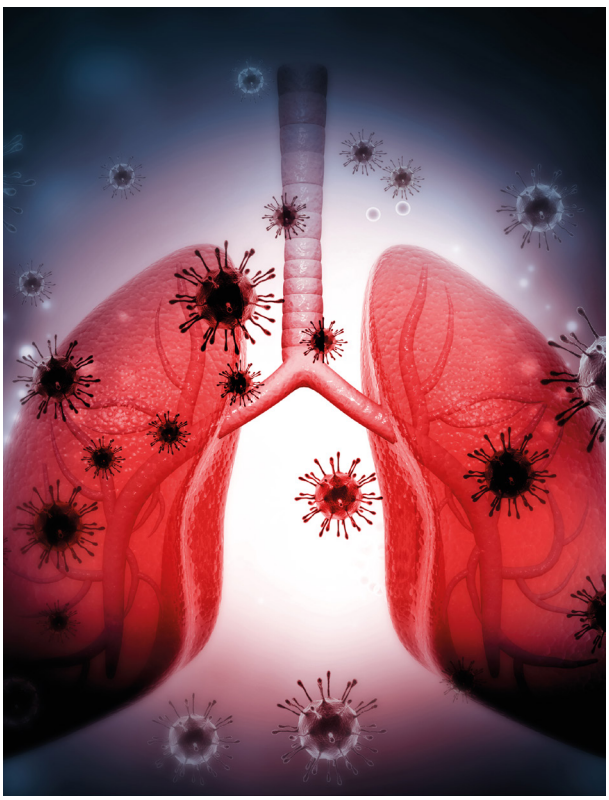

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EDITORIAL
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Editorial: Orchestration of an Immune Response to Respiratory Pathogens

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Editorial on the Research Topic

Orchestration of an Immune Response to Respiratory Pathogens

This issue of Frontiers deals with the complex series of events and long-term consequences of immune responses to respiratory pathogens. In this issue, the contributors discuss the earliest events following infection, the alternative paths that the adaptive immune response can take and long-term immunity that becomes established, both locally and systemically. The diversity of respiratory pathogens that present challenges to immune protection in humans is substantial and, as discussed in this issue, includes a large number of distinct respiratory viruses, including respiratory syncytial virus, metapneumovirus, rhinovirus and influenza virus (Ağaç et al.; Ascough et al.; Devarajan et al.; Fonseca et al.; Ivanciuc et al.; Richards et al.; Schmidt and Varga) as well as bacterial pathogens such as tuberculosis (Choreño Parra et al.; Orme and Henao-Tamayo). These human pathogens differ in their tropism, primary site of infection within the respiratory tract, degree of pathogenicity, seasonality and potential for chronic infection. Here, we summarize some of the key concepts that are discussed in this issue and cite recent reviews by others to provide additional background.

A consistent theme discussed by several contributors (Ascough et al.; Schmidt and Varga; Ivanciuc et al.) is the characteristic nature that the earliest innate and inflammatory response initiates upon respiratory infection with alternate pathogens (1–6). Particularly striking in this regard is the contrast in the elicited response between RSV and influenza virus, a topic considered in detail by Ascough et al. RSV and influenza viruses differ in their primary tropism within the respiratory tract, as well as the source and abundance of early pro-inflammatory cytokines, such as type I IFNs. These elements, together with TLR-mediated differences, lead to distinctive patterns in the subsequent adaptive immune response. Typical seasonal influenza virus is characterized by dominant and protective Th1 response, while RSV is dominated by Th2/Th17 responses. As discussed by Fonseca et al., the impact of diminished type I IFN is particularly exacerbated in infants infected with RSV, who also exhibit delayed IL-12 production and IFN-γ, relative to cytokines such as IL-6 and IL-23. Together, these early soluble mediators are associated with lung immunopathology rather than protective immediate and long-term immunity. This early pattern of CD4 T cell responses to RSV is also associated with fewer polyfunctional CD8 T cells and weak protective antibody responses. Together, this pattern of immune responses to RSV poorly protects the host from future infection with even the same strain of RSV and biases the character of subsequent responses throughout life (Ascough et al.; Ivanciuc et al.). In contrast, the highly diverse population of responding CD4 T cells to influenza infection exhibits robust IFN-γ production in the lung-draining lymph node and the cohort of influenza-specific CD4 T cells that leaves the lymph node and localizes to the lung tissue becomes even more enriched in the Th1-IFN-γ dominated phenotype (Richards et al.).

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A key feature in understanding and ultimately manipulating T cell immunity to respiratory pathogens requires insight into the factors and mediators that contribute to homing, extravasation, and establishment of tissue residence. Many of the contributors to this volume address this issue and pointed out the significant areas of uncertainty in these fate decisions (Reagin and Klonowski; Souquette and Thomas; Topham and Reilly). Particularly for CD4 T cells, the effector function and fate after priming is heterogeneous (5, 7), and includes follicular helper cells, that remain in the lymph node and facilitate B cell responses, prototypical Th1 cells either enter recirculation or home to the lung and cytotoxic CD4 T cells that are primarily detected in the respiratory tract. The elements within the lung draining lymph node that control commitment to these alternate fates are not well understood, but as discussed by Richards et al., it is clear that the repertoire of T cells that migrate to the respiratory tract are extremely broad in antigen specificity and in T cell receptor diversity, allowing for significant breadth in epitope recognition in the infected tissue. As discussed by Walling and Kim, integrins, such as LFA-1 and its ligand ICAM-1, play a key role in the multiplicity of T cell events involved in priming, extravasation, and delivery of effector functions in the respiratory tract. Chemokine receptors such as CXCR3, CXCR4, and CCR5 (Reagin and Klonowski; Richards et al.; Topham and Reilly) on T cells and on other effector cells, such as NK cells (Ascough et al.; Carlin et al.), control recruitment and positioning of lymphoid cells to lung in response to ligands produced within the respiratory tract (e.g., CXCR3 with its ligands CXCL9/CXCL10 and CCR5 with its ligands CCL3/CCL5/CCL4).

As discussed by Topham and Reilly, within the respiratory tract, an extensive degree of lymphocyte compartmentalization takes place, which we are only beginning to fully understand. Evidence to date suggests that differences in localization between CD4 and CD8 T cells exists, as well as among subsets of CD8 T cells. These issues are discussed in the context of the receptor ligand pairs between T cells and tissue, such as CD103-E-cadherin, VLA-1–collagen IV, and CD49b-Collagen I that contribute to these distinct patterns of localization. Within the lung, the expression of even prototypical markers of "resident" cells, such as CD69 and CD103 are heterogeneous, again suggesting microheterogeneity in the fate and localization of the effector cells that migrate to the lung and establish residency. The expression of these receptors on T cells that control localization, retention, and even survival of T cells within the tissue are in turn regulated by complex events at priming but also by cytokines within the tissue, such as TGF-β and chemokines such as CCL25 (Topham and Reilly).

The effector functions delivered to the respiratory tract can contribute profoundly to viral clearance and resistance to future infection (Devarajan et al; Richards et al.; Schmidt and Varga; Souquette and Thomas; Topham and Reilly). Schmidt and Varga in particular, consider the types of approaches that have been used to identify the role of CD8 T cells in viral clearance and the diversity of effector mediators in the lung. Molecules such as Fas, TRAIL, and granzyme have been shown to mediate direct contact-mediated killing of infected cells within the respiratory tract and to be mediators in ultimate viral clearance. As discussed (Schmidt and Varga), identification of key mediators involved in these functions, such as killing of infected cells, recruitment of innate effectors and production of IFN-γ have largely been derived from animal studies where genetic approaches can be used to eliminate candidate mediators and cell types. However, in humans, although many of the same mediators of protection are expressed in effector CD8 or CD4 T cells, their role in protection can only be inferred by correlative studies. There have been a limited number of such studies implicating cytotoxicity and IFN-γ in some human studies of infection. Human challenge models, although costly and labor intensive, are the most rigorous way to identify correlates of protection and sensitivity to infection for pathogenic organisms and for evaluating vaccine candidates (8–10). It is also important to appreciate that the factors that mediate protection, such as the cytotoxic mediator Fas and the cytokines IFN-γ and TNF also can contribute to immunopathology. The events that control T cell mediated immunopathology vs. protection in humans are poorly defined presently (11, 12). For the design and administration of future vaccine candidates, it is critical to gain further insight into these factors.

Many of the contributors discussed the long standing evidence for the role of memory CD8 T cells and CD4 T cells in protection from repeat infections by respiratory viruses (Ascough et al.; Devarajan et al.; Schmidt and Varga; Topham and Reilly) and tuberculosis (Orme and Henao-Tamayo), providing both homologous and heterologous protection (Souquette and Thomas). Choreno Parra et al. also present the increasing evidence of memory in natural killer cells and other innate cells in viral infection and tuberculosis. Priming via infection elicits distinct subsets of cells that persist well after pathogen clearance. As discussed by many of the articles in this issue (Choreño Parra et al.; Orme and Henao-Tamayo; Schmidt and Varga; Topham and Reilly), for the most part, these subsets are defined by the array of cell surface proteins expressed. Protective tissue resident memory CD4 and CD8 T cells have been widely implicated as important for long term protection from many pathogens (11, 13–20). However, there is evidence, discussed by Reagin and Klonowski, that within the lung, resident T cells, defined by the expression of CD69 and CD103, wane over a period of weeks to months, in both the airways and in the tissue. These authors also suggest that the lung is inherently immunosuppressive, and populated by regulatory T cells and cells that produce suppressive cytokines and that express molecules such as PD-1 ligands. Also playing a role in the diminished persistence of tissue resident cells in the lung is diminished expression of cytokines such as TGF-β that promotes expression of CD69 and CD103, the molecules that prevent egress from the tissue and promote adherence of T cells to epithelial surfaces, respectively. These authors suggest that the ultimate loss of tissue resident cells is necessary to prevent immunopathology. Clearly, in consideration of this issue, it is important to assess the normal inflammatory state of the human respiratory tract during its repeated exposure to respiratory pathogens (21). Also critical in assessing this issue is development of improved methods to quantify long term T cell memory in the respiratory tract, using such methods as *in situ* imaging that do not rely on successful extraction or expression of particular surface markers (22, 23). Ultimately, it will be

important to explore the costs vs. benefits of generating and sustaining resident T cell memory for long-term protection from lung pathogens such as influenza virus and tuberculosis in future vaccine strategies that seek to amplify lung-specific immunity.

Beyond the important insights and paradigms that are derived from the detailed studies in animals, there are additional points to consider in understanding, predicting, and manipulating protective immunity to respiratory pathogens in humans. First, the primary response to most respiratory infections, generally used in animal models of infection and vaccination, only occurs once in the very long life span of humans. The vast majority of respiratory pathogens infect repeatedly, typically seasonally, in the human host. Reinfection sometimes occurs with a genetically identical strain, such as with RSV and in other cases, with a variant strain, the most typical pattern of influenza virus. Therefore, in humans, the secondary response and all of the subsequent responses will be contributed in whole or in part by memory cells which will compete with or influence the subsequent response. Beyond recruitment of lymphoid cells specific for the same pathogen, the long life span and the diversity of infections suffered by the human host (21, 24), an additional factor that is likely to play a substantial role in human immunity to respiratory pathogens is the considerable cross reactivity that exists in the T cell repertoire. This antigen/epitope cross reactivity leads to elicitation of heterologous immunity (25). Cross-reactive immunity, occurring in both chronic and acute infections to unrelated pathogens, is considered in detail by Souquette and Thomas. The cross-reactive response perturbs the trajectory of the responses that characterize the primary response in animal models of infection and that are observed in infancy and early childhood. Even with our limited understanding of this complexity, it is apparent from some examples in both animal and human studies, that such effects of heterologous immunity, induced by pathogens or vaccines, can both enhance protection or exacerbate the pathological effects of subsequent infection. Heterologous immunity can involve either immunity specific for chronic pathogens such as EBV and CMV or that specific for acutely infecting pathogens such as RSV and influenza and such cross reactivity can modulate both the effector phenotype and the responding T cell receptor repertoire. A second factor to consider in understanding human immunity to respiratory pathogens is that the respiratory tract in humans may rarely exist in the quiescent state observed in animal models maintained in specific pathogen-free facilities. A number of recent studies have documented the abundance of tissue resident memory T cells in human lung that have diverse antigen specificity (26–28). It is likely that the sustained accumulation of T cells, with a core signature of gene expression (29–31) that endow these cells with the capacity to rapidly respond to invading pathogens reflects, at least in part, the consequences of repeated and periodic confrontation of humans with respiratory pathogens. The consequences of sustaining a diverse memory pool within the lung may have pathological as well as protective consequences to the host (25, 26), depending on the individual's genetic profile, immune history, and the existence of co-morbidities. Finally, with respect to interpretation of the studies discussed in this Frontiers Research Topic, Orchestration of an Immune Response to Respiratory Pathogens and in consideration of novel vaccine strategies, it important to emphasize that the presence and function of memory lymphoid cells, whether from the innate or adaptive immune system, should not be estimated solely on the basis of complete resistance to infection. While such sterilizing immunity is the most profound and easy to assess, the contribution of memory cells to protection from pathogenic organisms can be reflected in blunted replication rates, more rapid clearance, less pathogenic disease courses as well as shorter and less efficient transmission. It is important that in the future, these parameters are monitored as effectively as possible in humans and animal models of infection and that the design and evaluation of vaccine strategies consider these benefits to the host and for preventing the spread of respiratory pathogens.

AUTHOR CONTRIBUTIONS

AS and SV jointly recruited authors and scope for this issue. AS reviewed submissions and relevant background literature to formulate the forward to this issue.

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REVIEW
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Induction and Subversion of Human Protective Immunity: Contrasting Influenza and Respiratory Syncytial Virus

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Respiratory syncytial virus (RSV) and influenza are among the most important causes of severe respiratory disease worldwide. Despite the clinical need, barriers to developing reliably effective vaccines against these viruses have remained firmly in place for decades. Overcoming these hurdles requires better understanding of human immunity and the strategies by which these pathogens evade it. Although superficially similar, the virology and host response to RSV and influenza are strikingly distinct. Influenza induces robust strain-specific immunity following natural infection, although protection by current vaccines is short-lived. In contrast, even strain-specific protection is incomplete after RSV and there are currently no licensed RSV vaccines. Although animal models have been critical for developing a fundamental understanding of antiviral immunity, extrapolating to human disease has been problematic. It is only with recent translational advances (such as controlled human infection models and high-dimensional technologies) that the mechanisms responsible for differences in protection against RSV compared to influenza have begun to be elucidated in the human context. Influenza infection elicits high-affinity IgA in the respiratory tract and virus-specific IgG, which correlates with protection. Long-lived influenza-specific T cells have also been shown to ameliorate disease. This robust immunity promotes rapid emergence of antigenic variants leading to immune escape. RSV differs markedly, as reinfection with similar strains occurs despite natural infection inducing high levels of antibody against conserved antigens. The immunomodulatory mechanisms of RSV are thus highly effective in inhibiting long-term protection, with disturbance of type I interferon signaling, antigen presentation and chemokine-induced inflammation possibly all contributing. These lead to widespread effects on adaptive immunity with impaired B cell memory and reduced T cell generation and functionality. Here, we discuss the differences in clinical outcome and immune response following influenza and RSV. Specifically, we focus on differences in their recognition by innate immunity; the strategies used by each virus to evade these early immune responses; and effects across the innate-adaptive interface that may prevent long-lived memory generation. Thus, by comparing these globally important pathogens, we highlight mechanisms by which optimal antiviral immunity may be better induced and discuss the potential for these insights to inform novel vaccines.

Keywords: respiratory syncytial virus, influenza, respiratory disease, CD4$^+$ T cell, B cell, innate immunity, toll-like receptor, RIG-I like receptor

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INTRODUCTION

Acute respiratory tract infection is a leading cause of morbidity and mortality worldwide, 2.7 million deaths having been attributed to lower respiratory tract infections (LRTI) in 2013 (1). Recent mortality statistics for England and Wales indicate that influenza and pneumonia were among the principal causes of death among both under 4 year olds and adults aged over 80 years (2), while in the US, pneumonia is known to be a predominant cause of hospitalization among pediatric and elderly populations, accounting for 36 hospital stays per 10,000 people (3). The causes of both upper and lower respiratory tract illnesses can frequently be traced to viral infections. A recent analysis of community-acquired childhood pneumonia in the US found that two thirds of patients with a detectable pathogen were infected with one or more viruses, of which the most common was respiratory syncytial virus (RSV) (4). This is consistent with global mortality data, which indicates that two of the most important causes of pneumonia in under 5 years olds are RSV and influenza (1). During the first 2 years of life, all children will have been infected at least once with RSV, with approximately two thirds infected by the end of their first year; in the UK this translates to a peak risk of hospital admission at approximately 1 month of age (5). With increasing age, subsequent RSV episodes decrease in severity, with a mild-to-moderate upper respiratory tract infection (URTI) in healthy adults. However, in elderly patients RSV once again becomes a cause of severe disease (6). Unlike RSV, influenza shows a less dramatic bias toward pediatric infections, and the majority of seasonal influenza-related deaths occur amongst elderly patients (7–9), although in a USA study, Thompson et al. found that rates of influenza-related hospitalization among infants and young children were on a par with those in the over-65s (8). This indicates that each year both pathogens represent an enormous disease burden in vulnerable population groups.

Influenza and RSV both circulate seasonally, with annual outbreaks in temperate regions occurring during winter months, although in tropical climates the seasonality is less distinct and peaks of infection occur year round (10). Both pathogens, which are responsible for recurrent infections throughout life, are enveloped single-stranded negative-sense RNA viruses. Influenza belongs to the Orthomyxoviridae family, and unlike RSV, which belongs to the Paramyxoviridae family, the genomes of the influenza genus are divided into segments. This segmented nature aids the emergence of variant viruses through antigenic shift (by genome segment reassortment), which in combination with antigenic drift (by accumulated point mutation), has led to enormous genetic diversity. Naturally occurring infection by influenza viruses is known to induce long-lasting protective immunity, although this is strain-specific. Recurrent infections therefore occur due to the antigenic variation seen in circulating strains over time. In contrast, reinfection with antigenically similar strains of RSV occurs frequently throughout life, especially in children and the elderly, indicating that the immunity induced by natural infection provides little long-term protection against reinfection (11). The natural histories of these two viruses are a consequence of the divergent immune sequelae that they provoke, but the lack of persistently protective immunity following natural infection in each case can be seen as two sides of the same coin. The continued lack of a universal, effective vaccine against either virus means that both continue to cause huge burdens of mortality and morbidity across the globe.

CLINICAL, EPIDEMIOLOGICAL, AND VIROLOGICAL FEATURES OF RSV

Respiratory syncytial virus causes a spectrum of illness, which ranges from mild URTIs, or otitis media, to severe LRTIs, encompassing bronchiolitis. Bronchiolitis is associated with the infiltration of inflammatory cells into the airways, over-production of mucus, and edema of the respiratory tract (12). This leads to a progressive narrowing of the airways with increased airflow obstruction, causing tachypnea and hypoxia. The characteristic airway inflammation and epithelial necrosis associated with RSV-induced bronchiolitis has also been implicated in long-term impairment of lung function, as RSV infection during childhood is believed to be an important risk factor for the development of asthma and allergic wheeze during later life (13, 14). Indeed, Blanken et al. have shown that treatment with the anti-RSV monoclonal antibody (mAb) palivizimab, reduces postviral wheeze at 1 year of age (15). An estimated global burden of 33.1 million acute respiratory tract infections, resulting in 3.2 million hospital admissions in under 5 year olds, have been attributed to RSV (16). Approximately one third of deaths resulting from acute lower respiratory infection in the first year of life can be ascribed to RSV, with between 94,600 and 149,400 childhood deaths related to RSV every year (17, 18). However, with limitations in healthcare provision in low and middle income countries, accurate epidemiological assessment in many regions is difficult due to inconsistent diagnostics and under-reporting of cases. In addition to the pediatric disease burden, RSV also contributes to significant morbidity and mortality in the elderly, immunocompromised individuals, and those with pulmonary or cardiac comorbidities (see **Figure 1**) (6).

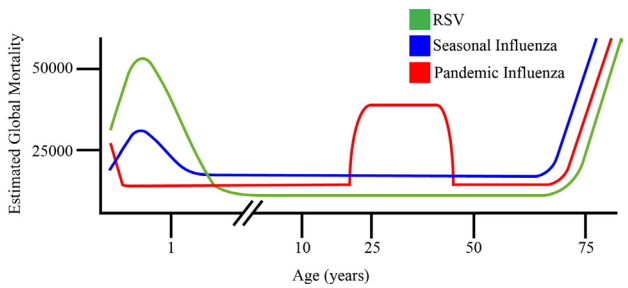

FIGURE 1 | Schematic representation of comparative age distribution of respiratory syncytial virus (RSV), 2009 pandemic influenza and seasonal influenza mortality. RSV is a leading viral cause of infant death (8), with maternal antibodies offering temporally constrained protection to neonates against seasonal flu and RSV. This is limited in the case of novel pandemic influenza strains not previously encountered by the maternal immune system. Young adults are disproportionately affected by pandemic influenza strains, relative to seasonal influenza and RSV. Pandemic and seasonal influenza, as well as RSV all cause dramatic mortality in elderly individuals, reflecting the age-related decline of immune function in this population (8, 19).

The 15.2 kb genome of RSV consists of 10 genes coding for 11 proteins, many of which have been targeted in attempts to induce pathogen-specific immunity. Eight of the proteins are internal and include the matrix (M) and two non-structural proteins (NS1 and NS2). Further internal proteins are all associated with the construction of the nucleocapsid. These include the products of the M2 gene, which are two polypeptides resulting from overlapping open reading frames: a nucleocapsid-associated transcription factor (M2-1) and the associated polypeptide involved in genome replication (M2-2). The nucleoprotein (N) and phosphoprotein (P) together interact with the RNA-dependent polymerase (L) to form the nucleocapsid. The 3 remaining transmembrane proteins are found in the viral envelope: the small hydrophobic protein (SH) which enhances membrane permeability in host cells through ion channel formation (20); the highly glycosylated major attachment protein (G), which mediates viral attachment to the host cell; and the fusion protein (F), which mediates both virus-to-cell and cell-to-cell fusion. RSV is relatively antigenically stable compared to influenza and only the G protein undergoes extensive variation akin to hemagglutinin (HA) and neuraminidase (NA) found on the surface of influenza. This has led to the classification of circulating RSV strains into two different antigenic groups (A or B) based upon divergent sequences within the G protein ectodomain. Furthermore, in addition to its genetic diversity, the virus also expresses G protein in a secreted form, which may function as an antigenic decoy (21).

The F protein has presented an attractive target for rational vaccine design since the early days of RSV vaccine development. While both G and F proteins are immunogenic targets of the antibody response, F is the only one essential for cell entry and (unlike G) is highly conserved across all known RSV strains. However, the F protein presents an unusual target for the immune system, as the metastable prefusion conformation present in the virus rapidly undergoes a number of structural changes both spontaneously and following host cell binding that results in a secondary, more thermodynamically stable postfusion form (22–24). Both forms of this protein display epitopes but only three of the antigenic sites (I, II, and IV) found on the prefusion protein are preserved following conformational change. These have been defined by mAbs, with site I recognized by mAbs such as 2F, 44F, or 45F and site IV by mAb19 and 101F (25, 26). Site II is targeted by the humanized monoclonal IgG antibody, palivizumab, currently the only clinically approved RSV immunoprophylaxis in use. In high-risk infants, monthly administration of palivizumab reduces RSV hospitalizations by at least 50%, showing that systemic IgG alone can confer protection against LRTI. However, the relatively modest efficacy, high cost and requirement for repeated dosing precludes its use in resource-constrained settings. Furthermore, all of the antibodies raised against these antigenic sites show at best moderate neutralizing activity and postfusion F protein-based vaccines have so far failed to demonstrate efficacy in clinical trials (27, 28). In contrast, the most potent RSV neutralizing antibodies are raised against the site Ø epitope, which is found exclusively in the pre-fusion conformation of the protein, the structure of which was only recently elucidated. The mAbs D25, AM22, and 5C4, which target this site, have a neutralizing potency up to 100-fold greater than palivizumab, giving rise to the possibility of more effective antibody-mediated protection if site Ø can be preferentially induced (24, 29).

While vaccination remains the most cost-effective health intervention for infectious diseases, there are currently no effective vaccines available against RSV. This underdeveloped state of vaccine development is due in part to the unenviable position that an early RSV vaccine candidate has in the history of vaccines. During the late 1960s, a clinical trial involving vaccination of children with an alum-precipitated formalin-inactivated RSV vaccine preparation (FI-RSV) led to exacerbated disease upon subsequent natural infection with live RSV. Although almost half (43%) of the infants receiving the vaccine displayed at least a fourfold rise in serum neutralizing antibody titer and almost all (91%) had at least a fourfold rise in complement fixing antibody, 80% of vaccinees required hospitalization for pneumonia and bronchiolitis on subsequent RSV exposure, resulting in two fatalities (30, 31). Considerable research effort over the decades since has sought to determine the mechanisms responsible for this immunopotentiation or "vaccine-enhanced disease." Subsequent studies have suggested that formalin-inactivation led to the deformation of B cell epitopes, which may have promoted the development of non-neutralizing antibodies (32). This in combination with $CD4^+$ T cell responses biased toward production of Th2-associated interleukins (IL) (IL-4, IL-5, and IL-13), may have exaggerated immunopathology upon later infection to RSV (33). There is also recent evidence that deficient toll-like receptor (TLR) stimulation resulting in a lack of antibody affinity maturation may have played a role (34).

The state of RSV vaccine development has drastically improved over the last decade in view of increased understanding of disease burden and antigenic targets. Almost 50 vaccine and mAb products are in development, of which several are currently in clinical trials (35, 36). However, the recent failure of a recombinant F protein nanoparticle vaccine candidate in a phase III trial of elderly adults despite showing immunogenicity in earlier clinical trials (37–40), demonstrates that there is still an urgent need to improve our understanding of immune correlates of protection for this pathogen.

CLINICAL, EPIDEMIOLOGICAL, AND VIROLOGICAL FEATURES OF INFLUENZA

Acute respiratory infections with seasonal influenza cause an estimated 3–5 million cases of severe illness and 250,000–500,000 deaths globally each year (17, 41), with an accumulated cost in the USA alone of ~$87 billion per annum (42). In addition to this ever present threat to public health, there is the constant possibility that a novel virus with a high case-fatality rate may emerge and prove capable of initiating a pandemic. Over the past 100 years there have been four such pandemics: the H1N1 Spanish flu in 1918 which was responsible for 50–100 million deaths; the H2N2 outbreak in 1957 which caused 100,000 deaths; the H3N2 outbreak in 1968 which caused 700,000 deaths; and the recent H1N1 pandemic in 2009 which claimed over 15,000 lives (43). Although large variations have been seen in the excess mortality caused by successive pandemic influenza strains, these appear to

disproportionately affect young adults relative to seasonal influenza and RSV (**Figure 1**), these deaths have been linked to an influenza initiated "cytokine storm" and a susceptibility to lethal secondary bacterial pneumonia (44, 45). Influenza viruses which have their origins within the avian viral reservoir have accounted disproportionately for many of these deaths, and continue to represent an emerging threat to human health as the progenitors of the next influenza pandemic. Currently, two strains of highly pathogenic avian influenza are circulating in birds and causing sporadic outbreaks in humans (H7N9 in South East Asia, and the panzootic H5N1), both of which have pandemic potential.

As with RSV, influenza viruses enter hosts either intranasally or less commonly *via* the eye, following exposure to infected secretions. Influenza infection is then initiated within the airway by the attachment of HA to sialic acid receptors on the surface of the host epithelium. While RSV is uniquely adapted to human cells, with attachment thought to be mediated by the chemokine receptor CX3CR1 (46), HA may be adapted to a number of species and specificity is thought to be a critical factor in host tropism. Avian influenza HA preferentially binds to α(2,3)-sialic acid linkages, while influenza viruses circulating in humans possess HA subtypes that recognize and attach to the α(2,6)-sialic acid linkages more commonly expressed in the human respiratory tract. It is possible to modify this binding specificity through the mutation of a single amino acid within the receptor binding domain, increasing the likelihood of the virus acquiring the capability to infect a new host species. This is of particular concern in pigs and certain birds, such as turkeys, which have both α-2,3 and α-2,6 linkages, and are thus capable of acting as mixing vessels to generate reassortant viruses (47).

Influenza viruses are divided into A, B, and C types. Influenza A viruses, which are the pathogens responsible for the majority of seasonal and all pandemic influenza infections, infect a range of mammals and birds, while types B and C typically infect humans. They all possess segmented genomes: influenza A and B contain eight RNA segments and influenza C seven. The influenza A genome encodes 11 core and accessory viral proteins. A further two proteins (negative sense protein and the N-terminal truncated variant N40) may have a role in late-stage infection but as yet their functions remain unclear (48, 49). In common with RSV there are two non-structural proteins (NS1 and NS2) and influenza also possesses two matrix proteins; M1 is found within the lipid bilayer surrounding the virus core and M2 is a transmembrane ion channel. The internal core of the virus is a ribonucleprotein RNA-dependent polymerase complex composed of a nucleoprotein (NP), polymerase acidic (PA), and two polymerase basic subunits (PB1 and PB2) along with an alternatively transcribed proapoptotic peptide, PB1-F2. Influenza viruses are divided into subtypes based on sequence variations in their main surface glycoproteins: HA (which is divided into two subunits, HA1 and HA2) and NA. These are involved in host cell attachment and host cell egress, respectively. Thus far, 18 different HAs and 11 NAs have been defined.

In common with RSV, the surface glycoproteins of influenza are the major targets of the protective humoral response. However, unlike RSV, both proteins are apt to vary greatly as a result of antigenic drift and shift (50). In comparison, the genes encoding the internal virus proteins such as the M gene, are highly conserved between influenza A viruses (50). While it is possible to generate effective vaccines which offer protective immunity against circulating strains, the changing nature of both seasonal and pandemic viruses means that individuals need to be vaccinated repeatedly as mutations are gathered and it is not possible to produce a tailored vaccine quickly and affordably in the quantities required to respond to a novel pandemic virus.

INNATE IMMUNITY FOLLOWING RESPIRATORY VIRUS INFECTION

Intrinsic and Immediate Barriers to Infection in the Respiratory Tract

During the initial stages of respiratory virus infection the naso- and oro-pharynx are the primary sites of exposure. These areas are covered by a thick mucus layer, which protects the epithelial cells. However, mucin macromolecules and sialic acid compounds found within this mucus throughout the respiratory tract can be cleaved by influenza NA, leaving HA free to bind to cell-surface sialic acid residues and initiate viral entry (51). The action of NA has been shown to be crucial in this regard, as oseltamivir blocks influenza infection of mucus-producing human bronchial epithelial cells (52). RSV lacks this sialic-acid cleaving capacity and host cells respond to infection by expression of mucins, the specific mucin protein MUC5AC is elevated in A549 human alveolar epithelial (53) and bronchial epithelial cells (54). MUC5AC is also linked to the innate immune response, being elevated through chemokine (C-X-C motif) receptor CXCR2 signaling in a mouse model of RSV infection (55). Although the exact extent to which mucus upregulation alters infection risk is unclear in humans, the protective effects of MUC5AC overexpression during influenza infection of mouse models (56) suggest that this often overlooked first line of defense plays an important role in warding off both respiratory viruses. Within mucus, other immediate mechanisms including cationic host defense peptides such as cathelicidin have also been shown to play a role in disrupting respiratory viruses at the point of virus encounter (57, 58). Both influenza and RSV envelopes are directly damaged by cathelicidin, which represents one of a number of ancient defense mechanisms that may act immediately to prevent entry of virus into nasal cells. Emerging evidence suggests that respiratory viruses may harness posttranslational modification of these defenses to abrogate their function. Indeed, it remains to be seen whether these early barrier mechanisms can be manipulated for the purposes of prophylaxis and for the time being the focus is therefore on the innate-adaptive immune axis for vaccine development.

The Role of Pattern Recognition Receptors in Respiratory Virus Infection

Airway epithelial cells, neutrophils, alveolar macrophages (AMs) and dendritic cells (DCs), and members of the innate lymphoid cell (ILC) family resident in the respiratory tract make up the first line of innate immune cellular defense. These cells are capable of sensing viral RNA, the predominant pathogen-associated molecular pattern (PAMP) of influenza and RSV,

as well as cell-generated danger-associated molecular patterns (DAMPs), through several families of highly conserved pattern recognition receptors (PRRs). Innate recognition through PRRs has a profound impact on the entire immune response and it is increasingly obvious that pathogens interfere with these pathways to modulate both short and long-term immunity.

In **Figure 2**, we highlight the mechanisms involved in the induction of innate and adaptive immunity to influenza and RSV, and how the disparity in protection may be related to the manner in which these pathogens engage PRRs.

The three key families of PRRs involved in sensing viral infection are TLRs, retinoic acid inducible gene-I (RIG-I), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). These function as critical components of the initial innate immune response, governing the release of cytokines including the type I interferons α and β (IFN α/β). This was recently shown by Forero et al., who found by transcriptomic analysis of human nasal epithelial cell cultures that the innate response to viral infections such as influenza is driven by type I and III IFN (59). This was also associated with induction of the chemokine (C-X-C motif) ligands for CXCR3; CXCL9, CXCL10, and CXCL11, indicating a coordinating role for the early innate epithelial response in the recruitment of monocytes and generation of effector and memory $CD8^+$ T cells (59). Large scale genetic studies have also demonstrated that susceptibility to RSV-induced bronchiolitis are associated with polymorphisms in innate immune genes (60, 61). However, the contribution of individual genetics to innate immune responses (both positive and negative) is yet to be clearly delineated. A recent study of common single-nucleotide polymorphisms in RLRs and IL-4 signaling genes have showed no link to susceptibility to severe RSV infection and there remains controversy in the field (62). Nevertheless, it is clear that innate recognition of PAMPs and DAMPs triggers the induction of the antiviral immune response and might reasonably be a point at which viruses attempt to evade detection.

Intracellular Toll-Like Receptors Recognizing RSV and Influenza

The intracellular receptors TLR3, TLR7, TLR8, and TLR9 all play a role in sensing viral infection. TLR3 is an important activator of type I IFNs; interacting with the toll/IL-1 receptor (TIR) domain-containing adaptor protein inducing IFN beta (TRIF) to activate serine-threonine kinases IκKε and TANK-binding kinase 1 (TBK1), phosphorylating the IFN regulatory factor 3 (IRF3) which induces the expression of IFNβ. TLR3 recognizes double-stranded RNA (dsRNA), a potent DAMP, which may be released from virus-infected phagocytosed cells (63). Within human respiratory epithelial cells, which constitutively express TLR3, downstream proinflammatory cytokine production may be protective, but can also lead to pathology (64). This is borne out by animal studies which indicate that, despite an elevated viral load, *tlr3*$^{-/-}$ mice show decreased infiltration of inflammatory cells into the lungs and increased survival rates following lethal influenza challenge (65). Conversely, in a *tlr3*$^{-/-}$ mouse model of RSV infection, alteration of the immune environment leads to increased IL-13 and an associated overproduction of mucus (66). In this context, it is interesting that both Influenza A and RSV infection lead to upregulated expression of TLR3 in pulmonary epithelial cells but that this may result in different outcomes (67, 68). Adding further to this complexity, influenza and RSV infection can promote increased production of CXCL8/IL-8 upon subsequent re-stimulation with TLR3 ligands, suggesting that previous virus exposure can induce "trained" immunity that may be both beneficial and harmful to the host (67, 68). However, despite the importance of TLR3 in the early immune response, *tlr3*$^{-/-}$ mice have been found to generate normal humoral and T cell responses to sublethal influenza infection (69), which suggests that, at least in the case of influenza, TLR3 stimulation is not essential for the induction of adaptive immunity.

This redundancy can also be seen in the activation of TLR7; neither activation of TLR3 nor TLR7 alone appear capable of stimulating IFN-mediated antiviral defense during infection. TLR7 is highly expressed in plasmacytoid dendritic cells (pDCs), being capable of detecting the single stranded RNA (ssRNA) present in viruses which are taken up into the endosome. It interacts with the adaptor protein, myeloid differentiation factor 88 (MyD88) (70, 71) leading to the activation of nuclear factor-κB (NF-κB) and IRF7, transcription factors that are responsible for stimulating the expression of proinflammatory cytokines and type I IFNs (72–74). Murine DCs deficient in either TLR7 or MyD88 show reduced levels of IFNα induction following influenza virus infection (70, 71). This is in accordance with animal models of infection, which found that naive *myd88*$^{-/-}$ *tlr3*$^{-/-}$ and *tlr7*$^{-/-}$ mice were more susceptible to influenza virus infection, demonstrating that MyD88 signaling is important in resisting primary challenge (75). Although MyD88 signaling was essential for optimal induction of Th1 cells, with Th2 skewing in MyD88 and TLR7 deficient mice (75, 76), Seo et al. found that virus-specific IgG and IgA titers were unaffected and that TLR deficient mice were protected against secondary lethal challenge (75). Other mouse models have found that, despite elevated hemagglutinin inhibition (HI) titers and protective immunity against rechallenge, *tlr7*$^{-/-}$ *myd88*$^{-/-}$ mice demonstrate impaired B cell responses (77). Indeed, TLR7 stimulation has proven critical for isotype class switching in influenza infection (69) and augments antibody secreting cell (ASC) differentiation (78). Taken together this evidence indicates that TLR7 plays an important role in the primary response to influenza, but is not essential to the development of a memory response. In an interesting caveat to this, TLR7 signaling appears to be crucial to the development of protective HI antibodies targeting the pandemic H1N1 (pH1N1) 2009 split vaccine (77), suggesting that the context (infection versus vaccination) in which PRRs recognize viral RNA directs their relative importance. In RSV, TLR7 appears to have an important role in promoting a non-pathogenic T cell response to infection. Engagement of this ligand elicits the IL-12 and type I IFN production required for Th1-type responses, and may skew immunity away from a harmful Th2 response (66, 70, 71). The absence of TLR7 expression in *tlr7*$^{-/-}$ mice does not affect the clearance of RSV from infected animals, but does promote the release of mucogenic cytokines IL-4, IL-13, and IL-17, and preferentially activating IL-23, which is associated with an immunopathogenic Th17 response to RSV in the lungs (79). TLR7 has also proven

to have an important role in the humoral response to viruses, inducing B cells to release antigen specific antibodies following sublethal doses of influenza virus (69, 77, 80).

In common with TLR7, the remaining "viral-sensing" intracellular receptors, TLR8 and 9, both initiate proinflammatory cytokine release through the MyD88 signaling pathway. TLR8, which is stimulated by the presence of ssRNA in monocytes and macrophages, induces production of IL-12, proinflammatory IL-6, tumor necrosis factor (TNF), chemokine (C-C motif) ligand/monocyte chemoattractant protein CCL2/MCP-1, and CXCL8/IL-8 but not IFNα (81–83). TLR9, which recognizes unmethylated CpG repeats within DCs, is a potent inducer of proinflammatory and Th1 responses, and upregulates the costimulatory molecules CD80/86. The action of these receptors plays a role in innate immunity and signaling, the impact of which upon viral replication and dissemination has only recently begun to be investigated. During influenza infection increased TLR7/8/9 expression is known to correlate with elevated production of Th1-related cytokines IFN-γ, CXCL10/IFN-γ induced protein 10 and CXCL9/monokine induced by IFN-γ (MIG). The administration of CpG as a TLR9 ligand during exposure to purified RSV F or G proteins or killed bovine RSV (BRSV) also results in Th1-associated immune responses (84–88). Mucosal administration of a TLR9 ligand CpG oligodeoxynucleotides/L18-muramyl dipeptide (CpG ODN/L18-MDP) with inactivated RSV viral particles potently activates NF-κB, shifting cellular immune responses toward a dominant IFN-γ-producing Th1-type response (89). Furthermore, mucosal immunization of BALB/c mice with inactivated RSV and TLR9 ligands induce local IgA responses and Th1-associated IgG2a high-affinity

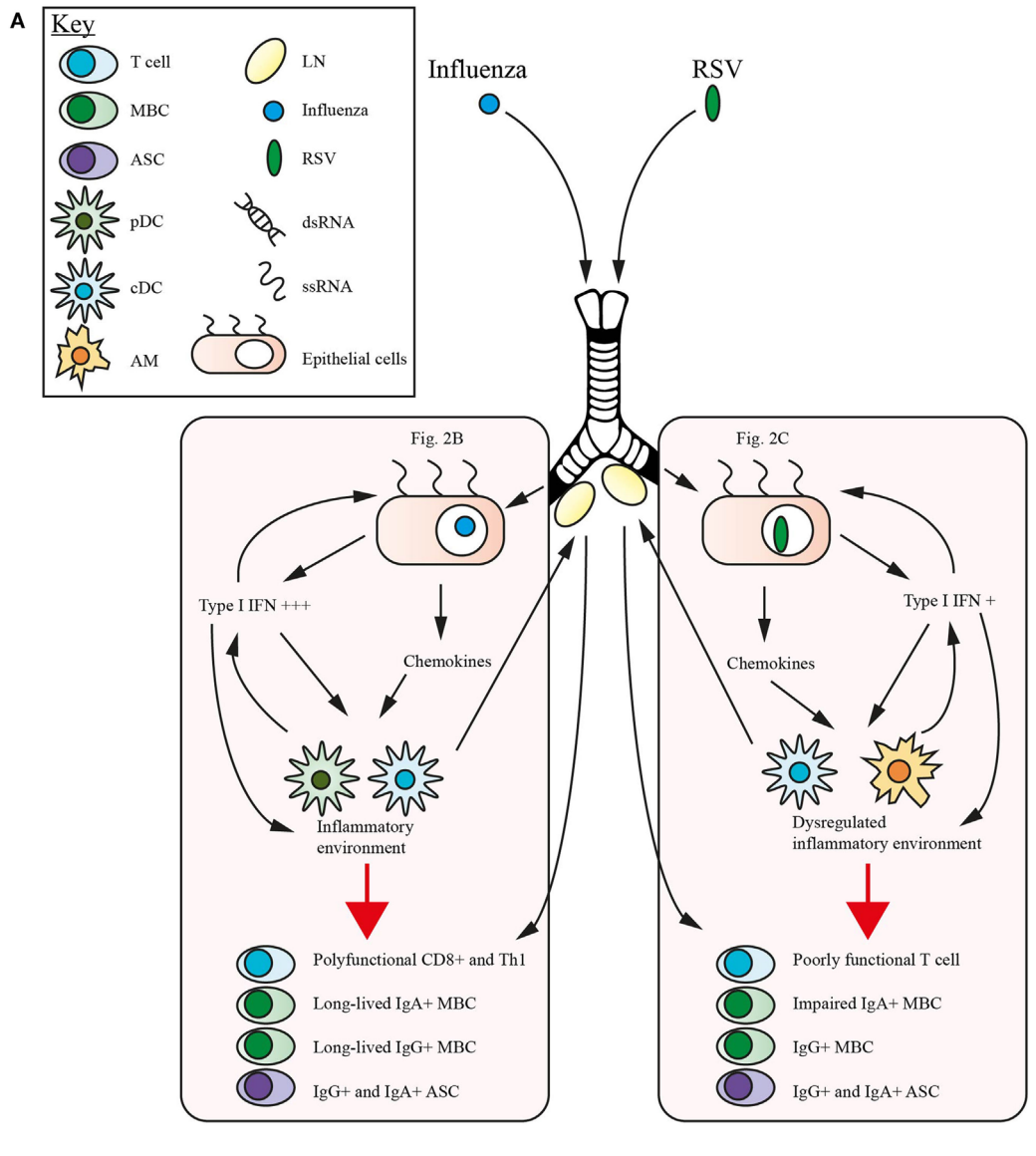

FIGURE 2 | Continued

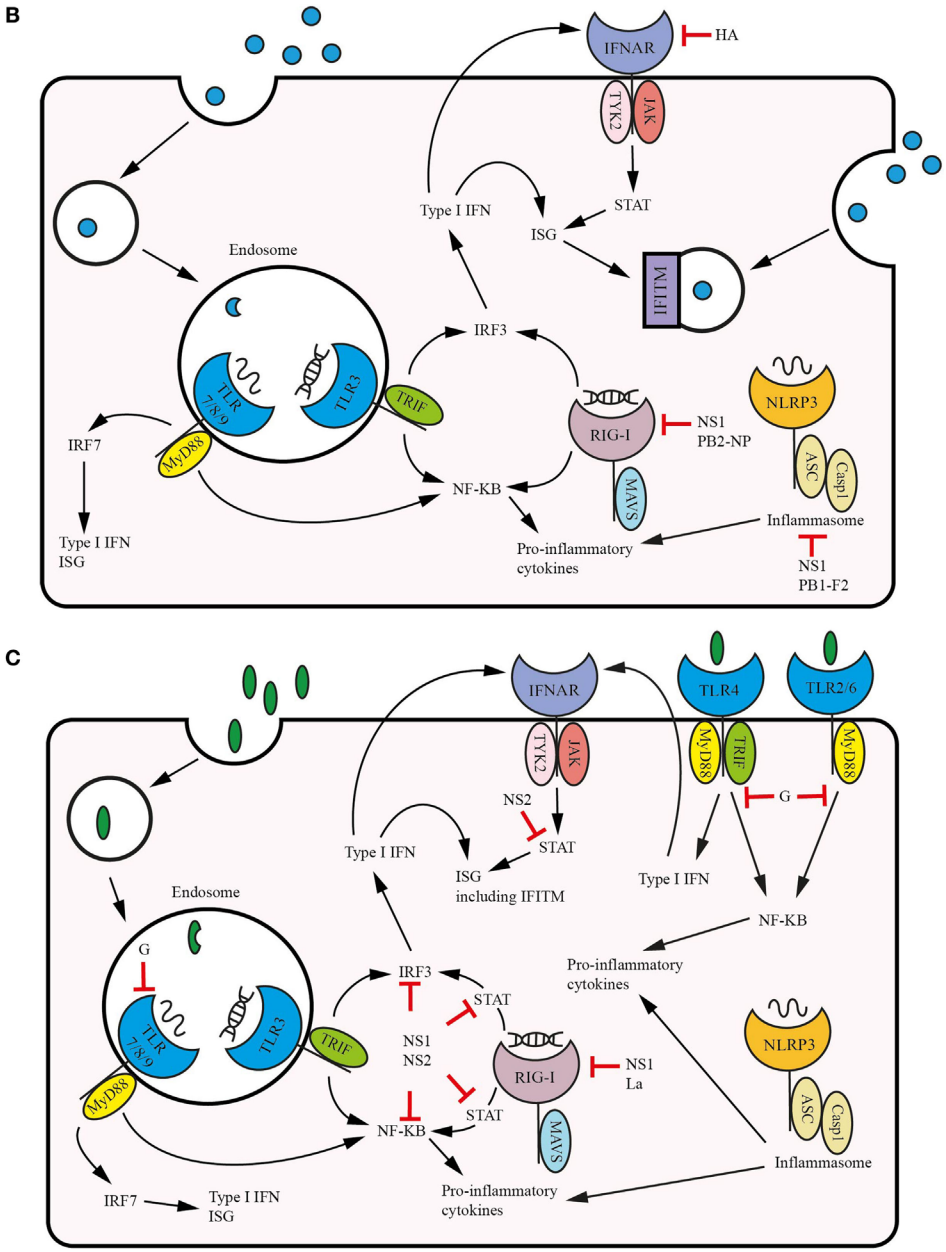

FIGURE 2 | Comparative immunity in respiratory syncytial virus (RSV) and influenza. Within the mucosal surfaces of the lung the host immune response to each virus feature intersecting and non-overlapping traits. **(A)** Influenza and RSV establish infection in the lung epithelial cells, initiating the release of type 1 interferons (IFNs) which act in a feedback loop along with release of proinflammatory cytokines such as interleukin (IL)-1β and IL-18, and upregulation of IFN-stimulated genes (ISGs) to promote an inflammatory environment. This inflammatory milieu, which in influenza recruits conventional dendritic cells (cDCs) and plasmacytoid DCs (pDCs) as the primary type 1 IFN producing antigen-presenting cells (APCs), promotes optimal signaling and memory generation. This includes polyfunctional $CD8^+$ and Th1 T cells, and immunocompetent IgG^+ and IgA^+ ASCs and memory B cells (MBCs). Following viral clearance T cells remain in the lung as T resident memory (Trm) and MBCs and T cells traffic into the systemic compartment. RSV infection of the lung follows a broadly similar pattern, although the primary type 1 IFN producing APCs are alveolar macrophage (AM) and cDCs. The lower levels of type 1 IFN produced during infection impact the generation of memory responses with poorly functional T cells, skewing toward Th2/Th17 generation in early life and a profound defect in the induction of an IgA^+ MBC subset. As the influenza virus establishes infection within a lung epithelial cell **(B)** it enters the endosome and following viral uncoating it triggers TLR3 signaling with double-stranded RNA (dsRNA) and TLR 7/8/9 with single-stranded RNA (ssRNA), in addition retinoic acid inducible gene-I (RIG-I) and the inflammasome are stimulated by contact with dsRNA and ssRNA, respectively. These signaling pathways individually stimulate nuclear factor-κB (NF-κB), IRFs and upregulate ISGs and the production of type 1 IFNs. These mediators are released by the cell and recognized by IFNα receptor (IFNAR), leading to positive feedback, which also stimulates the sequestration of virus by IFN-induced transmembrane protein (IFITM), preventing viral spread. Influenza acts to mitigate the actions of the pattern recognition receptors (PRRs), especially RIG-I and the inflammasome through NS1-mediated inhibition, while hemagglutinin (HA) has recently been found to trigger ubiquitination of IFNα receptor (IFNAR), downregulating IFNAR1 expression. In the case of RSV infection within a lung epithelial cell **(C)** the action of the intracellular PRRs is augmented by the engagement of the extracellular TLRs 4/2/6. RSV strategically targets signaling pathways at multiple points, most notably the action of G protein upon TLR7/8/9 and TLRs 4/2/6, and NS1 upon a number of pathways, particularly STAT signaling.

antibody responses (89). It is notable that the engagement of TLR9-induced signaling pathways during FI-RSV immunization is even capable of ameliorating vaccine-enhanced disease (90). TLR9 polymorphisms have also been associated with the risk of developing RSV linked post-bronchiolitis wheeze (91). Thus, it is likely that appropriate TLR-signaling will be required for an effective early vaccine-induced response.

Extracellular TLRs in RSV

The extracellular receptors TLR2, TLR4, and TLR6 are commonly recognized as bacterial sensors, given that they recognize lipoproteins, peptidoglycans and bacterial cell-surface lipopolysaccharides, respectively. Nevertheless, these TLRs, which signal through the MyD88 pathway, have been found to play an important role in governing the immune response to RSV. The release of NF-κB-related proinflammatory cytokines from airway epithelial cells is the primary mode of action for TLR2 and TLR6, which do not contribute to the production of type I IFN (92). Engagement of both TLR2 and TLR6 has an effect upon cytokine and chemokine production during RSV infection, and increased viral loads have been observed in mice deficient for these receptors, with impaired neutrophil migration and DC activation in *tlr2*$^{-/-}$ mice (93). The release of proinflammatory cytokines in response to heat-inactivated RSV exposure can also be inhibited by TLR2 blocking antibodies (94). The pathogenic effect is compounded in MyD88$^{-/-}$ mice, as multiple TLR pathways (TLR2, TLR4, and TLR7) are abrogated, leading to increased Th2 cytokine release, mucus hypersecretion, and eosinophilia (66).

However, it is primarily TLR4 which has drawn attention for its interaction with F protein during the early stages of RSV infection (93). Indeed, due to its extracellular nature it is difficult to escape the idea that TLR4 may be the first PRR activated during RSV infection, providing an early innate activation signal sensing infection. TLR4 activation has been associated with RSV clearance in several studies (95–98) and severe RSV disease in infants has been linked to TLR4 polymorphisms (99). In the cotton rat model, inclusion of the adjuvant monophosphoryl lipid A (MPL), which is a partial TLR4 agonist, in an FI-RSV formulation ameliorated the lung pathology associated with vaccine-enhanced disease. This was associated with a reduction in the levels of Th1- and Th2-type cytokines and chemokines normally released following RSV challenge (86, 100). This suggests that while TLR4 engagement is important in mounting an early antiviral response to RSV, it is also capable of provoking complex dysregulation of multiple cytokines. It is therefore not surprising that the RSV G protein may have evolved to target TLR2, 4, and 9 expression in human monocytes, suppressing innate immune responses through multiple downstream mechanisms (101, 102). However, conflicting studies have also suggested that depletion of TLR4 does not impact RSV clearance, nor its infiltration of pulmonary inflammatory cells (103). TLR4 has also been linked to the induction of acute lung injury (ALI) during influenza infection, with exposure to influenza derived antigens inducing nicotinamide adenine dinucleotide phosphate oxidase-dependent production of reactive oxygen species which generate oxidized host phospholipids. These were found to include the oxidized 1-palmitoyl-2-arachidonoyl-phosphaticylcholine, a potent activator of the TLR4-TRIF signaling pathway, which triggers production of proinflammatory cytokines, such as IL-6, by pulmonary macrophages, leading to lung injury (104). Abrogation of TLR4 expression, either through administration of a therapeutic antagonist or use of *tlr4*$^{-/-}$ murine strains, provided protection against virally induced lung injury in mouse models of influenza infection (104–106). Nevertheless, it is likely that, unlike RSV which is recognized by both intracellular and extracellular TLRs, pathogen sensing of influenza is predominantly mediated by intracellular TLRs.

Intracellular Sensing by RIG-I-Like Receptors and Innate IFNs

The RIG-I like receptor (RLR) family represent an important additional viral recognition pathway which governs type I IFN production in infected cells. Within the cytosol of the host cell a 5′ triphosphate dsRNA "panhandle" is formed by the conserved 5′ and 3′ end sequences of replicating viral ssRNA. This is detected by one of the RLR helicases, RIG-I (107, 108). RIG-I and melanoma differentiation-associated protein 5 (MDA5) (triggered by longer dsRNAs) both possess caspase activation and recruitment (CARD) domains. MDA5 predominantly senses positive-strand RNA, but can also be triggered by certain negative-strand RNA viruses, such as members of the Paramyxoviridae family (109) and influenza viruses (110). The CARD domains are lacked by the final RLR family member, the laboratory of genetics and physiology 2 (LGP2) molecule. The exact role of LGP2 in antiviral signaling has not yet been fully elucidated, as it has both negative and positive regulatory functions, depending upon the signaling context (111). These adaptor proteins interact with the mitochondrial antiviral-signaling protein (MAVS) or IFN-β promoter stimulator 1 (IPS-1) molecules and initiate signaling transduction cascades that leads to activation of downstream NF-κB and IRF3 pathways (112, 113). This stimulates the induction of type I IFNs. In the case of IFNβ this leads to transcriptional activation of numerous IFN-stimulated genes (ISGs), the most important of which are the IFN-induced transmembrane protein (IFITM) family, the myxovirus resistance proteins (Mx), the serine/threonine kinase protein kinase R (PKR), and the 2′–5′-oligoadenylate synthetase/RNase L system. The IFITM family member IFITM3 has recently been identified as an important receptor in antiviral response against influenza, as it blocks viral entry by impairing virus-host cell membrane fusion, impacting viral susceptibility (114, 115). Both IFITM3 and IFITM1 show a similar impact upon RSV activity, suppressing early viral entry, with IFITM3 in particular delaying the phosphorylation of IRF3 by sequestering RSV in vesicular compartments (116). The Mx family of large guanosine-5′-triphosphate hydrolase enzymes represents another important antiviral factor acting against RNA viruses. In human columnar epithelial cells, the Mx proteins interfere with nuclear transport of viral nucleocapsids to impair influenza transcription and replication (117, 118). The evolutionary pressure exerted by these proteins is capable of causing adaptive mutations in NP which have been observed in pandemic influenza A strains (119).

Given that it is widely conserved across vertebrates, the RLR family clearly plays an important role in sensing and responding

to viral infections. An instructive exception to this is demonstrated by the lack of RIG-I receptors within chickens, which has been posited as an explanation for the increased susceptibility of chickens to avian influenza strains, compared to naturally resistant waterfowl such as ducks, which possess a fully functional RIG-I signaling pathway (120, 121). Type I IFNs remain important in chicken antiviral immunity and recent research suggests that the role of sensing influenza infection is assumed by MDA5 in these animals (121). Indeed, animal models, which have long been used as surrogates for evaluating human influenza and RSV infection, show many such unique features, which can unfortunately complicate extrapolation of host and viral factors involved in the immune response to humans (122). This can be observed in the Mx family, as many of the inbred mouse strains used in research, such as C57BL/6 and BALB/c, carry defective genes for Mx1 and Mx2. This has led to a complex picture of MyD88-dependent immunity which long underestimated the role of pDCs in pulmonary defense (123, 124). Mice have been broadly employed to study host-genetic determinants and have helped to identify many host genetic candidates of the genetic host regions involved in antiviral protection (125, 126). However, these animals remain a poor model for respiratory virus transmission, in contrast to which the ferret model is better suited for studying both the pathogenicity and transmissibility of human respiratory viruses (127), despite suffering from a lack of specialized reagents. It is only recently that physiologically relevant human challenge studies have allowed researchers to elucidate in detail the course of disease within immune compartments such as the lung (128, 129). Further systems biology analysis of mucosal tissues obtained during *in vivo* respiratory viral infection will be critical for better understanding of these sensors in humans.

Evasion of PRR Recognition and Signaling by RSV and Influenza

The importance of PRRs in the antiviral response and their likely early evolutionary emergence as mediators of protection mean that both RSV and influenza have evolved potent immunomodulatory mechanisms to overcome these pathways, often in a highly species-specific manner (130, 131). During early influenza infection this takes the form of concealing the dsRNA "panhandle" recognized by RIG-I through the action of the nucleocapsid polymerase complex (132). Avian adapted strains which have a reduced PB2-NP affinity are consequently sensed more effectively in cells possessing RIG-I (133). The RSV leader sequence performs a similar function, utilizing the host cellular RNA-binding protein La to inhibit the early RIG-I binding and thus detection of RSV (134). The non-structural proteins derived from both viruses impact the innate immune signaling at multiple points, interacting with the ubiquitin E3 ligases, Tripartite motif-containing protein 25 and Riplet, to prevent RIG-I ubiquitination (135, 136). Influenza NS1 prevents the activation of PKR and the nuclear translocation of transcription factors NF-κB and IRF3 to suppress type I IFN synthesis (131, 137–144). In addition, recent evidence has suggested that influenza HA also targets the positive feedback loop of IFN upregulation by interfering with the IFN receptor (145). Zhang et al. also found that RSV-derived NS1 specifically inhibits IFN-β production by decreasing RIG-I interaction with MAVS (116). The NS2 protein of RSV also inhibits RIG-I activation of IFN promoters by binding to the N-terminal CARD domains of RIG-I and inhibiting MAVS interactions (131). In addition, NS2 induces degradation of the signal transducer and activator of transcription 2 (STAT2) signaling pathways involved in MAVS activation (MAVS) (131, 146). RSV strains deficient in NS1 and NS2 expression show incomplete suppression of type I IFN production during human macrophage and epithelial cell infection, leading to a diminished ability to replicate *in vivo*. Thus respiratory viruses rely to a great extent upon these immunomodulatory proteins to evade the early antiviral response and establish infection. Whether these mechanisms are more potent in RSV than influenza and have a greater impact on downstream impairment of host immunity is difficult to test but they certainly contribute to an immediate failure to control RSV infection as well as probably the inability to elicit long-term protection.

Plasmacytoid DCs in RSV and Influenza

Another clue to the reasons for divergence in immunity against the two viruses can be found in the response of neonates and previously uninfected infants to their first encounter with RSV. Until recently, in both murine influenza and RSV, the infiltration of pDCs was thought to play the major role in type I IFN-dependent virus control (147, 148). *In vitro*, while pDCs from adults are capable of responding to cytosolic delivery of RSV-derived dsRNA in a RIG-I–dependent manner (149) by producing type I IFN independent of endosomal TLR activation and PKR (150), similar responses are profoundly impaired in neonates (151). In neonatal mice especially, the limited pDC function and associated type I IFN deficit contribute to Th2-skewed immunopathology in RSV infection (152). Marr et al. not only found that RSV-related IFN-α release is impaired in neonates, but also that peripheral blood cells from children aged between 12 months and 5 years show similarly reduced responsiveness to RSV and synthetic 5′ppp-dsRNA RIG-I agonists (151). This is less clear *in vivo*, where expression levels of MDA-5, RIG-I, TLR7 and TLR8 were all highly elevated in the respiratory tract of infants with bronchiolitis (153). This may have been partially confounded by other respiratory viruses being responsible for bronchiolitis in some of these infants, activating alternative pathways. Plasmacytoid DCs primarily sense ssRNA viruses, including influenza, through the endosomal TLR7/8 pathway (66, 101). In the case of RSV, endosomal maturation and acidification is not required for viral fusion, which may lead to bypassing of the activation of such receptors entirely (154, 155). In contrast, exposure to a low endosomal pH is necessary for the conformational changes that trigger the release of influenza viral ribonucleoproteins from the M1 protein, and subsequent viral-endosomal fusion (156). A fusion pore is then formed, through which viral RNA exits the endosome to the cytosol and then on to the nucleus, where viral replication and mRNA transcription occurs (122, 156, 157). It is possible that pDC exploit this requirement to enter the acidified compartment by initiating proteolytic cleavage and TLR activation. Work in mice lacking asparagine endopeptidase suggests that peptidase-dependent activation of TLR7 is essential for cross priming of $CD8^+$ T cells in influenza infection (70, 158). In comparison,

RSV not only evades recognition by pDC TLRs, but is capable of abolishing TLR7 and TLR9-dependent IFN-inducing pathways in pDC (150, 154). This difference in the routes taken by the two viruses through the host cells and therefore which PRRs are differentially activated may represent a key divergence. Recent studies have suggested that, *in vivo*, the primary source of type 1 IFN is AMs and not pDCs (see below). It is unclear whether differences in mechanisms of viral entry play a role in this dichotomy.

NLRs and Inflammasome Activation by RSV

The cytosolic receptor NLR family consists of members NOD1, NOD2, and NACHT, LRR and PYD domains-containing protein 3 (NALP3, also known as cryopyrin), which are capable of recognizing PAMPs and contributing to the immune response in collaboration with membrane-bound TLRs (159). Conventionally these receptors are activated by molecules, such as muramyl dipeptide, which are produced during the degradation of bacterial peptidoglycan, inducing transcription of immune response genes through NF-κB and mitogen-activated protein kinase signaling pathways. Recent studies have indicated that NOD2 may play a role in sensing viral ssRNA, facilitating MAVS-dependent IRF3 activation and type I IFN production in RSV infection (160). However, it is cryopyrin (encoded by the *Nlrp3* gene), which has garnered attention for its antiviral activity *via* formation of the inflammasome, a caspase-1 activating molecular complex (161). Upon exposure to virally derived ssRNA the NALP inflammasome recruits ASC (apoptosis-associated speck-like protein containing a CARD) and procaspase-1, which is then activated by autocatalytic cleavage to caspase-1. This catalyzes proteolytic processing of pro-IL-1β, pro-IL-18, and pro-IL-33 into active proinflammatory cytokines.

In RSV infection, the SH protein accumulates in lipid rafts of the Golgi apparatus, acting as a viroporin to enhance membrane permeability and promote the entry of ions and small molecules into host cells. This enhances ion-sensitive transcriptional factors promoting viral replication and has also been implicated in NLRP3 inflammasome activation (162, 163). Depletion of SH leads to defective NLRP3/ASC inflammasome activation and IL-1β secretion (163). The M2 protein of influenza also acts as a viroporin, promoting viral uncoating within endosomes and stimulating the activation of the NLRP3 inflammasome (164). *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice were more susceptible to influenza infection, with decreased neutrophil and monocyte recruitment and an associated reduction in cytokine and chemokine production (165). This did not, however, translate into an effect on induction of the adaptive immune response. Ichinohe et al. expanded upon this and found that ASC and caspase-1, but not NLRP3, were required for the induction of CD4$^+$ and CD8$^+$ T cell responses, mucosal IgA secretion and systemic IgG responses (166). Influenza has therefore evolved several mechanisms to evade the activation of the NLRP3 inflammasome. PB1-F2 translocates into the inner membrane space of the mitochondria, acting to decrease the membrane potential and trigger mitochondria disintegration, which blocks formation of the inflammasome. Partial deletion of the amino-terminal RNA-binding domain of NS1 from influenza A rescues the production of IL-1β and IL-18 during infection, suggesting that this domain antagonizes the activation of caspase 1 (167). Intriguingly, Goritzka et al. recently showed that while early lung infiltration of immune cells and levels of proinflammatory mediators were abrogated in *Myd88*/*Trif*/*Mavs*$^{-/-}$ mice (which lack TLR, RLR, and IL-1R signaling), there was no subsequent effect upon the induction of RSV-specific CD8$^+$ T cells (168). This implies that caspase-1 and TLR7 are essential to the development of adaptive immune responses to influenza virus, while the RIG-I pathway is more important in RSV but that redundant downstream pathways exist to induce cell-mediated immunity even in its absence.

Production of Innate IFNs by DCs and AMs

As discussed earlier, the pDC subset, which express TLRs at a high level, is known to be a potent producer of type I IFNs crucial to antiviral defense, and as such both RSV and influenza have developed mechanisms, such as NS directed antagonism to subvert this immunity (169, 170). This allows the viruses to act upon pDC directed immunity; during the acute phase of infection, patients hospitalized with H1N1 during the 2009 pandemic showed depletion of pDCs, as well as myeloid DCs (mDCs), with pDC counts remaining persistently low for up to 16 weeks (171). The consequences of this are seen in mouse models, which indicate that pDC depletion reduces influenza virus-specific serum antibodies during convalescence (172, 173). The engagement of pDCs in influenza infection, particularly involving TLR7 stimulation, leads not only to induction of type 1 IFNs, Th1 and cytotoxic responses, but also enhances B cell expansion and differentiation into CD27^{high} plasmablasts (174). However, it is clear that pDCs are not essential for the effective induction of CD8$^+$ T cell responses or viral clearance (175), and may contribute to immune pathology (176).

Research into the effect of RSV upon pDCs has revealed a contradictory picture of immunity; work in mice has indicated that RSV infection inhibits the ability of lung resident pDCs to respond to TLR activation, reducing IFNα, IL-6, TNF-α, CCL2, CCL3, and CCL4 production, and impairing antigen presentation to T cells (169). This is supported by work in human cord blood derived pDCs exposed to RSV infection, which show reduced IFNα production (151). Conversely, human peripheral pDCs are capable of producing IFNα upon *in vitro* stimulation with RSV (177). While depletion of pDC, in the lungs of mice, affected IFNα levels following influenza, this was not the case with RSV (178), suggesting that pDCs play a less important role in type 1 IFN production during RSV infection than in influenza.

Previous murine studies have implicated AMs, rather than DCs, as major producers of IFNα following RSV infection (172). Monocyte-derived AMs, which make up 95% of all leukocytes found in the airways, are important in antiviral defense, especially within the lower respiratory tract and orchestrate RSV immunity through type I IFN production (173). However, type I IFN deficient murine AMs are still capable of controlling RSV replication (179), suggesting that this may not be their sole function. In concert with studies which throw light onto the involvement of TLR signaling in RSV immunity, these results illustrate the difficulty in extrapolating from mouse models of infection to humans and indicate that peripheral and lung derived pDCs may differ markedly in their function and response to the same viral pathogens.

ILCs at the Interface with Adaptive Immunity

Innate lymphoid cells are a recently described family of effector cells which bridge innate and adaptive immune systems as well as having a crucial role in mucosal immunity. The prototypic group 1 ILC members, the natural killer (NK) cells, are the only currently known cytotoxic cells within the group and there is accumulating evidence that they have a role in antiviral immunity within the lung. These cells, which develop under the influence of IL-15 released by virally infected bronchial epithelial cells, clearly must achieve a delicate balance in influenza and RSV infection. Numerous studies have highlighted the role NK cells play in both viral clearance and in the exacerbation of virus-induced lung pathology. Mouse models of influenza infection have shown that the presence of NKp46$^+$ cells are important in the control of infection (180), with the peak of the antiviral response governed by NK cells displaying an activated phenotype. Human studies have also demonstrated that severe influenza infection is associated with diminished frequencies of circulatory NK cells (181, 182). Welliver et al. found extremely low levels of CD8$^+$ T cells and NK cells within pulmonary tissues taken from infants with fatal RSV and influenza LRTI (183). Indeed, these infections were characterized by uncontrolled viral replication in individuals with an inadequate NK response, possibly suggesting a vital antiviral role for NK cells within respiratory tissues. Furthermore, a clue to the lethality of H5N1 influenza strains may be found in their avoidance of NK cell control. The HA protein of human and swine influenza binds to 2,6-linked sialic acid residues on the NK cell receptor, inducing NKp46-mediated killing, while H5N1 (which binds preferentially to 2,3-linked residues), only initiates targeted cell death during human infection following activation of both NKp46 and NKG2D (184, 185). This impaired NK cell activation may help to explain the differential pathogenicity seen in the human and avian strains.

With both viruses, NK cell-derived IFNγ has an important function in the protective response, although the signaling pathways are not yet clearly understood. In the case of RSV, NK cell depleted mice have been found to produce lower levels of IFNγ, with early IFNγ release promoting the development of a Th1 type response. Ordinarily this suppresses production of IL-25, which can drive the development of a Th2 polarized response, characterized by IL-4, IL-13 and mucus production (186). During influenza infection NK cell-derived IFNγ is also required for optimal cytotoxic T lymphocyte (CTL) function and recall responses are augmented by NK cell IFNγ production (187). It is therefore unsurprising that both viruses have developed methods of subverting the NK response, with the G protein of RSV displaying a capacity for CX3C chemokine mimicry and acting as an antagonist to inhibit the trafficking of CX3CR1$^+$ NK cells (188). Influenza virus meanwhile, affects killer cell immunoglobulin-like receptors (KIR)/HLA-C interactions, increasing the binding of inhibitory receptors KIR2DL1 and the leukocyte Ig-like receptor, which act to inhibit NK cell function (189). The immunopathogenic effects of NK cell accumulation and IFNγ release within delicate lung tissues are well known in both influenza and RSV infection and may go some way to explaining the increased survival and lowered morbidity seen in NK cell depleted mice infected with influenza (190). This is echoed by a reduction in ALI and morbidity (191) observed in NK cell depleted mice infected with RSV.

Apart from ILC1s, increasing evidence indicates that the other predominant innate effectors involved in respiratory viral infections are the ILC2s. Recent work in mouse models has demonstrated that during the early stages of RSV infection the IL-13 producing ILC2 subset are activated (192). If this finding is emulated in humans, it may be one mechanism by which type 2 immunopathology is promoted in some individuals. The dysregulation of the antiviral immune response can also be seen in influenza infection, in which IL-33 driven secretion of IL-13 by ILC2 cells contributes to airway hyperactivity and may exacerbate disease in asthmatic individuals (193). ILC2s are also the primary source of IL-5, another type 2 cytokine induced by IL-33 secretion, which has been shown to promote the accumulation of eosinophils and the exacerbation of disease in influenza infected lungs of mice (194), and these lung resident ILC2s contribute to the induction of an immunopathogenic type 2 response in RSV and influenza infections. This picture is somewhat complicated by emerging evidence that ILC subsets exhibit plasticity depending upon the inflammatory milieu. ILC3 cells are known to differentiate into ILC1 cells (and vice versa), and TLR2 promotes the production of IL-5 and IL-13 by ILC3 cells, suggesting that they are capable of differentiating into ILC2 cells. The ILC3 subset, which express retinoid-related orphan receptor γt and produce IL-17A/F and IL-22 has not been previously implicated in antiviral responses. However, work by Stier et al. has found that during RSV infection, reduced numbers of antiviral IFN-γ$^+$ ILC1 and increased numbers of pathogenic IL-5$^+$ and IL-13$^+$ ILC2 and IL-17A$^+$ ILC3 accumulate in the lungs of STAT1-deficient mice (195). This raises the possibility that the inflammatory ILC3 subset may play a role in virally induced lung pathology. Alongside the newly described ILC regulatory subset, it remains to be seen, however, whether ILC3 cells have a functional role in the antiviral response.

The inflammatory milieu within the lungs during viral infections is heavily composed of neutrophils. These short-lived, terminally differentiated, phagocytic cells have been implicated in the early response to RSV, especially in infants which go on to develop bronchiolitis (196). Their recruitment to sites of infection within the mucosal tissues is associated with viral killing by oxygen dependent and independent mechanisms. In addition to this secretion of antimicrobial products through degranulation, activated neutrophils also release neutrophil extracellular traps (NETs), which consist of chromatin and granule proteins such as elastase. Neutrophil-derived elastase and chemoattactants such as IL-8 are elevated during initial RSV infection, and neutrophils are capable of ameliorating disease during influenza infection (197, 198). During the course of infection with both viruses an influx of neutrophils can stimulate antiviral IFNγ release by CD8$^+$ T cells (199, 200). In mouse models of influenza infection, administration of the mAb 1A8 (which specifically targets the Ly6G antigen expressed by neutrophils), enhances viral replication and exacerbates pulmonary inflammation, edema, and respiratory dysfunction (201). Depletion of neutrophils with 1A8 also impairs the cytotoxic function and cytokine release of influenza

specific pulmonary CD8+ T cells (202), and can lead to increased susceptibility to lethal challenge with virulent influenza strains (198). However, contradictory evidence suggests that excessive infiltration of neutrophils into the airways is associated with a fatal outcome in H7N9 and H1N1 infected patients, with NETs playing a key role in influenza induced lung damage (203). The importance of neutrophil infiltration and formation of NETs in ALI has also been observed in mouse models of sublethal influenza challenge. Following administration of 1A8 mAb, mice exposed to H1N1 on a PR8 backbone strain demonstrated milder lung pathology than macrophage depleted mice (204). Research by Brandes et al. suggests that neutrophil infiltration establishes a damaging feed-forward loop which is dependent upon the viral strain and inoculating dose (205), which may go some way to explaining the discrepancies observed in the pathogenic role neutrophils play during influenza infection. It appears that while recruitment of these destructive cells to the delicate tissue of the lung may be crucial in early clearance of viral particles, their proinflammatory nature can also cause substantial immune pathology.

Antigen Presentation and Induction of Proinflammatory Cytokines by DCs

Dendritic cells play a crucial role in linking the innate and adaptive immune systems, especially at mucosal surfaces. The majority of immature DCs fulfill a sentinel function in the periphery, including the respiratory system. Here, they may remain for up to two weeks before leaving tissues *via* the lymphatics, although the turnover of DCs in the mucosa is much faster. Engagement of PRRs expressed by DCs leads to the upregulation of costimulatory molecules, and the generation of proinflammatory cytokines, such as IL-6, IL-12, and TNF, which trigger a maturation program determining how a naive T cell responds to antigen stimulation. The wide range of pulmonary DC subsets fulfill distinct, occasionally overlapping functions, influencing viral clearance from the lung and induction of T cell responses. Following PRR stimulation, DCs downregulate the expression of tissue retaining chemokine receptors, CXCR1, CCR1, CCR2, and CCR5, and upregulate CCR7 and CD11c, allowing the DC to home to secondary lymphoid organs. Within the lymph node or spleen the mature DC, which has massively upregulated the expression of both peptide-loaded major histocompatibility complex (MHC) and costimulatory molecules at the cell surface, is capable presenting antigen to cognate T cells. Murine lung DCs can be divided into CD103+ conventional DCs (cDCs) and CD11b+ cDCs (in humans these correspond, respectively, to the CD141+ and CD1c+ DC subsets), and plasmacytoid DCs (pDCs). Monocyte-derived DCs are also generated in the lung during inflammatory episodes.

Influenza and RSV infection have both been reported to cause CD103+ cDCs to migrate from the intraepithelial basement layer to draining mediastinal lymph nodes for antigen presentation to naive T cells (175, 206). However, severe influenza infection particularly leads to lymph node accumulation of CD11b+ cDCs in the lymph nodes, with these DC subsets dominating antigen presentation at the peak of infection (207). As priming during early infection is characterized by a more even balance of CD11b+ and CD103+, it is unclear whether severity of infection, inoculating dose or temporal kinetics govern the subsets activated. In the context of viral immunity it is important to note that these DCs tend to differ in their antigen presentation. CD11b+ cDCs readily present viral antigens to CD4+ T cells through MHC class II, promoting both Th2 and Th17 responses. CD103+ cDCs, meanwhile are capable of biasing toward a Th1 response, but also specialize in the cross-presentation of antigen through MHC class I pathways, driving CD8+ T cell priming. This is further complicated by studies indicating that differential expression of CD24 by CD103+ DCs and CD11b+ DCs lead to preferential induction of effector and central memory CD8+ T cells, respectively (208). Bearing this in mind, it is unsurprising that depletion of CD11c+ cDCs results in the impaired development of influenza virus-specific CD8+ T cells in the mouse model (175, 209),.

Significantly, studies in neonatal mice have described a fundamental shift in the balance of cDC subsets in early life. While both CD103+ and CD11b+ cDCs are substantially less effective in infants than adults, a functionally limited CD103+ population dominates the response in favor of a diminished CD11b+ cDC subset in early life (210). In mice, this shift in cDC subsets influences the CD8+ T cell epitope hierarchy during RSV infection, dramatically changing the immunodominant epitopes between infancy and adulthood (210, 211). Recent work by Ruckwardt et al. suggests in the neonatal mouse model that both RSV and influenza infection lead to the production of a distinct $CD103^{low}$ population which predominates over $CD103^{high}$ in early life. These express lower levels of lineage-defining markers and costimulatory molecules, as well as more limited antigen uptake and processing capabilities (211). This highlights the complex and subtle changes undergone by the immune system at different ages, confounding our understanding of antiviral immunity in patients.

DIFFERENTIAL INDUCTION OF LONG-TERM HUMORAL IMMUNITY BY INFLUENZA AND RSV

Natural infection by influenza is known to induce long-lived high affinity antibodies that are associated with complete protection from symptomatic infection for many years (212). However, the defining characteristic of influenza vaccination is the requirement for reformulation on a regular basis to match predominant circulating strains. This is due to the majority of human antibodies induced by influenza being directed toward the immunodominant globular head domain of HA. Following infection, high levels of strain-specific anti-HA antibodies confer long-lasting protection that can endure for many years, but rapid accumulation of mutations in the globular HA head leads to antigenic escape. Recent work to develop a cross-strain protective vaccine has therefore focused on inducing antibodies against the more conserved HA stalk. Mouse studies have demonstrated that sequential exposure to divergent influenza strains can trigger the production of broadly neutralizing anti-stalk antibodies (213). In humans, infection with pH1N1 boosted the production of

HA stalk antibodies and these neutralizing antibodies could in principle be replicated by vaccination (214). However, while neutralizing antibodies are understood to correlate with protection against influenza infection, it has been suggested that heterosubtypic stalk-binding mAbs may be less effective and display limited activity against cell-bound viruses (215). Nevertheless, conflicting evidence has revealed that not only may these antibodies actually outperform conventional neutralizing antibodies within a more physiologically relevant polyclonal condition (216), but also that *in vivo* these antibodies are able to activate antibody-dependent cellular cytotoxicity which correlates with enhanced antiviral protection (217).

Historically vaccine efforts against RSV have also focused on the induction of neutralizing antibody titers, and an association between systemic antibody levels and protection from severe disease has been shown both in hospitalized patients and in animal models (218). Indeed, the efficacy of the mAb palivizumab in reducing RSV-related hospitalization in high-risk infants proves the efficacy of serum IgG alone in preventing RSV LRTI (219). However, it has long been known that while higher levels of neutralizing antibody and F protein-specific IgG do correlate with protection from severe disease, it is still possible to experimentally re-infect adults with RSV regardless of their systemic antibody levels (11). This contrasts with influenza, where re-challenge after an interval of as much as 7 years fails to induce any viral replication or symptoms (212), and pH1N1 antibodies are long-lasting (220). This has led to difficulty in identifying a threshold of protective titers for anti-RSV antibodies. Furthermore, levels of antibody show a precipitous decline post-infection and do not provide incremental protection during subsequent RSV seasons (221).

As respiratory viruses, both RSV and influenza are capable of inducing mucosal IgA, which acts as first-line antiviral defense. Mouse models have confirmed that these antibodies are secreted rapidly in the upper airways following primary infection with RSV (222), and in both viruses secretory IgA (sIgA) has been shown to confer protective immunity in mice (222–224). In humans the role of sIgA in protection is relatively poorly understood. However, recent RSV challenge studies have revealed that, while serum antibody does not significantly affect the likelihood of infection, nasal anti-RSV and anti-F protein IgA titers are significantly higher in individuals who are resistant to experimental challenge (225). This is supported by findings by Bagga et al. confirming that pre-existing nasal IgA is a predictor of lower infectivity and viral replication, although in this model, serum neutralizing antibody titers played a greater role in predicting immunity (226).

Antibody producing B cells can be generated in a T cell dependent or independent manner. The majority of IgA^+ memory B cells (MBC) and long-lived IgA^+ plasma cells develop within the germinal centers (GC) of peripheral lymphoid organs, undergoing affinity maturation and somatic hypermutation, predominantly requiring T-cell help *via* CD40L and transforming growth factor (TGF) β1, although T-cell independent B-cell class switching has been observed in the GC, mediated by follicular DCs (fDC). DCs are derived from mesenchymal rather than hematopoietic origin, and unlike pDCs and mDCs they do not express MHC molecules at the cell surface; instead antigens complexed to antibodies or complement are displayed at the cell surface for recognition by B lymphocytes. These antigen presenting cells (APCs) are a key component in the generation of long-lived MBC and plasma cells, but mature fDC networks are absent in early life, which may limit responses of T cell dependent ASCs. MBCs and plasma cells have distinct phenotypic characteristics, which may be impacted by early life experiences with enduring consequences upon immune responses. Human challenge models have demonstrated that while IgG^+ MBC frequencies increase following both RSV and influenza infection these cells did not influence the outcome of viral challenge (225). There was however a profound defect in the induction of RSV- and F-specific IgA-producing MBCs which showed no detectable increase in frequency following RSV infection (225). This can be contrasted with individuals naturally infected during the 1918 influenza pandemic, who were found to have functional strain specific MBCs circulating up to 70 years following exposure (227) with long-lived plasma cells producing high affinity antibodies against not only 1918 strains, but also contemporary pH1N1 from 2009 (228).

Antigen-specific IgG and IgA producing MBCs also develop at extrafollicular mucosal sites in a T cell dependent or independent manner, involving B cell activating factor of the TNF family (BAFF) and a proliferation-inducing ligand (APRIL), which in influenza infection leads to protection against reinfection with similar strains (229). BAFF protein expression has been found in the upper airways of children with influenza virus, as well as in the lower airways of infants with severe RSV bronchiolitis (230). However, recent work in the closely related BRSV has suggested that the SH protein not only inhibits NF-κB expression in APCs (231), but also blocks phosphorylation of STAT1 thereby inhibiting BAFF expression and potentially interfering with induction of B cell responses. Infection of APCs with an SH deletion mutant led to increased production of BAFF and APRIL. It remains to be seen whether this action of the human RSV SH protein may contribute to the peculiar defect in MBC induction seen in RSV, but not influenza.

GENERATING EFFECTIVE T CELL HELP IN INFLUENZA

Animal models have clearly shown that both $CD4^+$ and $CD8^+$ T cells are essential for effective resolution of viral infection and protective memory. Influenza vaccination strategies may therefore be improved by targeting conserved regions of HA and NA antigens for presentation by MHC molecules to expand the $CD4^+$ T cell repertoire. This is an attractive strategy as $CD4^+$ T cells are capable of supporting both humoral responses and enhancing $CD8^+$ T cell immunity to generate high affinity and cross-protective responses. The predominant $CD4^+$ population capable of inducing the generation of high-affinity, class-switched antibodies are the T follicular helper (TfH) cells which reside within the GC. These cells are involved in the production of IL-2, IL-4, IL-10, TGFβ, and IL-21, which promotes the generation of plasma cells and MBCs (232). TGFβ and IL-21 in particular, have been found to promote class-switching and stimulate the

generation of IgA$^+$ plasmablasts (233). These cytokines also act to downregulate CXCR5 and upregulate CCR10 on plasmablasts, enhancing migration toward local mucosa. TfH cells expressing CXCR5, programmed cell death protein 1 (PD-1), inducible costimulator (ICOS), CD28, CD40L, the adaptor protein SAP, and the B cell lymphoma 6 (Bcl-6) transcription factor are abundant in the nasopharynx-associated lymphoid tissues during early childhood. Stimulation by live attenuated influenza vaccine induces a marked increase in TfH frequency which correlates with anti-HA IgA IgG and IgM antibodies in human tonsillar cells (234). Work by Leon et al. in mice has furthered our understanding of the GC TfH response to viral antigens, suggesting that FoxP3$^+$ Tregs play a critical role in the differentiation of influenza-specific TfH cells by regulating the availability of suppressive IL-2 *in vivo* (235). Miyauchi et al. has also posited that mice deficient in Bcl-6, and consequently TfH cells, are capable of producing low affinity IgG2 neutralizing antibodies, which nevertheless provide protective immunity against lethal challenge with pathogenic H5N1 and pH1N1 strains (236). It is possible, in this case, that the role of promoting B cell proliferation and differentiation into plasma cells was assumed by IL-21 and IFNγ-secreting CXCR3$^+$CD4$^+$ Th1 cells. The existence of a circulating compartment of TfH cells within the periphery has also been recently demonstrated in humans. This ICOS$^+$ PD-1$^+$ CXCR3$^+$ population correlates with the generation of high-avidity antibodies following influenza vaccination (237, 238).

There is a paucity of work demonstrating the role of CD4$^+$ T cells in human RSV. The first work exploring the impact of RSV upon TfH suggested that this virus is capable of upregulating expression of programed death ligand 1 (PD-L1) on murine DCs and B cells to decrease TfH production of IL-21 and expression of IL-21R (239). It is already known from coculture experiments that blocking PD-L1 on RSV-infected bronchial epithelial cells enhances effector-memory and terminally differentiated CD8$^+$ T cell secretion of IFNγ, and granzyme B, enhancing viral clearance (240). It is currently not known whether Tregs affect TfH differentiation in response to RSV, with the impact of Tregs during an RSV infection related to their recruitment of activated CTLs into the lungs, increasing viral clearance (241). RSV may also act to inhibit Treg differentiation, stimulating a pathogenic Th17/Th2 response (242, 243). If defects in CD4$^+$ T cell help of B cells, such as a dysregulated PD-L1/IL-21 axis or failure to promote class switching to IgA in the secondary lymphoid tissues, can be demonstrated in human RSV infection, this may provide fresh clues regarding mechanisms by which this virus modulates the protective adaptive response.

IMPAIRED CELL-MEDIATED IMMUNITY IN HUMAN RSV

The protective and pathogenic effects of T cells in respiratory virus infections have been extensively reviewed elsewhere (244–247). Mouse models have shown that both CD4$^+$ and CD8$^+$ T cells mediate RSV clearance (248), and studies in hospitalized infants have demonstrated a robust antigen-specific CD8$^+$ T cell response peaking at convalescence (249, 250). In addition, heterosubtypic immunity against influenza in mice is promoted by crossreactive CD8$^+$ T cells (251), while antibody production is impaired in T cell deficient mice (252). In humans, both CD4$^+$ and CD8$^+$ influenza specific T cells have been shown to correlate with reduced disease severity in experimental challenge and natural infection (129, 253).

However, in human RSV, impairment in T cell function has been implicated in susceptibility to recurrent infection. For example, population-based studies have shown an association between reduction in T cell numbers and function and susceptibility to more severe disease. This is perhaps more obvious in older adults, where an age-related decreases in the numbers of RSV-specific T cells and ratio of CD8$^+$/CD4$^+$ memory T cells expressing IFNγ is associated with waning immunity observed in elderly adults (254–256). However, confounding difficulties with confounders in natural infection studies have meant that there are also studies which do not show impaired CD8$^+$ T cells activation in response to RSV infection in the elderly (257).

Human challenge studies of young adults have more directly demonstrated the limited polyfunctionality of circulating RSV-specific CD8$^+$ T cells compared to influenza-specific CD8$^+$ T cells from the same individuals (128). During RSV infection cDCs and pDCS are capable of migrating to draining lymph nodes bearing antigens from the lung, but these cells have proven poor inducers of CD4$^+$ T cell proliferation (177). *In vitro* studies have also suggested that RSV infection causes suboptimal DC activation and that these DCs are defective in antigen presentation *via* the immune synapse (258). Given that primary expansion, CTL activity and effective long-term memory of antiviral CD8$^+$ T cells are all optimally dependent upon CD4$^+$ T cell help, this may suggest a mechanism for RSV impaired CD8$^+$ T cell function as well as B cell memory. Indeed, in an *in vitro* coculture system, DCs pulsed with recombinant RSV NS1-deletion mutant resulted in an increased activation and proliferation of tissue homing CD103$^+$ CD8$^+$ T cells and antiviral Th17 cells, suggesting an NS1-mediated mechanism of immune modulation being responsible for CD8$^+$ T cell impairment (259). RSV infection of DCs has also been shown to inhibit the production of proinflammatory cytokines, including IFNγ (260). The result, it has been speculated, is suboptimal signaling to T cells and therefore failure to optimally stimulate a full program of differentiation for the generation of long-lived polyfunctional T cells.

In mouse models of influenza Tem cells proliferate in the lungs contributing to heterosubtypic immunity (261, 262). However, Jozwik et al. did find that local virus-specific CD8$^+$ T resident memory (Trm) cells in the airway (that express the canonical markers CD103 and CD69) correlated significantly with reduced RSV disease severity, suggesting that while T cell functionality might be impaired, their presence at the site of infection could still lead to improved outcome (128). The role of Trm cells in immunity against respiratory viruses has only recently begun to be unraveled, with these cells differentiating in the lymph node following antigen presentation by CD103$^+$ DCs. Given that both RSV and influenza infection promote CD103^{low} DCs in early life, it is tempting to speculate that this represents an attempt by the

TABLE 1 | Comparative immunity against influenza and respiratory syncytial virus (RSV).

	Influenza	RSV
Clinical outcome of natural infection	Robust strain-specific protection	Recurrent symptomatic infection throughout life
Virology	Highly variable surface glycoproteins Segmented genome	Major surface target F protein highly conserved Non-segmented genome
PRR recognition	TLR 3/7/8/9 sensing RIG-I sensing NLRP3 inflammasome partial evasion	TLR 3/7 sensing TLR 2/4/6 sensing RIG-I less important? NLRP3 inflammasome activation
Primary sources of innate IFN	Plasmacytoid DCs Epithelial cells	Alveolar macrophages Epithelial cells
Suppression of innate IFN	NS1 block PKR	NS1 and NS2 block RIG-I/MAVS interaction NS2 degrades STAT2
Recruitment of immune cells	Th1-promoting environment	CX3C chemokine mimicry dysregulates inflammation
Antibodies	Protective and long-lasting	Protective but short-lived
B cells	Robust IgG^+ and IgA^+ MBCs	IgG^+ MBCs Poor IgA^+ MBC response Inhibition of BAFF?
T cells	Th1 and $CD8^+$ dominated Highly polyfunctional	Th2/Th17 bias in early life Poor polyfunctionality

viruses to subvert Trm production. Recent work by Zens et al. has also shed light upon the inefficient generation of Trm cells observed in early life (263). It appears that the reduced generation of Trm may be a factor intrinsic to infancy, as mouse models of influenza infection and vaccination demonstrate ineffective generation of lung Trms and promotion of long term memory, despite the generation of $CD4^+$ and $CD8^+$ effector T cells capable of viral clearance (263). In contrast to immunity in adults, in infants the distinct transcriptional profile of these lung-homing T cells favors enhanced T-bet expression and reduced expression of the survival factor CD127, leading to pathogen clearance by terminally differentiated T cells. Furthermore, it is clear from influenza mouse models that $CD4^+$ T cell help is required for the promotion of $CD103^+$ $CD8^+$ Trm cells (264) and so both play an important part in the induction of heterosubtypic immunity (265). Interestingly, unlike most other tissues where Trm cells have been investigated, lung Trm cells are not persistent and undergo attrition over time (265, 266). In view of the work by Jozwik et al. this suggests that while Trm can be important in the early clearance of viruses from the lung, RSV-specific impairment of T cell functionality contributes more rapidly to the downregulation of protective immunity, compared with influenza.

It remains to be seen whether stimulation of Trm cells by influenza or RSV vaccination can enhance protection but if indeed they can, a careful balance is likely to be required. Data from healthy young adults in experimental challenge and natural infection studies as well as in infants with severe RSV and influenza LRTI all suggest that lower $CD8^+$ T cell numbers may be associated with more severe disease (183). H owever, induction of T cell immunity in the delicate mucosal structures of the lung has also been associated with greater severity of disease, with a higher frequency of activated T cells in both experimentally infected mice (248, 267) and RSV-infected adults who required hospitalization (257). This dysregulation of the immune system may also be seen in influenza infection with an increased release of proinflammatory cytokines. Thus, cell-mediated immunity is critical in clearing viral infection and coordinating the adaptive immune response as a whole. However, the exact role of each subset, their anatomical location and how they can be induced and maintained at optimum levels must be better understood before harnessing them to improve protection.

CONCLUSION

It has long been recognized that influenza and RSV infection lead to highly distinct clinical and immunologic outcomes in humans. While the long-lived antibody and cell-mediated protection conferred by influenza infection is the prototype of a complete and robust antiviral response, immunity against RSV is poor in all settings and unable to fully protect against recurrent symptomatic infection even with an identical strain. It is increasingly clear from natural infection and human challenge studies that the immediate reason for this is impairment in quantity, quality and durability of antibodies, B cells and T cells. However, the underlying mechanisms leading to this remain poorly understood. It seems likely from the wealth of *in vitro* and animal data that both influenza and RSV subvert innate immunity in order to establish infection (**Table 1**). However, RSV additionally impairs long-term memory generation. The relative contribution of mechanisms such as alteration of the inflammatory environment, IFN inhibition, and impaired antigen presentation is still unclear. By further understanding of how influenza induces robust immunity whilst RSV evades it, a more general understanding of the critical components of an effective immune response is now coming through but many knowledge gaps remain. Development of novel vaccination strategies are likely to require these insights so that immunogenicity can be optimized through avoiding or overcoming pathogen-induced immunomodulatory mechanisms and thus successfully elicit long-term protective immunity.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the manuscript and approved it for publication.

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Factors Affecting the Immunity to Respiratory Syncytial Virus: From Epigenetics to Microbiome

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Respiratory syncytial virus (RSV) is a common pathogen that infects virtually all children by 2 years of age and is the leading cause of hospitalization of infants worldwide. While most children experience mild symptoms, some children progress to severe lower respiratory tract infection. Those children with severe disease have a much higher risk of developing childhood wheezing later in life. Many risk factors are known to result in exacerbated disease, including premature birth and early age of RSV infection, when the immune system is relatively immature. The development of the immune system before and after birth may be altered by several extrinsic and intrinsic factors that could lead to severe disease predisposition in children who do not exhibit any currently known risk factors. Recently, the role of the microbiome and the resulting metabolite profile has been an area of intense study in the development of lung disease, including viral infection and asthma. This review explores both known risk factors that can lead to severe RSV-induced disease as well as emerging topics in the development of immunity to RSV and the long-term consequences of severe infection.

Keywords: respiratory syncytial virus, neonatal immunity, epigenetics, microbiome, metabolites

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CURRENT STATUS OF RSV DISEASE AND TREATMENT

Respiratory syncytial virus (RSV) is an omnipresent virus that infects nearly all children by age 2 years (1). It is also the most common cause of hospitalization in children under 2 years of age, causing severe lower respiratory tract infection, bronchiolitis, and pneumonia, and is associated with an increased risk of developing childhood asthma and recurrent wheezing (1, 2). Severe RSV infections often exhibit an inefficient activation of innate immunity and skewing of the acquired immune response toward a Th2 and/or Th17 phenotype leading to airway mucus overproduction, with both an excessive Th2 response and with IL-17 highly upregulated in infants (1, 3–6).

Considerable effort has been expended to develop an effective vaccine against RSV infection without success. The prophylactic use of a neutralizing monoclonal antibody against the RSV F envelope protein (palivizumab) prevents progression of RSV severity in the high-risk patient with RSV infection (7). It is also used as a therapy to control the infection in patients who present with RSV-related bronchiolitis (7, 8). However, this treatment is expensive, and most infants who develop severe infection do not exhibit risk factors, necessitating a better understanding of what drives severe disease in order to develop new therapeutics or effective vaccines.

In this review, we cover several aspects that contribute to the immune response to RSV, including the development of the immune system and the lung environment, the role of epigenetics

in this development, and the contribution of the microbiome to long-term pulmonary health. These factors are summarized in **Figure 1**.

THE NEONATAL IMMUNE SYSTEM AND RSV

The age at which a child is infected with RSV plays a critical role in disease outcome. Infants who are infected before 6 months of age are at higher risk for developing bronchiolitis that requires hospitalization, even when compared with bronchiolitis caused by other viral infections (9). One of the primary reasons for this is that the neonatal immune system has a number of functional differences when compared with the immune response of adults.

Early in life, immune protection is mediated primarily by the innate immune system, as adaptive immunity does not begin to develop until after birth and continues developing through early childhood (10). Furthermore, the immune system is skewed away from a pro-inflammatory state, as this can lead to spontaneous abortion and damage to the developing tissue, especially the lungs (11). Thus, neonatal immune cells exhibit low levels of pro-inflammatory cytokines including the type I interferons (IFN), IFN-α and IFN-β, as well as low levels of IL-12 and TNF-α, but higher levels of anti-inflammatory cytokines such as IL-10 and TGF-β (12–14).

Because the neonatal system is reliant on innate immune recognition, signaling through toll-like receptors (TLRs) is relatively well developed in neonates. These pattern recognition receptors are a critical component of the innate sensing of pathogens in infants. However, although TLR sensing and signaling in infants occurs on a similar level to that in adult cells, the immune mediators that result from these signaling pathways are impaired in infants. While type I IFN levels rise rapidly in the first month of life, IL-12, which is important for driving a Th1 response, is one of the last cytokines to reach adult-like levels (13, 14). This

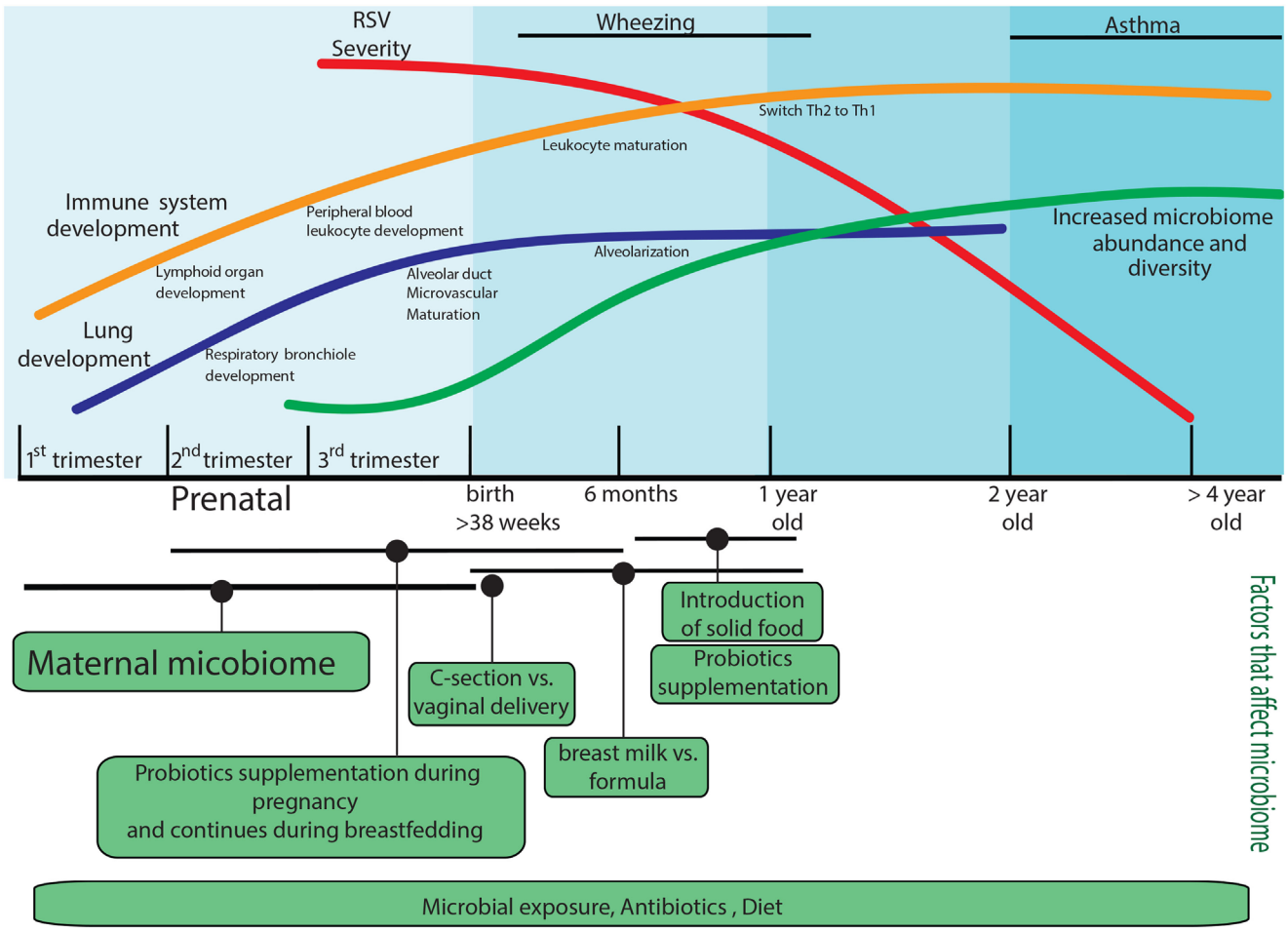

FIGURE 1 | Factors that predispose to the development of severe respiratory syncytial virus (RSV) disease. Factors that can impact the immune response during RSV infection are age; lung development is not complete in preterm infants, and the immune system development will continue until the first years of life, and it will be impacted by the microbiome composition of the mother and infant. The infant microbiome would be shape since the prenatal stage by the mother microbiome, and it will continue modifying by postnatal factors such as environmental microbial exposure, mode of delivery, diet, and antibiotic use. The infant microbiome would have long-acting effects on RSV immune responses. All these components independently or together represent the most common elements that are involved in the development of RSV severe disease.

increase in pro-inflammatory cytokines is mirrored by a decrease in anti-inflammatory IL-10 following TLR signaling (13). IL-6 and IL-23 are also enhanced in TLR-stimulated neonatal cells compared to adults (13, 14). As these cytokines are potent inducers of IL-17, neonates have an increase in Th17 cells (15). Because increased IL-17 is often associated with enhanced RSV-induced pathology, the prevalence of this cytokine in infants may contribute to enhanced disease (3, 4, 6). An additional level of TLR regulation in neonates is the high level of adenosine found in the plasma of umbilical cord blood (16). Adenosine is a metabolite that is released in response to inflammatory events and acts as a feedback mechanism to decrease TLR-dependent Th1 inflammation (17–19). This combination of both cell-intrinsic factors such as decreased cytokine production and exogenous factors such as plasma adenosine limits the function of TLRs in the neonates.

Dendritic cells (DCs) express a number of TLRs that are essential for dictating T cells responses based on the ability of DCs to present antigen to T cells causing their activation, and subsequent cytokine production. Myeloid DCs (mDCs) are the primary source of the IL-12 needed to drive a Th1 response. As mentioned above, IL-12 levels are reduced in neonates compared to adults. mDCs are also a significant source of other pro-inflammatory cytokines including IFN-β, which is robust in adult cells but impaired in neonatal DCs (20, 21). Neonatal and adult mDCs also exhibit a number of other differences including lower expression on neonatal cells of MHC-II for antigen presentation and CD80/86 for costimulation of T cells (21, 22). Activation through TLR signaling or cytokine addition fails to upregulate these surface molecules to a level seen in adult mDCs (21). Plasmacytoid DCs (pDCs) are another subset of DCs involved in directing inflammatory responses. While in adults, the number of mDCs is higher than pDCs, neonates have approximately three times the amount of pDCs as mDCs (23). However, even with this increased predominance, pDCs from neonates fail to adequately mature upon stimulation, including decreased production of type I IFNs that are critical for the antiviral response (12, 24).

Neutrophils are another critical innate cell type, which function independently of antigen recognition. While neutrophil infiltration into the lungs in response to RSV may contribute to tissue damage, these cells also help limit the spread of the virus and participate in the induction of the adaptive immune response (25–28). Infants have a higher number of neutrophils in the blood than adults, but these cells have decreased function (29). Neutrophils can control viral infection through phagocytosis and release of cytotoxic granules for killing the infected cells; both of these features are impaired in neonatal neutrophils (29, 30). During RSV infection, neutrophils can phagocytose RSV-infected cells as well as protect epithelial cells from infection, providing a protective role against viral replication (25, 31, 32). Although damaging effects of neutrophils are thought to contribute to a decline in lung function following infection, they are an essential part of the innate immune response to RSV (28).

Natural killer (NK) cells are one of the more prominent cells types in the blood of neonates, with levels exceeding those of adult blood. However, like many other cell types, the function of these cells is drastically reduced early in life. NK cells bind to target cells, such as virally infected cells, and release cytotoxic granules to kill the infected cells. The cytotoxic ability of neonatal NK cells is decreased compared to adults, as cells from infants have fewer cytotoxic granules as well as a decreased capacity for degranulation to release their contents (33, 34). Whether these cells have dysfunctional migration is unknown; however, infants with severe RSV infection have been noted to have low numbers of NK cells, underscoring the need for these cells in a productive response to the infection (35). NK cells also produce IFN-γ, and neonatal NK cells produce less of this Th1 cytokine than adult cells (36). However, unlike other cell types, these defects are not intrinsic to neonatal NK cells but are instead due to decreased signaling and activation from pro-inflammatory cytokines made by other immune cells. Exogenous addition of IL-12 to neonatal NK cells restores their cytotoxic effects and ability to produce IFN-γ (36–38). As mentioned above, IL-12 production from DCs is delayed in neonates, thereby resulting in an environment that is less permissive for NK cell activation, as well as Th1 cell differentiation.

Neonatal $CD4^+$ T cells are skewed away from a pro-inflammatory Th1 response and instead favor a Th2 or Th17 response, both of which have been implicated in severe RSV infection (4, 39). Like NK cells, this is at least in part due to extrinsic factors such as decreased DC-derived IL-12 in the environment, as these cells can produce IFN-γ when transferred into an adult environment (40). However, there are T cell-intrinsic factors that limit Th1 responses as well, including a number of epigenetic factors that will be described later in this review. Additionally, many of the circulating T cells in neonates are known as recent thymic emigrants, which do not proliferate as well as other circulating T cell subsets and produce lower levels of IFN-γ (41). Finally, neonates have increased numbers of regulatory T cells (Tregs), which limit inflammatory responses early in life (42–44). As previously noted, this is important for preventing tissue damage. In addition, maternal immune cells can cross the placenta and reside in developing fetal lymph nodes. As some of these cells can recognize proteins derived from paternal genes, Tregs are a critical mechanism for suppressing this maternal immunity and preventing fetal rejection (45).

The decrease in $CD4^+$ T cells in neonates translates to altered B cell function early in life. As these T helper cells are needed to drive antibody production from B cells, neonates have delayed antibody responses compared to adults, including antibody responses to RSV (46, 47). Neonates also have a different distribution of IgG isotypes, with lower levels of Th1-associated IgG2 (48). Antibody responses in neonates are also of lower titer, lower affinity, and persist for less time, but begin to reach adult-like function after 6 months of age (49–51). In general, the primary antibody protection in infants comes from maternal antibody transfer, both *in utero* and through breast milk following birth. This latter aspect has led to the support of developing a vaccine for RSV that would target mothers in order to transfer maternal protective antibodies to infants to provide early life protection from severe infections (52–54).

PRETERM BIRTH AND RSV RISK

As previously mentioned, infants under 6 months of age are at a higher risk of severe RSV infection requiring hospitalization

for RSV-induced bronchiolitis. This is especially true of infants who are born prematurely (<37 weeks gestation) (55, 56). There are number of reasons why premature birth results in increased risk for severe RSV infection. The differences in the immune response between infants and adults are more pronounced in preterm neonates. As mentioned above, maternal antibodies are a significant source of immune protection in neonates and are able to cross the placenta during pregnancy, and the majority of this occurs during the third trimester (57). Babies born to mothers with high levels of RSV neutralizing antibody are protected from severe diseases. However, this protection is to an extent lacking in preterm infants (58). Thus, treating preterm infants with palivizumab has become more standard for these infants born within RSV season, which occurs during the winter months (58).

As noted earlier, neutrophils are critical regulators of innate immunity and are especially important in early life when the adaptive immune response has not fully developed. However, neutrophils in preterm neonates exhibit reduced function compared with those from term infants. It has long been known that neutrophils from preterm neonates have decreased migratory ability compared with the cells from term neonates (59, 60). Neutrophils from these infants release lower levels of bactericidal/permeability-increasing protein than cells from term infants, which may result in increased susceptibility to infection (61). Recently, it has been shown that neutrophils from preterm infants have decreased pathogen recognition and antimicrobial ability as well (62). One study found that nasal washes from preterm infants have fewer leukocytes and lower levels of IL-8 than washes from term infants, which is a key cytokine in the recruitment of neutrophils, indicating a role for neutrophils in RSV infection of preterm neonates (63).

Numerous other innate immune defenses are also deficient in preterm neonates compared to term neonates. A small study of infected infants found that preterm infants infected with RSV had fewer pDCs in the bronchoalveolar lavage fluid than those born at term (64). In addition to decreased cell number in preterm infants, monocyte activity is also decreased. A recent study found decreased inflammatory gene expression in monocytes of preterm cord blood compared with both term cord blood and adult peripheral blood (65). Upon exposure to RSV, lowere IL-6 production was noted in monocytes from the cord blood of preterm infants compared to term and adult blood (66). The overall reduction in the innate immune cell responsiveness to RSV infection may be intrinsic and therefore difficult to overcome by vaccination, especially if muted adjuvant reactivity is also a lingering issue (66).

In addition to immunologic deficiencies in preterm neonates compared to those born at term, complications also arise due to differences in lung development. The initial capacity for oxygen exchange occurs late in development when alveolar septal formation begins at approximately 34 weeks of gestation (67, 68). This increases the number of alveoli in the distal airways, thereby increasing the surface area available for oxygen exchange. As this process continues into the postnatal period, premature birth before the process is fully underway can have serious consequences. The primary pathology that occurs in the lungs from premature birth is bronchopulmonary dysplasia (BPD), which results in a decrease in the number of alveoli (69). RSV infection of infants with BPD results in a much higher rate of hospitalization than for RSV-infected infants without BPD, even when comparing infants born at similar gestational ages (70). Treatment of these patients with appropriate antiviral therapy or palivizumab, although complex, would greatly reduce the hospitalization rates due to RSV infection (70).

EPIGENETIC MODIFICATIONS AFFECTING THE IMMUNE RESPONSE TO RSV

Many of the differences in immune function between neonates and adults are due to epigenetic modification of genes that control inflammation. Epigenetics refers to mechanisms that alter gene expression without changes to the sequence of underlying DNA, thereby controlling many aspects of development. The three primary types of epigenetic regulation are histone modifications, DNA methylation, and microRNA expression. Histone modifications can regulate gene expression by altering residues on the histone tails, such as methylation, acetylation, and phosphorylation. In general, histone modifications "fine tune" the gene expression by altering chromatin structure. This change in structure can wind the chromatin more tightly to prevent access of transcription factors and repress gene transcription. Alternatively, the change in chromatin structure can result in increased DNA accessibility to transcription factor binding, therefore promoting gene activation. These modifications are referred to as "activation marks" or "inhibitory marks." For example, methylation of lysine (K) 27 of histone (H) 3 (i.e., H3K27) is associated with repression of gene transcription, whereas H3K4 methylation is permissive for active gene transcription (71–73). This process is orchestrated by methyltransferases that catalyze the addition of methyl groups and demethylases that remove them. Other modifications such as acetylation and phosphorylation have similar mechanisms of action. On the other hand, DNA methylation results in the inhibition of gene transcription, resulting in genes being turned "off" (74–76). This primarily occurs on cysteine residues in regions rich in cytosine and guanidine, known as CpG islands, in which methylation of the cysteine residues results in suppression of gene transcription (77). Finally, microRNAs are small non-coding RNAs that bind to messenger RNA. miRNA recognition of target mRNA is not always 100% complementary, so a single miRNA can target multiple mRNAs (78). miRNA interaction with mRNA results in either mRNA degradation (in the case of complementary recognition) or translational repression (in the presence of base pair mismatches), but both with the final result of inhibiting protein synthesis (79, 80). Together, these mechanisms are able to alter gene expression on multiple levels, as summarized in **Figure 2**.

Epigenetic Regulation of Immune Cell Development

Several recent studies suggest that epigenetic modulation is important in dictating immune cell phenotype and function and

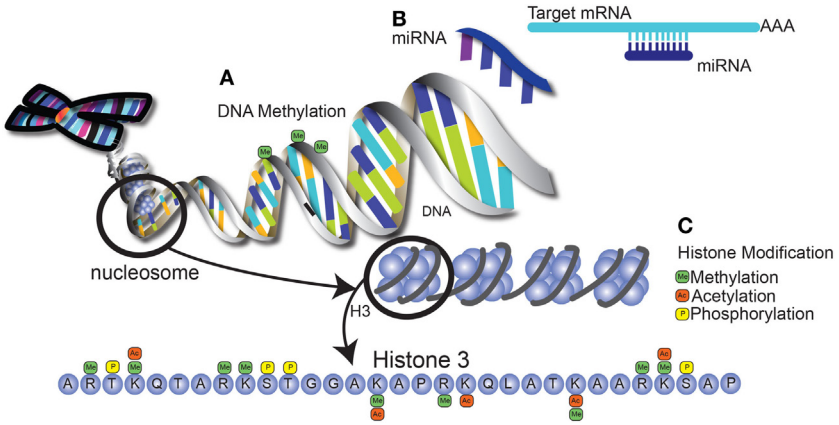

FIGURE 2 | Epigenetic modification alters gene expression through multiple mechansims. **(A)** DNA methylation occurs at regions that are rich in cytosine and guanine (CpG islands), leading to the repression of gene transcription. **(B)** MicroRNAs are short, non-coding RNA molecules encoded in the genome that bind to mRNA, leading to mRNA degradation or translational suppression. These matches can either be fully complementary or may contain mismatched bases, allowing one miRNA to target several mRNA molecules. **(C)** Histone modifications include methylation and acetylation of lysine and arginine residues on histone tails, as well as phosphorylation of serine and threonine residues. Potential modifications of the tail of histone 3 are shown as an example. These modifications result in changes in chromatin structure that can be either repressive or permissive for transcription factor binding.

allows the immune environment and/or the external environment to influence the outcome of the immune response. For example, IL-4, IL-5, and IL-13, which are produced by Th2 cells, are silenced in Th1 cells through histone modification (81). Conversely, Th17 cells have increased levels of the activating mark H3K4 methylation at the Th17 promoter (82). In DCs, decreased H3K4 methylation at the IL-12 promoter has been measured following severe sepsis, thereby altering subsequent immune responses and leading to higher susceptibility to subsequent infections (83). The epigenetic alteration of immune cells allows a more stable phenotype to develop in response to the environment, however sometimes in negative contexts (83).

In the neonatal immune system, further epigenetic regulation is present and prepares the immune cells for subsequent responses. As discussed above, neonatal immune cells are poised toward Th2 effector function. There are a number of epigenetic mechanisms behind this Th2 bias. Many studies have identified differential DNA methylation patterns in human neonatal cells compared to that of adults. In differentiated adult Th2 cells, the Th2 locus is hypomethylated, leading to a more permissive transcriptional state of Th2 cytokines (84, 85). In neonatal naïve $CD4^+$ T cells, a similar hypomethylation is observed, indicating that these cells are poised for a Th2 response even before activation (86). Hypomethylation of the *IL13* distal promoter has also been observed in naïve human neonatal $CD4^+$ T cells (87). Increased methylation of Th2 genes begins in the first year of life and continues out to 5 years of age (88, 89). Conversely, naïve human neonatal $CD4^+$ T cells have increased CpG methylation at the *IFNG* promoter compared with adult cells, which correlates with decreased IFN-γ production (90, 91). Furthermore, hypermethylation has been observed at numerous Th1-associated genes in neonatal mononuclear cells compared with adults, indicating increased regulation of Th1 responses in neonates (92). These phenotypes must be actively altered to allow a more balanced response to occur, including moving the responses to environmental and infectious agents toward a more appropriate, nonpathogenic response (92).

MicroRNAs contribute another level of regulation of immune development early in life. Many miRNAs have been found to be differentially regulated in human cord blood samples compared to adult bone marrow or peripheral blood samples (93, 94). For example, monocytes isolated from cord blood express more miR-146a following LPS stimulation compared to cells from adult peripheral blood (95). As miR-146a is a negative regulator of TLR4 and increased expression of this miRNA results in a decrease in TLR4 responsiveness in neonatal monocytes, this prevents a robust pro-inflammatory response to bacterial infection (96). T cells are also differentially regulated by miRNAs in neonates. In neonatal $CD8^+$ T cells, two miRNAs, miR-29 and miR-130, drive cells to become short-lived effector cells instead of memory effector cells, thereby altering the immunological memory to an antigen (97). Similarly, cord blood $CD4^+$ T cells express higher levels of miR-181a and miR-184 compared to adult peripheral blood cells, which decrease maturation and activation of these cells (98, 99).

Fewer studies have identified changes in histone modifications in neonatal immune cells, although some work has been done in monocytes. In particular, altered nucleosome remodeling in neonatal monocytes has been linked with defective DC development and impaired IL-12 production (100, 101). More recently, an in-depth analysis of H3K4 methylation on neonatal monocytes was performed (65). Increased methylation of H3K4 is associated with increased gene transcription, and cord blood monocytes have dramatically lower levels of H3K4 methylation compared with adult cells. Furthermore, it was noted that H3K4 methylation is nearly absent in preterm neonates and is even lower than cord blood from term infants, indicating that DNA methylation is continuously increasing during development and that monocytes

from premature infants are not "poised" for an appropriate innate immune response (65).

Epigenetic Regulation by Environmental Exposures

There are a number of epigenetically regulated factors that may affect the severity of RSV infection in infants. Environmental exposure both *in utero* and following birth results in alterations of the lung environment that may be detrimental to developing the appropriate response to RSV infection. One of the best-studied exposures is that of tobacco smoke. Infants who are exposed to tobacco smoke *in utero* are more likely to be admitted to the intensive care unit with bronchiolitis (102). This risk is further increased when these infants continue to be exposed to tobacco smoke following birth (102). A number of studies have shown that *in utero* smoke exposure results in detrimental epigenetic changes. Prenatal exposure has been shown to alter the DNA methylation status of a number of genes into childhood (103). In particular, differential methylation of genes involved in T cell development has been observed in infants exposed to tobacco smoke prenatally compared with those that were not (104). A recent study has also identified miRNAs that are altered in the lungs of mice exposed to prenatal tobacco smoke, including miRNAs that regulate immune pathways (105).

Maternal diet is known to alter the epigenetic profile *in utero*. In particular, women are encouraged to take a folic acid supplement during the first trimester of pregnancy to prevent neural tube defects. However, folate also acts as a methyl donor, and children born to women who begin folic acid supplementation 6 months before pregnancy and continue supplementation throughout pregnancy have increased methylation at CpG islands in cord blood DNA (106). These children have an increased incidence of wheezing during childhood, as well as increased risk of hospitalization with respiratory infection early in life (107, 108). These early studies set up new paradigms to focus on the role of epigenetic changes that have long-term effects on the development of lung pathology.

Epigenetic Modifications in Response to RSV

A number of studies have shown that inflammatory stimuli can alter histone methylation of immune cells, including influenza and sepsis (83, 109). We have previously found that RSV infection of DCs leads to alterations in histone methylation by the H3K4 demethylase KDM5B, resulting in decreased pro-inflammatory cytokine production and a subsequent increase of Th2 cytokines from T cells (110). We have also found that methylation of H3K4 in regulatory T cells by the histone methyltransferase SMYD3 is necessary to control inflammation in the lungs following RSV infection (111). Another group recently identified that IFN-γ stimulation of epithelial cells resulted in changes in H3K9 methylation status of the RIG-I promoter, thereby upregulating RIG-I (112). As RIG-I is an important component of the immune response to RSV, this resulted in decreasing viral load (112, 113). Other histone modifications induced by RSV infection include histone acetylation. Infection of bronchial epithelial cells results in increased histone deacetylase 2 (HDAC2) that appears to alter innate immune responses; inhibition of HDAC2 results in increased production of type I IFN and decreased viral replication both in cultured cells and following RSV infection of mice (114).

Respiratory syncytial virus infection has also been shown to alter DNA methylation status, as shown by a number of recent studies. In humans, cultured bronchial epithelial cells infected with RSV upregulate Nodal, a member of the TGF-β superfamily, resulting in increased Th2 and Th17 skewing of T cells (115). A study in the cord blood of children found that those who had increased methylation in the enhancer region of the perforin-1 gene had increased risk of developing lower viral respiratory tract infection, including an increased risk of RSV infection (116). Perforin-1 is an essential cytotoxic protein in $CD8^+$ T cells and NK cells in the control of viral infection. A follow-up study found that children at 3–4 years of age who had been hospitalized with severe RSV-induced bronchiolitis as infants had decreased methylation of the perforin-1 enhancer, indicating a role for RSV in altering the immune response even years after infection (117).

Studies in mouse models suggest that RSV can alter miRNA expression in the airway and affect how the tissue responds to future stimuli, especially in the development of post-viral allergic asthma. Infection of allergic mice with RSV results in the development of steroid-insensitive allergic airway disease, and this event is driven by the upregulation of a miRNA that inhibits steroid sensitivity (118). Another model using pneumonia virus of mice (PVM, a rodent-specific RSV homolog) found that neonatal infection changed the expression of a set of miRNAs that altered the way the mice responded to allergen challenge as adults (119). The regulation of how these miRNAs are expressed and what cell populations are expressing the specific miRNAs is not clear, but this regulation likely dictates how they alter the subsequent allergic responses (119). Together, these studies demonstrate the many ways in which the immune system is regulated during RSV infection and the potential development of allergic asthma.

MICROBIOME, RSV, AND ASTHMA

As previously described, a number of studies have linked early severe RSV infection and the consequent RSV-bronchiolitis immunopathology to the development of chronic allergic diseases like asthma in the later years of life (120, 121). We have discussed some factors that contribute to the development of severe RSV disease such as prematurity (immature immune system), congenital conditions like bronchopulmonary dysplasia, and age (from premature infants to 6 months old). There are many additional known factors that predispose to develop severe RSV disease, including Cesarean (C)-section delivery and breastfeeding for less than 1 month, which are factors that are also known to affect the composition of the microbiome and to contribute to the development of allergic disease (122–124). The microbiome assembles during the first year of life and critically sets the characteristics of the adult microbiome, as well as contributes to healthy immune responses and metabolic processes in infants (124–126).

Our lab and others have shown that microbiome composition can impact the development of RSV pathogenesis and

allergic asthma in mice previously exposed to enriched microbial suspension or probiotic prior to RSV infection or allergen challenge (127, 128). In this section, we will discuss the impact of the infant microbiome in the development of allergies and severe lower respiratory tract infection (LRTI).

Infant Microbiome and the Development of the Immune System

The human microbiome has been shown to be involved in several essential processes in humans, from organ development to modulation and fine tuning of the immune system. The microbiome is formed by microbial communities, collectively termed as microbiota. Their composition is in constant change during life, with variations of the microbiota in a number of environments, such as the lung, gut, or skin. Several factors affect the diversity of the microbial communities: the use of pharmaceuticals, nourishment, microbial exposure, and infection. Changes in the microbial diversity can impact the general homeostasis of the organism (122–126).

Impact of Prenatal Microbiome: Respiratory Virus and Allergies

Historically, the uterus has been thought to be a sterile environment, although studies have questioned whether there is microbial exposure during the prenatal period. Microbes have been described in the placenta, amniotic fluid, fetal membrane, umbilical cord blood, and meconium (129, 130). The possibility of the impact of this microbial community to the development of the immune system, metabolite production, and fetus health, in general, is an active area of investigation for many groups (129, 131–134). For example, in a study where pregnant mothers received probiotic supplements, a microbial alteration was detected in infant meconium compared to controls (130). Others have found that changes in microbiome during pregnancy are linked to different outcomes in the offspring from behavior alterations to allergy predisposition (130, 135).

Atopic dermatitis is one of the first and most studied diseases in infants used to predict their allergy susceptibility and the early manifestation of atopic dermatitis-like eczema has been associated with an increased risk of asthma (136, 137). A double-binding study showed that administration of probiotics to mothers during the last trimester of pregnancy and during lactation increased the immune-protective potential for the breast milk, by increasing TGF-b2, which is a known immunoregulatory factor, in the milk compared with placebo-receiving mothers. In this case, the infants from probiotic treated mothers had a significantly reduced risk of developing eczema during the first years of life compared with the children from placebo-treated mothers (130, 132).

Increased microbial exposure has been shown to modify the microbiome (127, 131). In a longitudinal study, a cohort of infants was followed from birth to 2 years of age and IgE was measured in blood samples. Infants born to mothers who owned pets (parental indoor pet exposure) had a lower titers of total systemic IgE from 6 months old than the children lacking prenatal pet exposure and the difference was significantly stronger at 2 years of age (134). In a double-blind trial, mothers from families with allergic disease received probiotic supplements in the last trimester of pregnancy. After birth, their babies continued with supplementation until 1 year old, and these babies had decreased allergic incidence compared to control groups. Specifically, these infants had less eczema associated with IgE-associated at 2 years of age and reduced risk of developing later respiratory allergic disease (136). Another longitudinal study found that infants who were at risk of developing asthma had a transient disruption of the gut microbiome during the first 100 days of life, with significant decreases of *Lachnospira, Veillonella, Faecalibacterium,* and *Rothia* genera (138). The reduction of these bacterial taxa also impacted the levels of acetate in the feces, as well as dysregulation of liver metabolites. Germ-free mice inoculated with these four bacteria showed decreased airway inflammation demonstrating the importance and impact of the early microbiome composition in preventing the development of asthma and possibly other allergic diseases in children (138). Together, the microbiome during pregnancy has been proposed as a critical component for the overall health of the infant. A healthy microbiome during pregnancy may modify microbial communities in the mother's gut, vagina, and uterus and would transfer the healthy microbiome in gut, lung, and skin to the fetus/infant *in utero* and during vaginal delivery. Evidence suggests that this is correlated with a healthy microbiome in the infant.

Mother to Offspring: Microbiome Alteration

It has been widely described that the mode of delivery strongly affects the microbiome species found in the neonates, specifically that C-section delivery increases the risk of several health conditions in the newborn including increases in the likelihood of respiratory distress even at term (139, 140). In addition, C-section deliveries are associated with an increased risk of developing asthma and gastrointestinal diseases, among others (141). Industrialized countries have experienced a rise in the rate of Cesarean delivery in the 20 last years, correlated with an increase of autoimmune diseases and also allergic disease including asthma, eczema, atopic dermatitis, and food allergies (142, 143). The implication of the microbiome modification by C-section and the impact in the early and adult life of the offspring to develop asthma is currently an area under investigation.

Using 16S rRNA sequencing to define the bacterial microbiota in stool specimens of a birth cohort collected during the first year of life, one group evaluated the associations between pre- and postnatal environmental and sociocultural factors and the composition of the early life gut microbiome. This study found substantial age-related taxonomic variation in the microbiota (144). At birth, the primary bacterial communities were *Bifidobacteriaceae* or *Enterobacteriaceae* taxa, while infants (<1-year olds) were typically dominated by either *Bifidobacteriaceae* or *Lachnospiraceae*, the latter of which represents a typical dominant family in adult gut microbiomes (144). The impact of delivery mode in the composition of the infant microbiome has been previously described, with infants delivered by C-section presenting significant different diversity in the gut microbiome compared with vaginally

delivered infants (145). Besides compositional variation by mode of delivery overall (vaginal vs. C-section), significant differences were observed whether the C-section was planned or unplanned, indicating that even early labor may affect the microbial composition of the infant (139).

In a population-based national register cohort study, the effects of acute or elective C-section vs. vaginal delivery in children from 0 to 23 months of age were assessed. Hospitalization for RSV disease, with adjustment for external factors such as birthrate and maternal smoking during pregnancy, showed a positive association between delivery by C-section and increased risk of hospitalization for RSV infection, with this effect continuing out to 2 years of age (123). Together, these results suggest a correlation between RSV hospitalization and microbiome diversity modification by mode of delivery, although more research is needed in this area.

Breastfeeding: Microbiome, Respiratory Virus, and Allergies

The microbiome and immune system are actively modulated by specific factors within breast milk, including IgA that confers passive immunity. Breast milk also includes several metabolites that can impact metabolism, development, and maturation of the immune system in the neonates (146, 147). As we described above, the neonatal immune system is not entirely mature during the first months of life, and thus the interaction with the different organisms, foreign proteins and chemicals will set the basis for a successful interaction between the innate and adaptive immune systems. The infant's interaction with components of the commensal bacteria population helps to stimulate the maturation of the immune system. A critical component of the immune development driven by the microbiome is immune suppression at the gut mucosal surface, which induces oral tolerance to food antigens (145). Breast milk is known to contribute commensal bacteria to the infant gut microbiome, and several studies have shown that breast milk contains approximately 10^3–10^4 colony forming units per milliliter, thus being an excellent source of probiotics (147).

A number of studies have investigated the link between breastfeeding and the development of respiratory disease. In a prospective cohort study, newborns were followed up for 1 year to monitor hospitalization for bronchiolitis (148). These infants were classified as "never breastfed" and "ever breastfed," with the second group further divided into those "exclusively breastfed" and "breastfed associated with milk formula." The risk of hospitalization at 1 year of age for bronchiolitis was significantly higher in the "never breastfed" group. Infants who were exclusively breastfed or breastfed associated with formula milk demonstrated a similar, reduced risk of bronchiolitis-related hospitalization, thus confirming the protective effect of maternal milk in the development of severe bronchiolitis (148).

In another study, infants who tested positive for RSV were divided into full, partial, or never breastfed as above, and were followed for 10 days after the initial diagnosis to analyze the incidence and duration of hospitalization and the frequency of requiring oxygen therapy. While there were no differences in the hospitalization rate among the three groups, those infants who were never breastfed had an increase in the duration of hospital stay and required oxygen therapy at a greater rate than the breastfed groups (149). This study agrees with another cohort of infants (<1-year olds) hospitalized for bronchiolitis, which found that the lack of breastfeeding correlated with higher rates of bronchiolitis (150).

We describe above that the prenatal microbiome composition impacts the development of allergies in the offspring and that probiotic supplementation infants received from their breastfeeding mothers decreased the risk of atopic dermatitis (146), indicating that the microbiome composition of the mother during breastfeeding also impacts the immune system of the infants. It is known that human milk composition can be modulated by the maternal environment and also that breast milk is unique between mothers (146). These data suggest that probiotic supplementation by the mother during breast feeding could be a best strategy aimed at decreasing further the incidence of allergy development and LRTI (RSV) in breastfed infants.

Microbiome Modification in Infants: Supplementations and Microbial Exposure

In addition to probiotic supplementation of pregnant women, supplementation of children has also been studied. These studies show a similar effect to those observed in mothers on probiotic supplements during both pregnancy and breastfeeding, reinforcing the idea of the gut microbiome shaping the immune response to asthma and LRTI. The lung microbiome has also been studied. A shift in the microbial composition of the lung during respiratory diseases suggests a link to the host immune response to viral infection (151). An experimental study in mice that were exposed nasally to two different strains of *Lactobacillus rhamnosus* showed that the intranasal administration of these commensal bacteria modulated the TLR3/RIG-I antiviral respiratory immune response, where both strains demonstrated increased resistance of infant mice to RSV infection compared to controls (152). Another study in which young mice (3 weeks old) were orally supplemented with *Lactobacillus rhamnosus* CRL1505 before a RSV challenge showed improved resistance against RSV infection with decreased immunopathology, along with altered expression of IFN-γ and IL-10 in bronchoalveolar lavage, suggesting that the modulation of the immune response by probiotics early in life could favor protective immunity against RSV (153).

In humans, a randomized study with preterm infants was grouped to receive oral probiotics (*Lactobacillus Rhamnosus* GG) or placebo, between days 3 and 60 of life. These infants were followed to assess the effects on viral respiratory tract infection. The results showed a significant decrease in the detection of rhinovirus in nasal swabs in the children that were treated with probiotic compared with the placebo group, suggesting that the supplementation of probiotics in premature infants may prevent rhinovirus infection, although there were no significant differences in the detection of RSV (154). Prenatal characteristics were described but not analyzed (154), and as mentioned above, the prenatal microbiome regime likely modified the immune response of the infants positively (130, 132). These results suggest

that the supplementation in infants after birth may not be as an effective as prenatal intervention.

Household microbial exposure during the first years of life can shape the microbiome of children, and it has been suggested that the increased microbial exposure during the prenatal stage and the early years of live affect maturation of the immune system and help to reduce the risk for the development of allergic diseases (155). The protective effect of allergic diseases by microbial interaction with farm animals and pets during early life has been studied by a number of groups (155, 156). In a cross-sectional survey in rural areas in Europe, children from farming families who were exposed younger than 1 year to farm animals had significantly decreased allergy sensitization with lower frequencies of asthma, hay fever, and atopic sensitization (155).

In another study demonstrating the importance of microbial exposure in early life, a birth cohort study of healthy, full-term infants were followed until the age of 6–7 years. These children had a significantly decreased risk of atopy when exposed during the first year of life to two or more dogs or cats, suggesting that local household microbial exposure in the early years of life confers protection against allergic disease development (156). Following these studies, the same group determined whether the presence of pets in the household increased the microbial communities and if this microbial material conferred protection (126). They examined the diversity of bacterial and fungal communities in dust collected from the homes with pets and observed significant enrichment of bacterial communities in the dust samples from houses with pets compared to the dust from houses without pets. A vast majority of the enriched bacteria found were also detected in the human gut microbiome, suggesting that the exposure to enriched microbial communities conferred a protective effect on the development of allergies (126, 156). Thus, it appears that multiple factors during pregnancy and early infancy impact the development of the microbiome, which can shape immune function throughout childhood.

MICROBIAL METABOLITES AND THEIR ROLE IN RSV AND ASTHMA

The gut microbiota is necessary for proper food digestion, and a number of metabolites are a result of digestion by these bacteria. Several studies support the concept that airway immune status is impacted by the metabolite profile produced by the gastrointestinal microbiome (128, 157, 158). The metabolites from intestinal microbiota are the result of the microbial-host interactions, which are a complex system dependent on the food intake of the host, the external environment, the use of antibiotics, and the other microbial genera occupying a person's microbiome. Together, these interactions generate a host of metabolites that can impact the immune response systemically and locally in organs of the host, including the lung and gut (128, 157, 159).

Using animal models, the effect of the dietary fermentable fiber content has been studied in the composition of the lung and gut microbiota (157). In one study, mice were raised on a diet with standard, low or high fiber, followed by intranasal exposure to house mite extract. The mice that had low fiber diet showed increased inflammatory cell infiltration and increased Th2 cytokines in the lung, with elevated total IgE serum levels compared with the control, standard diet. The authors also observed that DC isolated from the lung of the low fiber diet mice exhibited a more active phenotype, as determined by increased CD40 and CD80 (157). Furthermore, the mice that were fed high fiber diets demonstrated reduced inflammatory cell infiltration and decreased Th2 cytokines in the lung, as well decreased levels of total IgE. The isolated lung DCs were also less activated, with reduced expression of CD40 and CD80. Finally, the authors observed that increasing the fiber in the diet modified the composition of the microbiota, with a difference in the ratio of *Firmicutes* to *Bacteroidetes*. The gut microbiota degraded the fiber and increased the concentration of systemic short-chain fatty acids (SCFAs). Increased circulating levels of SCFAs were observed in mice fed a high fiber diet, resulting in protection from allergic airway inflammation. Conversely, mice fed a low fiber diet had decreased circulating SCFAs, correlating to increased allergic disease in the lungs (157). Supplementing mice with the SCFA propionate altered hematopoiesis and the phenotype of the DCs. The authors concluded that fermentable dietary fiber and SCFAs could modify the immunological environment in the airway and influence the severity of allergic inflammation (157). In a human study, bacteria were grown from the intestinal microbiota of infants who were at high risk of atopic diseases, followed by gas-liquid chromatography to detected bacterial cellular fatty acids (158). The authors found that the bacterial cellular fatty acid profile in fecal samples was significantly different between atopic and healthy infants, and that the atopic infants had increased *Clostridium* cells in their feces compared to non-atopic, healthy infants, concluding that increased *Clostridium* numbers were associated with childhood allergic sensitization and that differences in neonatal gut microbiome and bacterial fatty acids precede the development of atopy in infants (158). These data also suggest the key role that the microbiome and their metabolites play in the development and maturation of the immune system.

We have previously shown that oral supplementation of mice with *Lactobacillus johnsonii* resulted in significantly reduced airway allergic sensitization and RSV-induced pulmonary immunopathology (127). The gut microbiota was modified considerably in the supplemented mice, but *L. johnsonii* was not detected in the airways, and furthermore, only viable *L. johnsonii* was able to generate protection (127). Following this study, we tested the metabolic potential of *L. johnsonii* for its impact on the lung immune response against RSV, and we found that *L. johnsonii* supplementation of the mice diet modified the systemic metabolic profile. In plasma samples analyzed by liquid chromatography/mass spectrometry, we were able to detect more than 50 different metabolites altered at baseline in supplemented mice (128). Following RSV infection, the plasma samples from *L. johnsonii* supplemented animals exhibited substantial metabolic reprogramming in response to RSV infection, involving significant increases in a broad range of lipid-, bile-, amino acid, and peptide-derived metabolites (128).

We also observed that RSV-infected bone-marrow derived DCs from *L. johnsonii* supplemented mice had altered cytokine secretion, reduced expression of co-stimulatory molecules, and modified $CD4^+$ T cell cytokine production. In this study, we outlined a potential mechanism for immune alteration by manipulating the microbiome and facilitating the availability of a complex and broad profile of microbial and mammalian-derived immunomodulatory metabolites that collectively may play a regulatory role (128). This result supports the idea that the metabolic potential of the gut microbiome is substantial and extends beyond fatty acid production, and may extend to immune regulation at tissues other than the gut.

In a US birth cohort of neonates, stool samples were analyzed to identify differences in microbiota-composition and associated metabolites. The authors observed that the microbiome composition of the infants that developed multi-sensitized atopy at the age of 2 years, present significant different compositions of microbial communities than those who did not. The group of children who developed atopy showed lower relative abundance of specific bacteria (*Bifidobacterium*, *Akkermansia*, and *Faecalibacterium*) and higher relative abundance of fungi (*Candida* and *Rhodotorula*) (160). Furthermore, the authors observed a distinct set of fecal metabolites, which were tested to generate pro-inflammatory responses using sterile fecal water from the samples of the infants with higher risk of developing allergies. When adding this sterile fecal water to the growth medium of adult human peripheral T cells, the authors observed an increased proportion of $CD4^+$ IL-4^+ cells and reduced numbers of regulatory T cells (160). Interestingly, one of the metabolites identified in the fecal samples of the high-risk infants was 12,13-DiHOME, which can suppress Treg cells. Addition of 12,13-DiHOME recapitulated the effect of the fecal water on T cell culture. This suggests that the gut microbiome in infants can modify the metabolic profile and that those metabolites can potentially impact the immune response to cause the development of allergies in later life (160). This study correlates with another study in which bronchoalveolar lavage fluid (BALF) was collected from adult pollen-allergic patients with mild asthma (age 22–42 years) and healthy non-allergic people, in basal conditions or under inhaled-pollen challenged (161). 87 lipid mediators were screened, and a significant difference in lipid metabolites was observed between allergic and healthy patients, including increases in both 12,13-DiHOME and 9,10-DiHOME in the BALF of allergic patients (161). These data highlight the importance of metabolites in lung allergic disease through modulation of the immune response, and the impact of the microbiome on the metabolic profile of the host. These pioneering studies suggest a young field ripe for further scientific investigation bringing together the microbiome and its metabolites and exploring their relationship to a healthy immune response.

CONCLUDING REMARKS

Severe RSV infection followed by the development of allergies and asthma is determined by the interaction of environmental and inherited factors (162, 163). Nevertheless, genetics cannot explain the continued increased in allergies and asthma, and any change in population genetics would require multiple generations to occur (162). On the other hand, epigenetics could be induced more rapidly and can be generated by environmental changes. Importantly epigenetic modifications can be passed down from parents to offspring or can result from *in utero* modification (162). As we describe above, epigenetic changes can modify the immune phenotype, with alterations inherited from the mother are fivefold more significant than the paternal factors (140, 164).

The ability of epigenetic modifications to impact gene expression to regulate or dysregulate the homeostasis of the organisms has been widely studied from cancer to modulation of the immune system (165). The concept of modulation of the immune response through epigenetic modification caused by environmental factors, diet, microbiome, and metabolites may have clinical therapeutic implications for human health (165). Future studies will now focus on how the microbiome metabolite profiles impact immune responses in the long term through epigenetic alteration of not only ongoing immune responses but also progenitor cell populations. These changes would have long acting effects on future immune responses. Whether these changes can be reversed will be a significant challenge for future therapeutic progress (165).

Many known factors that predispose to develop severe RSV disease are linked, from the immune system and lung development through microbiome composition (**Figure 1**). In this review, we summarize the role of diverse factors that can impact the immune response during RSV infection. The further pursuit of the interactions among the environment, the microbiome, and its metabolites, and their impact on epigenetics in the context of severe RSV infection has the potential to reveal new therapeutics to alter the development of this disease and the predisposition to asthma. Ideally, immune development would be altered favorably early in childhood prior to the initiation of pathogenic immune phenotypes including the development of severe disease caused by RSV infection, and this is likely to be a future focus of many studies. The complex interactions among the numerous factors reviewed herein will likely also have far reaching effects on other aspects and types of disease.

AUTHOR CONTRIBUTIONS

WF, NL, and CP conceived of and wrote the manuscript.

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Incomplete Memories: The Natural Suppression of Tissue-Resident Memory CD8 T Cells in the Lung

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The yearly, cyclic impact of viruses like influenza on human health and the economy is due to the high rates of mutation of traditional antibody targets, which negate any preexisting humoral immunity. However, the seasonality of influenza infections can equally be attributed to an absent or defective memory CD8 T cell response since the epitopes recognized by these cells are derived from essential virus proteins that mutate infrequently. Experiments in mouse models show that protection from heterologous influenza infection is temporally limited and conferred by a population of tissue-resident memory (T_{RM}) cells residing in the lung and lung airways. T_{RM} are elicited by a diverse set of pathogens penetrating mucosal barriers and broadly identified by extravascular staining and expression of the activation and adhesion molecules CD69 and CD103. Interestingly, lung T_{RM} fail to express these molecules, which could limit tissue retention, resulting in airway expulsion or death with concomitant loss of heterologous protection. Here, we make the case that respiratory infections uniquely evoke a form of natural immunosuppression whereby specific cytokines and cell–cell interactions negatively impact memory cell programming and differentiation. Respiratory memory is not only short-lived but most of the memory cells in the lung parenchyma may not be *bona fide* T_{RM}. Given the quantity of microbes humans inhale over a lifetime, limiting cellular residence could be a mechanism employed by the respiratory tract to preserve organismal vitality. Therefore, successful efforts to improve respiratory immunity must carefully and selectively breach these inherent tissue barriers.

Keywords: respiratory immunity, influenza infection, CD8$^+$ T cells, CD8 memory, heterologous immunity, tissue-resident memory cells

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INTRODUCTION

Respiratory infections continue to be one of the leading causes of morbidity and mortality worldwide (1). Approximately four million annual outpatient visits are associated with viral respiratory infections, including influenza and respiratory syncytial virus (RSV) (2, 3). While a RSV vaccine remains elusive, available influenza vaccines induce specific antiviral neutralizing antibodies that recognize the external antigens hemagglutinin and neuraminidase and are protective against a homologous infection. However, host immune pressure promotes mutations of these antigens between seasons rendering the elicited antibodies and those derived from a natural infection ineffective at providing long-term cross-protection against mismatched or heterologous viral strains (3).

Activated CD8 T cells lyse infected lung epithelial cells and produce antiviral cytokines, ultimately eliminating viral reservoirs (4). In the case of influenza infection, CD8 T cells recognize epitopes derived from internal viral proteins that are conserved across 80–100% of circulating influenza strains (4–8), indicating that elicitation of CD8 T cell immunity could offer a broad range of protection against heterologous influenza infection. This protection would rely on the development of memory CD8 T cells (T_{mem}) capable of responding rapidly upon challenge (9). However, evidence from murine (6, 10–12) and human (13, 14) studies suggest that long-lived protective T_{mem} does not form in response to influenza infection. While human studies are lacking, murine models indicate that respiratory anti-influenza T_{mem} numbers wane coordinate with loss of heterosubtypic immunity to influenza infection (10). This observation, paired with the knowledge that humans are susceptible to seasonal infections following both natural infection and vaccination with the live, attenuated vaccine (3) shows that respiratory T_{mem} are not stable which we believe is partly due to the incomplete generation of a specific population of T_{mem} in the lung.

T_{RM}: THE OTHER MEMORY CELL

Infection with various pathogens elicits a heterogeneous T_{mem} pool that was previously thought to consist of predominately two distinct populations: central memory cells (T_{CM}) located primarily in lymph nodes and effector memory cells (T_{EM}) which circulate through lymphoid and non-lymphoid tissues (15). The preferential localization of T_{CM} is due to expression of CD62L and CCR7 (15), whereas T_{EM} express low levels of these molecules. T_{mem} develop under a transcriptional program regulated by Eomes (16) and require IL-7 signaling for their survival through T cell contraction (17). However, IL-15 and IL-2 signaling bias T_{mem} toward a T_{CM} or T_{EM} lineage, respectively (18). In many cases, T_{EM} provide initial pathogen control at portals of entry, while T_{CM} are positioned to broadly patrol lymph nodes (19). Indeed, T_{CM} provide protection against systemic lymphocytic choriomeningitis virus infection (20), while T_{EM} protect against respiratory Sendai virus challenge (21). However, often this is not a true division of labor and, even in the case of non-lymphoid infections, reactivated T_{CM} will also contribute to the generation of new effector cells, albeit with delayed kinetics.

Subsequent studies using parabiotic mice demonstrated the existence of stationary, non-migratory populations of T_{mem} within the brain and small intestine, and to a lesser extent, other tissues like the lung and liver (22). These cells are now commonly referred to as tissue-resident memory cells (T_{RM}). T_{RM} have a core transcriptional profile that distinguishes them from their T_{CM} and T_{EM} counterparts (23), including expression of transcription factor Hobit (24). How T_{RM} cells developmentally diverge from other T_{mem} is unclear; however, it is likely to involve early programming followed by acquisition of tissue-specific factors that promote survival and tissue retention (23, 25). In most cases, $CD8^+$ T_{RM} have been identified by expression CD69 and CD103 (αE integrin) which are upregulated on T_{RM} in both humans (26, 27) and mice (28, 29). The ligand of CD103, E-cadherin, is expressed exclusively by epithelial cells and CD69 expression limits tissue egress (30, 31), suggesting these markers are responsible for locking T_{RM} within tissues. In fact, T_{RM} fail to develop in the intestines of $CD103^{-/-}$ mice, and absence of CD69 and CD103 limits T_{RM} formation in the skin (23), indicating that upregulation of CD103 and CD69 are crucial steps for the establishment of T_{RM}. Expression of CD103 and CD69 is regulated by TGF-β (32), which is highly expressed in mucosal sites such as the gut (33) where stable populations of T_{RM} cells have been observed (34). In most cases, T_{RM} are maintained through IL-7- and IL-15-mediated homeostatic proliferation (35, 36). T_{RM} are confirmed to exist in the skin (28, 37), brain (38), liver (39), and female reproductive tract (40, 41) where they are stably maintained. T_{RM} can persist for up to 120 days in the brain following vesicular stomatitis virus (VSV) infection (38), and skin-resident T_{RM} are the most durable, up to a lifetime in mice following cutaneous herpes simplex virus infection (42).

While a secondary, recall response can be delayed by several days for the activation of T_{mem} and recruitment of new effectors to the infection site, T_{RM} respond immediately to pathogen re-exposure (12). Upon antigen re-encounter, T_{RM} produce IFN-γ (9) to recruit circulating T_{EM} and other immune cells from the blood (43). In addition, T_{RM} can directly kill target cells *ex vivo* (44), suggesting a cytotoxic potential. T_{RM} have been shown to mediate long-term protection *in vivo* to infections in the intestine (34), female reproductive tract (40, 41), brain (45), and skin (28, 37). Regarding the latter, the smallpox vaccine, administered by skin scarification, generated T_{mem} which survived for decades (46). While the specific role of T_{RM} in the success of this vaccine is unclear, mice vaccinated *via* scarification of recombinant vaccinia virus (VacV) generate skin-resident T_{RM} that mediate protection against subsequent VacV infection (47). However, not every infection generates stable T_{mem} pools. While T_{RM} cells populate the lung and lung airways after influenza infection (12), protection between influenza seasons following natural infection or vaccination with the live-attenuated vaccine is lost (3), suggesting T_{RM} responses may be uniquely regulated in the lung.

T_{RM} IN THE LUNG

T_{RM} cells exist within the lung in two distinct compartments: the lung airways and the lung parenchyma. Influenza-specific airway-resident T_{RM} are $CD11a^{lo}CXCR3^{hi}$ (48, 49) and can be isolated by bronchoalveolar lavage. It is estimated that anti-influenza T_{RM} in the lung airways have a half-life of only 14 days, and for some period of time are continually replenished from the circulating T_{EM} pool (48). Interestingly, airway T_{RM} have a low cytolytic capacity and fail to proliferate upon antigen re-encounter but rapidly produce antiviral cytokines such as IFN-γ (44). T_{RM} embedded in the lung parenchyma are $CD11a^{hi}CXCR3^{lo}$, highly cytolytic and undergo rapid proliferation after antigen re-exposure (44). We have known for some time that regional T_{mem} are responsible for limited heterologous immunity after respiratory infection (10). A careful study of the kinetics of T_{mem} decay after Sendai and influenza virus infections demonstrated a rapid decline in T_{mem} numbers in the lung and lung airways by 90 days postinfection. Importantly, this loss of influenza-specific T_{mem} in the lung coincided with loss of heterosubtypic immunity

to influenza infection (10). The attrition of influenza-specific cells is restricted to the lung, as splenic memory cell numbers do not decline, indicating this is likely loss of the T_{EM} or T_{RM} pools. Subsequent experiments demonstrated that airway CD103$^+$ cells are responsible for protection against a secondary, heterologous virus challenge. However, this pool declines rapidly after infection and is undetectable within 7 months postinfection (12), in part due to the inhospitable environment of the lung airways.

T_{RM} in the airways reside at the frontline, adjacent to influenza-susceptible epithelial cells. However, lung parenchymal T_{RM} and circulating T_{EM} are also available within the lung tissue and can serve as a secondary line of defense. Recent evidence indicates that over time, T_{RM} cells in the lung airways wane and are replaced by circulating T_{EM} cells; however, these T_{EM} also decline and lose the ability to convert to T_{RM} (50). This, coupled with a loss of T_{RM} in the lung parenchyma, results in a gradual decline in the overall T_{RM} population in the lung. Decline in the lung parenchymal T_{RM} pool could be due to increased cell death, limited proliferation, or emigration. Unlike T_{RM} in other sites (28, 34, 38), most lung T_{mem} do not undergo homeostatic proliferation (50, 51). However, a small pool is replenished from proliferating T_{mem} that have recently emigrated from secondary lymphoid tissues (50). In addition, there is no evidence that T_{RM} cells in the airways egress from the lung or re-enter circulation (48). Therefore, we propose that T_{mem} embedded in the lung tissue are either eventually lost to the airways or do not represent a *bona fide*, protective T_{RM} pool. Our opinion that lung parenchymal T_{RM} do not exist is based on two observations. The first is that few T_{mem} truly penetrate into the tissue and the second is that those T_{mem} that do, are not CD103$^+$CD69$^+$.

Many techniques can identify T_{RM} (**Table 1**) and each has pros and cons. We believe that the most effective methodology is the combination of two of these approaches: intravascular staining and CD103/69 phenotyping. Intravascular staining distinguishes between cells circulating through the blood and those embedded within a tissue (52). Approximately 99% of the T_{RM} within the epithelial layer of the small intestine are protected from the intravascular staining (**Figure 1**) (52, 53), validating similar results observed in parabiotic mice (22). In contrast, the majority of the memory cells within the lung parenchyma 35 days after respiratory infection with either influenza, VSV, or *Listeria monocytogenes* are part of the circulating T_{EM} pool, with only 10–20% of the cells in the lung parenchyma truly within the tissue (52) (**Figure 1**). These data do contrast with other respiratory infections that are skewed toward the upper respiratory tract (54) or are chronic (55), both cases generating CD103$^+$CD69$^+$ T_{RM}. With regard to the latter study, it is possible that persistent antigen and inflammation is required for the successful development of T_{RM} within this site. In addition to antigen access, antigen competition can regulate T_{RM} populations at the clonal level (56). Moreover, many studies identify lung T_{RM} *via* CD103 and CD69 expression on isolated lymphocytes (57, 58), independent of intravascular staining. However, expression of these markers does not always correlate with tissue residency. For example, some T_{RM} cells in the lamina propria of the gut (59), the liver (39), and the brain (60) are CD103$^-$, and human splenic T_{mem} can be CD69$^+$ (26). In fact, less than 30% of the IV protected T_{RM} cells isolated from the lung

TABLE 1 | Common methods used for the identification of T_{RM} cells in peripheral sites.

Technique	Strengths	Weaknesses
Intravascular staining (Intravascular staining followed by flow cytometry)	• Identifies cells circulating within the bloodstream, eliminating contamination of parenchymal T_{RM} by T_{EM} within the intervening vessels, and eliminating the need for tissue perfusion (65) • Methodology highlights cellular location, which defines T_{RM} (52, 54, 58)	• Labor intensive (requires careful timing of Ab injection and animal sacrifice) (65) • Extensive tissue digestion protocols can result in inefficient cell isolation that can skew T_{RM} representation • Differential kinetics of antibody vascular extravasation or blood flow rates within specific tissue can affect antibody penetrance (66, 67) • Identifies localization at a single point in time; cannot eliminate transient migration through tissue
CD69/CD103	• Simple method of detection by flow cytometry on isolated tissue lymphocytes *ex vivo* (29)	• Extensive tissue digestion protocols (see above) • Not exclusively expressed on cells in tissue parenchyma (59) • CD69 expression is enriched in conditions of antigen persistence (68) • Requires perfusion to eliminate tissue-associated cells in vasculature (69) • Cells are not uniformly CD69/CD103$^+$ in all tissues (59)
Confocal microscopy	• Clearly identifies cells directly embedded in parenchyma or epithelium while excluding those in small vessels (57, 58) • Can reveal T_{RM} tissue niche (58) • Can identify which cells T_{RM} are interacting with (59)	• Cryosectioning can damage or distort tissue architecture (70) • Information is only a snapshot and limited tissue depth (70)
Parabiosis	• Identifies the proportion of circulating T_{mem} in a given tissue (using congenic markers of partner) in the steady state (22)	• Requires surgical procedure and extensive animal monitoring (71) • Unclear how much inflammation due to surgery changes T_{mem} cell migration/redistribution of subtypes (71) • Cannot distinguish between host T_{RM} and T_{EM} without pairing with other technique (22, 72)
FTY720 treatment	• Eliminates the ability of circulating T_{mem} to traffic into tissues and supplement the T_{RM} pool (enriches for T_{RM}) (12, 28)	• Does not eliminate the contribution of circulating memory cells (T_{EM}) in the blood before lymph node sequestration (73)

A summary of some of the commonly used immunological techniques that have been used to study T_{RM} cells in various peripheral sites, as well as the strengths and weaknesses of said techniques. With the exception of confocal microscopy, these techniques do not consider lung compartmentalization, which requires additional processing of BAL and subsequently lung tissue to identify the different T_{RM} pools.

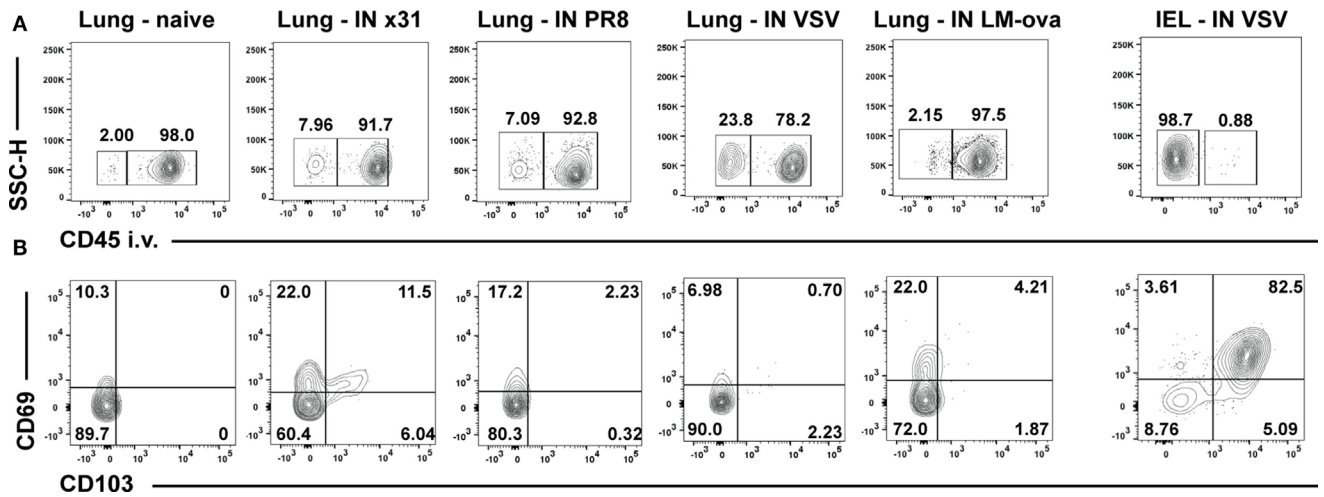

FIGURE 1 | Lung T_{RM} cells express low levels of CD69 and CD103 after respiratory infection with various pathogens. Age- and sex-matched C57/BL6 mice were infected intranasally with a 50-µl inoculum of PBS alone (naïve) or containing sublethal doses of either influenza (10^3 pfu of strain HKx31 and 10 pfu of PR8), VSV (10^4 pfu, Indiana strain), or *Listeria monocytogenes* expressing the recombinant ovalbumin (ova) (LM-ova) (10^4 cfu). One group of mice was additionally intravenously (i.v.) infected with 10^4 pfu VSV. Animals were sacrificed 35 days later and T_{RM} assessed by intravascular staining. Briefly, mice were injected i.v. with 3 µg FITC labeled αCD45 antibody 3 min before sacrifice, lungs or small intestine were harvested, and lymphocytes isolated as previously described (22). **(A)** Representative i.v. staining of lymphocytes isolated from the lungs or intraepithelial lymphocytes (IEL) of naïve mice or following the indicated infections. All samples were first gated on $CD8^+CD44^+$ memory phenotype cells and gates in **(A)** were set by FMO controls within each experiment. For the influenza and VSV-infected animals, an additional MHC-class I tetramer gate was applied to identify antigen-specific CD8 T cells [as in Ref. (61)]. Numbers in the right box represent the frequency of the gated cells that stained with the i.v. injected antibody ($\alpha CD45\text{-}FITC^+$) and are in the vasculature (IV^+). **(B)** Representative CD103 and CD69 staining of IV^- [resident cells, left box in **(A)**] cells from the various infections.

parenchyma express CD69 and CD103 (**Figure 1**) compared to T_{RM} isolated from other mucosal sites, where expression ranges from approximately 50–99% (59). Therefore, T_{mem} located in the lung parenchyma after respiratory infection lack one of the key attributes associated with *bona fide* T_{RM}, expression of CD69 and CD103. $CD103^-$ T_{RM} in the brain are maintained for a few months (60) which may be due to modified tissue localization and enhanced access to IL-15. However, lung parenchymal T_{RM} are maintained independent of IL-15 (61), at least in the short-term, so gained proximity to IL-15 may not matter. However, acquisition of other survival signals dependent on CD103 positioning may be altered, leading to either cell death or assimilation into the T_{EM} pool. Coupled with loss of airway-associated T_{RM}, this situation leaves circulating T_{EM} as the only viable responders. Whether the T_{EM} temporally supplementing the T_{RM} pool are CX_3CR1^{hi} and classified as the recently described "peripheral" memory cells (T_{pM}) (62, 63) is unknown. Nonetheless, as T_{EM} induced from respiratory infection decline over time (64), hosts will then be susceptible to infection. Therefore, an inferior $CD69^+CD103^+$ T_{RM} response underpins loss of heterosubtypic immunity in the lung and raises the question of why long-lived, stable T_{RM} does not form in the lung following respiratory infection.

THE RESPIRATORY ENVIRONMENT SUBVERTS THE DEVELOPMENT OF T_{RM}

As the lung is exposed to both infectious agents and innocuous environmental antigens, immune responses must be tightly controlled to prevent immunopathology (25). Similar regulation is also required in the liver and brain, additional tolerogenic sites. In part, this regulation is accomplished *via* tissue segregation. Indeed, liver T_{RM} are exclusively segregated from tissue stroma, retained within the sinusoids (74), whereas brain T_{RM} are preferentially localized in the meninges and perivascular areas (60), sequestered from the parenchyma. The lung is no different, with the development of BAL T_{RM} and parenchymal T_{RM}. However, unlike T_{RM} in the brain and liver, BAL T_{RM} are directly exposed to the external environment and easily lost, whereas the lung parenchymal T_{RM} are imbedded in the parenchyma and require an additional level of regulation to prevent immunopathology.

One potential mechanism is through altered mammalian target of rapamycin (mTOR) signaling within the respiratory tract. mTOR is responsible for regulating cellular metabolism, proliferation, and differentiation (75), including memory cell development (76). High levels of mTOR activation reduces the total number of antigen-specific cells expressing CD127, required for the development of memory precursor cells (77), and the subsequent T_{CM} pool (76). While reducing mTOR signaling with rapamycin reverses the effects on T_{CM} (76), T_{RM} formation and retention within the intestinal mucosa was also increased *via* enhanced expression of gut-specific homing molecules (78). To date, no study has linked reduced mTOR signaling to enhance lung homing and/or *respiratory* T_{RM} formation. However, evidence from viral respiratory infection models support a role for mTOR in T_{RM} formation. Rapamycin treatment during influenza infection increases the total number of antigen-specific CD8 T_{mem} circulating in the blood (79) similar to studies in the gut (78). In

addition, activated CD8 T cells isolated from infants infected with RSV and treated with rapamycin during *in vitro* re-stimulation express higher levels of CD127 compared to those cells stimulated without rapamycin. Rapamycin treatment also enhanced the effector response of RSV-specific cells by increasing their proliferation and production of granzyme B (80). While increased infiltration of RSV-specific effector cells into the lung may be important for viral clearance, this can also result in damaging pathology within the lung tissue itself. This indicates that perhaps careful regulation of mTOR signaling during respiratory infection is important for limiting potential immunopathology (80) and T_{mem} development; however, further studies are needed to directly implicate mTOR as a player in lung T_{RM} formation.

The lung environment is inherently immunosuppressive. In the steady state, a large reservoir of T_{regs} populate this tissue and contribute to significant IL-10 post-influenza infection (81). Moreover, bronchial and alveolar epithelial cells are known to express moderate levels of the programmed death-1 (PD-1) ligands PD-L1 and PD-L2, both of which are significantly upregulated upon RSV (82) and influenza infection (83). In addition, antigen-specific CD8 T cells infiltrating the lung following RSV and influenza infection have an increased expression of PD-1 (83, 84). Both IL-10 and PD-1 signaling can modulate CD8 T cell activation both individually (85, 86) and cooperatively (87) by tuning TCR signaling. IL-10 suppresses IL-12 signaling which, like PD-1 signaling, activates mTOR. However, PD-1 signaling is not exclusively through mTOR and can affect transcriptional networks and other cell cycle regulators which can impact the fate and function of $CD8^+$ T cells (86). Memory phenotype cells isolated from PD-$1^{-/-}$ versus wild-type mice are preferentially T_{EM} (88). Reciprocal adoptive transfer experiments demonstrated this bias was inherent to the T cell. As PD-1 blockade during RSV infection results in enhanced inflammation and lung injury, PD-1/PD-L1 expression in the respiratory tract may serve to limit the expanding $CD8^+$ T cell pool, thereby restricting developing T_{RM}. Thus, while enhanced PD-1 expression within the respiratory tract may be important for regulating inflammation, this may create an environment that is inhospitable to the formation of T_{RM}.

It is also possible that respiratory infections alter T_{RM} programming *via* inhibition of CD103 and CD69 expression, which negatively affects the formation and/or retention of T_{RM} cells in the respiratory tract. Constitutive expression of TGF-β in mucosal sites such as the gut (33) is crucial for the development of long-lived T_{RM} through induction of CD103 expression (89). Epithelial cells also provide survival signals such as IL-15 (90), thus high CD103 expression may not only facilitate T_{RM} retention but aid in their development or survival *via* tissue positioning. However, high levels of TGF-β in the respiratory tract can be detrimental, leading to the development of cystic fibrosis within the lung (91). Although TGF-β expression is induced by influenza infection (92, 93), it may only be transiently expressed to limit immunopathology, albeit at the expense of T_{RM} formation. In fact, the T_{RM} in peripheral sites can cause semi-permanent scarring in tissues that worsens after T_{RM} re-activation and production of IFN-γ *in situ* (94). Since high levels of IFN-γ production (95), in addition to scarring and fibrosis in the lung, can cause respiratory failure (96), the retention of T_{RM} long term may be inherently limited to maintain host fitness. If this is the case, promoting T_{RM} formation within the respiratory tract could have severe consequences for host respiratory health. Therefore, by reducing TGF-β, and coordinately CD103 expression, lung memory precursor cells would perhaps be ill positioned to receive homeostatic signals responsible for the development, survival, and/or retention of T_{RM} and could be either be lost or assimilated into the T_{EM} pool.

While airway-resident T_{RM} cells confer protection against secondary influenza infection, they rapidly wane, leaving only parenchyma resident T_{RM} and circulating T_{EM} to maintain protection against subsequent infection. However, T_{EM} also wane over time (64) and the formation of *bona fide* T_{RM} in the lung parenchyma is limited (**Figure 1**). These incomplete memories leave the host susceptible to recurring influenza infection. We believe the lung evokes a form of natural immunosuppression whereby inhibitory signals in the site protect the host from debilitating tissue damage while simultaneously suppressing the formation of *bona fide* T_{RM} within the lung tissue. While the exact mechanisms that underlie altered T_{RM} formation within the respiratory tract are still not fully understood, future efforts to improve the maintenance and stability of this population must bear caution due to potentially negative, long-term effects on the host. Moreover, in developing vaccines against respiratory pathogens, it will be important to identify strategies that will prevent re-infection with respiratory viruses without compromising host respiratory health.

ETHICS STATEMENT

All animal studies were conducted under guidelines approved by the Institutional Animal Care and Use Committee of the University of Georgia.

AUTHOR CONTRIBUTIONS

Both KK and KR conceived and wrote the perspective.

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Natural Killer Cell Recruitment to the Lung During Influenza A Virus Infection Is Dependent on CXCR3, CCR5, and Virus Exposure Dose

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Natural killer (NK) cells are vital components of the antiviral immune response, but their contributions in defense against influenza A virus (IAV) are not well understood. To better understand NK cell responses during IAV infections, we examined the magnitude, kinetics, and contribution of NK cells to immunity and protection during high- and low-dose IAV infections. Herein, we demonstrate an increased accumulation of NK cells in the lung in high-dose vs. low-dose infections. In part, this increase is due to the local proliferation of pulmonary NK cells. However, the majority of NK cell accumulation within the lungs and airways during an IAV infection is due to recruitment that is partially dependent upon CXCR3 and CCR5, respectively. Therefore, altogether, our results demonstrate that NK cells are actively recruited to the lungs and airways during IAV infection and that the magnitude of the recruitment may relate to the inflammatory environment found within the tissues during high- and low-dose IAV infections.

Keywords: natural killer cells, influenza virus, cell trafficking, lung, CXCR3, CCR5

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INTRODUCTION

Natural killer (NK) cells, innate lymphocytes with cytolytic activity against infected and transformed cells, have known roles in protection from DNA virus infections, including members of the herpesviridae, poxviridae, and papillomaviridae families (1–3). More recently, NK cells have been shown to participate in the immune response against the orthomyxovirus influenza A virus (IAV), whose segmented genome is composed of negative sense RNA. However, it is currently less clear as to what contribution NK cells play [i.e., protective (4–11) vs. immunopathologic (12, 13)] in immunity during IAV infections. While differences in animal models, virus strains, route of infection, and dose of virus may explain the discrepancies between protective and immunopathologic effects, overall, these studies all suggest that NK cells are a component of normal immunity to IAV.

Natural killer cell-mediated protection from infections requires effective NK cell recruitment to the sites of lymphocyte activation and infection. NK cell recruitment to the liver is critical for

Abbreviations: BAL, bronchoalveolar lavage; BrdU, bromodeoxyuridine; CFSE, carboxyfluorescein succinimidyl ester; DLN, draining lymph node; IAV, influenza A virus; mAb, monoclonal antibody; NK, natural killer; PBS, phosphate buffered saline; p.i., post-infection; PT, pertussis toxin.

the control of both *Toxoplasma gondii* and murine cytomegalovirus, while recruitment to the draining lymph nodes (DLNs) in ectromelia virus infection is important for priming an effective CD8 T cell response (14–16). These data suggest that proper NK cell trafficking is important for both the initial control of infections at the replication site and the subsequent priming of an adaptive immune response against the pathogen. Currently, the mechanisms and molecular networks controlling NK cell trafficking and accumulation in the lungs and draining lymphatics during IAV infection remain unclear. In general, NK cells express many chemokine receptors, including CCR2, CCR5, and CXCR3 that have been linked to NK cell migration (17). In the lung tissue, CCR2 has been shown to be important for NK cell recruitment and subsequent protection from invasive *Aspergillus fumigatus* infections (18). While CCR2 has been shown to influence NK cell accumulation in the IAV-infected airway, its absence had no effect on NK cell recruitment to the IAV-infected lung parenchyma (19). This suggests that the mechanism of NK cell recruitment may differ between pulmonary infections. CXCR3 is known to be important in NK cell recruitment to the lung in homeostasis, as CXCR3$^{-/-}$ mice have significantly fewer NK cells in the lungs than WT mice (20). While NK cell expression of CXCR3 can result in an increased NK cell accumulation in the lungs during pulmonary inflammation (20, 21), the importance of CXCR3 in recruiting NK cells to the lung during IAV infection has not yet been determined. In addition to recruitment to individual organs, chemokine receptors can localize cells within an organ. For example, while CXCR3 expression is important in CD8 T cell recruitment to the lung, CCR5 expression on CD8 T cells is required for the localization of memory CD8 T cells to the IAV-infected epithelium (22, 23). While it has been shown that CCR5$^{-/-}$ NK cells are better able to proliferate in the IAV-infected lung compared to those in WT (24), the role of CCR5 in NK cell recruitment to and localization within the IAV-infected lung has not been directly examined.

As the dose of virus may affect how NK cells contribute to IAV immunity, we herein examined if IAV infection dose alters NK cell recruitment to lungs, lung DLNs, and spleen. Given the importance of CXCR3 in NK cell homeostasis, and the role of CXCR3 and CCR5 in recruiting and localizing CD8 T cells to the lung during IAV infection, we specifically determined if CXCR3 and CCR5 are required for NK cell recruitment during both high- and low-dose IAV infections. Our results demonstrate that while NK cells accumulate in the lung and DLN during both high- and low-dose IAV infections, a greater NK cell accumulation occurs in the lungs during high-dose infections and in the DLN during low-dose infections. CXCR3 expression on NK cells increased NK cell recruitment to the lungs, and the increased NK cell recruitment in high-dose IAV infections correlated with a higher expression of CXCR3 ligands in the lungs. CCR5 ligands were also upregulated in the lung and correlated with an increased recruitment of WT NK cells to the lung tissue and airways compared to CCR5$^{-/-}$ NK cells. Overall, our data suggest that in addition to infection-dependent mechanisms of NK cell recruitment to the lung, the severity of infection may also influence the magnitude of NK cell recruitment, thus influencing disease outcome.

MATERIALS AND METHODS

Mice

Six- to eight-week-old BALB/c and C57Bl/6 mice were purchased from the National Cancer Institute (Frederick, MD). BALB.B6-CT6 (i.e., CT6) mice were obtained from Dr. Anthony Scalzo (Nedlands, Australia). CXCR3$^{-/-}$ (B6.129P2-*Cxcr3*^{tm1Djen}/J), CCR5$^{-/-}$ (B6.129P2-*Ccr5*^{tm1Kuz}/J), and C57Bl/6 CD45.1 (B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and bred in house. The C57Bl/6 CD45.1 colony was started prior to 2009 (25). C57BL/6 and C57Bl/6 CD45.1 mice were bred together to generate the CD45.1^{+}/CD45.2^{+} mice used in adoptive transfer experiments. All animals were housed, bred, and maintained in the animal care facilities at the University of Iowa. All procedures were approved by the University of Iowa Animal Care and Use Committee.

Influenza Virus Infections

Mouse-adapted IAV A/PR/8/34 was grown in the allantoic fluid of embryonated chicken eggs as previously described (26). Mice were anesthetized with isofluorane and then infected i.n. with 50 μL of virus diluted in Iscove's media as indicated. All experiments with IAV were undertaken using BLS2/aBLS2 containment.

Influenza Virus Titers

Lungs were removed from infected mice at 2, 4, 6, and 8 days post infection (p.i.) and homogenized. Viral titers were determined as previously described by end point dilution assay and expressed as TCIU. Briefly, 10-fold serial dilutions of lung homogenates from IAV-infected mice were mixed with 2.5×10^4 Madin–Darby canine kidney cells incubated at 37°C. After 24 h, culture supernatants were removed and fresh media were added to each well. After 4 days of incubation, culture supernatants were mixed with 0.5% chicken RBC and the agglutination pattern was determined, and the TCIU values were calculated using the Reed–Muench accumulative method.

NK Cell Depletion

CT6 mice were administered 300 μg of anti-NK1.1 (PK136) monoclonal antibody (mAb) intraperitoneally (i.p.) 2 days before and on the day of IAV infection to deplete NK cells. NK depletion was verified by flow cytometry using mAbs against NKp46 (clone 29A1.4) and CD3ε (clone 17A2).

NK Cell Purification

Spleens were processed with frosted glass slides to create a single-cell suspension. Lymphocytes were separated from red blood cells using a Ficoll gradient before NK cells were purified using the EasySep Mouse NK Cell Enrichment kit (STEMCELL Technologies, Vancouver, Canada) as per manufacturer's instructions. For the competitive trafficking assay WT (CD45.1^{+}/CD45.2^{+}) and CXCR3$^{-/-}$ or CCR5$^{-/-}$ (CD45.2^{+}) NK cells were mixed 1:1 before transferring 100 μL i.v. to recipient mice (CD45.1^{+}) ($1–5 \times 10^5$ each NK cell population). Mixed donor cells were analyzed by flow cytometry prior to injection to determine the actual ratio for that individual experiment. Endogenous,

transferred WT, and transferred CXCR3$^{-/-}$ or CCR5$^{-/-}$ NK cells were identified by CD45.1 and CD45.2 expression by flow cytometry. For calculations of the ratio of donor NK cell trafficking, the values observed for each of the donor NK cell populations were normalized to the actual input ratio that was observed for the mixed donor cells for that experiment.

Flow Cytometry

Lungs were digested in DNAse and Collagenase IV for 10 min at 37°C. Organs were processed through a 60-μm screen, and single-cell suspensions were blocked in 2% rat serum. The following antibodies were used for these studies: rat anti-mouse CD3ε (17A2), hamster anti-mouse CD27 (LG.3A10), rat anti-mouse CD11b (M1/70), rat anti-mouse CD49b (DX5), and mouse anti-mouse CD45.2 (104) purchased from BD Biosciences (San Jose, CA, USA); mouse anti-mouse NK1.1 (PK136) purchased from eBioscience (San Diego, CA, USA); and mouse anti-mouse CD45.1 (A20) purchased from Biolegend (San Diego, CA, USA). For surface staining, 10^6 cells were incubated with antibody for 25 min at 4°C and then fixed in FACS lysis buffer (BD Biosciences, San Jose, CA, USA). All flow cytometry data were collected with a BD Canto II and data analyzed with Flow Jo software (Tree Star, Inc., Ashland, OR, USA).

NK Cell Proliferation Assay

For proliferation assays, A/PR/8/34-infected BALB/c mice were administered 8 mM carboxyfluorescein succinimidyl ester (CFSE) (50 μL) and 2 h later were given 80 μg bromodeoxyuridine (BrdU) i.n. as previously described (27). Four hours after the BrdU administration, lungs were harvested and single-cell suspensions were prepared for flow cytometric analysis with anti-CD49b (DX5) and anti-CD3ε mAbs as described above. Following cell fixation, BrdU incorporation was determined with the BrdU Flow Kit (BD Biosciences San Jose, CA, USA) according to manufacturer's instructions. NK cells proliferating within the lung were identified as DX5$^+$CD3$^-$CFSE$^+$BrdU$^+$ cells.

Drug Treatments

For the NK cell trafficking assay, A/PR/8/34-infected BALB/c mice were administered 500 ng pertussis toxin (PT) (Sigma-Aldrich, St. Louis, MO, USA) in 200 μL phosphate buffered saline (PBS) i.p. on days 2, 3, 4, and 5 post IAV infection. For the competitive NK cell trafficking experiment, purified NK cells from C57Bl/6 mice (CD45.2$^+$) were incubated with 200 ng/mL PT in RPMI supplemented with 2% FBS for 20 min at 37°C while NK cells from C57Bl/6 mice (CD45.1$^+$/CD45.2$^+$) were incubated in media + 2% FBS as previously described (28). Cells were washed three times in PBS and then mixed at a 1:1 ratio before transfer. Mixed donor cells were analyzed by flow cytometry to determine the actual input ratio for that individual experiment. For calculations of the ratio of donor NK cell trafficking, the values observed for each of the donor NK cell populations were normalized to the actual input ratio that was observed for the mixed donor cells for that experiment.

ELISA

Lungs from A/PR/8/34-infected BALB/c and C57Bl/6 mice were harvested at indicated days and were homogenized in 3 mL Iscove's media, aliquoted, and stored at −80°C. DuoSet ELISA kits (R&D Systems, Minneapolis, MN, USA) were used to determine CCL5, CXCL9, CXCL10, and CXCL11 (BALB/c only) per manufacturer's instructions.

RESULTS

NK Cells Accumulate in the Lung and Lung DLNs During IAV Infection

Prior studies examining the contribution of NK cells to IAV immunity have shown disparate results; studies using higher infection doses found that NK cells contribute to lethal immunopathology, while studies using lower doses of IAV showed a protective role for NK cells (4–9, 12, 13). Therefore, to determine if the kinetics and location of NK cell trafficking during IAV infection are altered by the severity and initial dose of IAV, BALB/c mice were infected with either a high- (1 LD_{50}) or a low (0.1 LD_{50})-IAV dose, and NK cells were subsequently quantified in the lung, lung DLN, and spleen by flow cytometry (see **Figure 1A** for representative gating strategy). Following infection, the number of NK cells found in the lung was significantly increased at days 2, 4, 6, and 8 p.i. in both the high- and low-dose infections when compared to naïve mice (day 0) ($p < 0.05$, Student's unpaired *t*-test), with maximal accumulation at day 6 p.i. Moreover, NK cell numbers in the lung were significantly greater in high- vs. low-dose IAV-infected lungs on days 4 and 6 p.i. (**Figure 1B**, left panel). Within the DLN, the number of NK cells present was significantly higher for IAV-infected mice than in naïve mice at days 2, 4, and 6 p.i. NK cell accumulation peaked at day 4 p.i. for both doses of IAV (**Figure 1B**, middle panel). However, while NK cell accumulation in the lungs was greater in high-dose IAV-infected mice, accumulation in the DLN was instead significantly higher for the low-dose IAV infection. In the spleen, NK cell numbers initially increased at day 2 p.i. before returning to homeostatic levels in low-dose IAV-infected mice and were significantly decreased relative to naïve mice in high-dose IAV-infected mice (**Figure 1B**, right panel). In summary, similar kinetics of NK cell trafficking were observed between high- and low-dose IAV infections; however, the overall magnitude of NK cell accumulation differed with a greater NK cell accumulation in the lungs during high-dose IAV and in the DLN during low-dose IAV infection. Importantly, C57Bl/6 mice infected with high and low doses of IAV showed similar kinetics and organ preference in NK cell trafficking as BALB/c mice (**Figure 1B**), suggesting that the pattern of NK cell trafficking during IAV infection was not mouse strain-specific.

Natural killer cells can be subdivided into subsets by CD27 and CD11b expression (CD27$^+$CD11b$^-$, CD27$^+$CD11b$^+$, and CD27$^-$CD11b$^+$) to correspond to different stages of maturation (29). These NK cell subsets differ in effector molecule expression as well as chemokine receptor expression (17, 29, 30). Therefore, differential recruitment of the various NK cell subsets could correlate with their different roles in immunity (31) or in protection vs. immunopathology during IAV infection. When we quantified the recruitment of NK cell subsets within the lung, DLN, and spleen of high- and low-dose IAV-infected mice, we observed increases in all three NK subsets in the lung and DLN. In the lung, mature

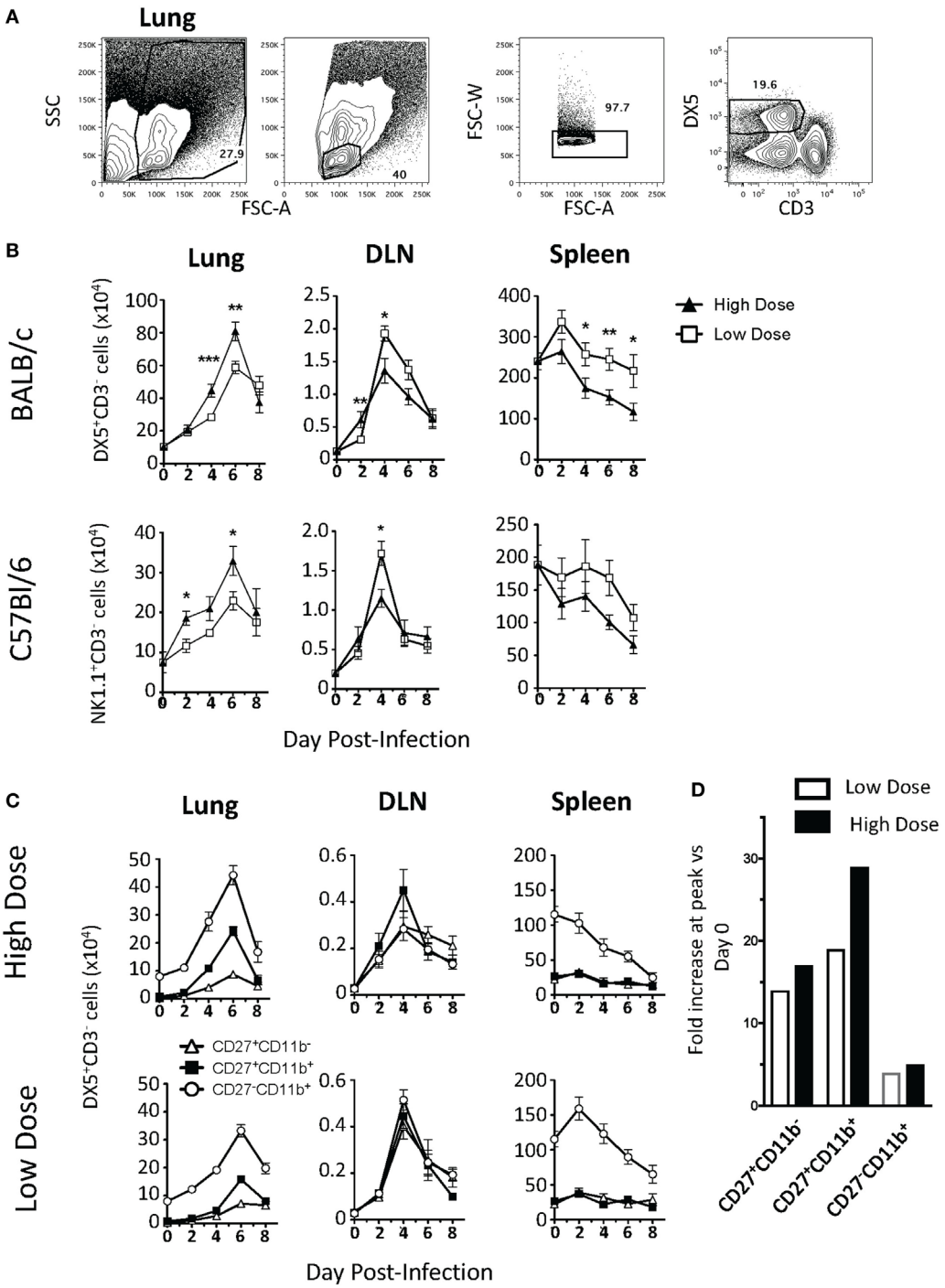

FIGURE 1 | Kinetics of natural killer (NK) cell accumulation during high- and low-dose influenza A virus (IAV) infections. **(A)** Representative gating strategy for $DX5^+CD3^-$ NK cells in the lungs. **(B)** BALB/c (top) or C57Bl/6 (bottom) mice were infected with a high (1 LD_{50}) or low (0.1 LD_{50}) dose of A/PR/8/34. NK cells were enumerated in the lung, lung-draining lymph node (DLN), or spleen at the indicated time points by flow cytometry. Shown are $DX5^+$ or $NK1.1^+CD3\varepsilon^-$ cells. The number of NK cells in the DLN is expressed as NK cells per node. **(C)** NK cells ($DX5^+CD3\varepsilon^-$ gated) within the lung, DLN, and spleen in BALB/c mice were further characterized by CD27 and CD11b expression to determine the distribution of functional subsets during both high- (top) and low (bottom)-dose IAV infections. Shown are *p*-values for high- vs. low-dose IAV infections. $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$ (two-tailed unpaired Student's *t*-test). The number of NK cells within the lung of high- and low-dose IAV-infected mice was found to be significantly greater than the number of NK cells in naïve lungs at days 2, 4, and 6 p.i. ($p < 0.05$). The decrease in NK cell numbers in the spleen was found to be significantly greater in high-dose-infected mice by two-way ANOVA ($p < 0.001$). Data are pooled from at least two separate experiments, with five mice per time point, per experiment. **(D)** The fold increase in $CD27^+CD11b^-$, $CD27^+CD11b^+$, and $CD27^-CD11b^+$ NK cells within the lungs was calculated by dividing the cell number found with the lungs at the peak of the response by the cell number found within the lungs at day 0.

$CD27^{-}CD11b^{+}$ NK cells accumulated in the greatest number in both the high- and low-dose infections (**Figure 1C**); however, the intermediate $CD27^{+}CD11b^{+}$ subset showed the greatest relative increase in magnitude (**Figure 1D**). The immature $CD27^{+}CD11b^{-}$ NK cells also increased in number within the infected lung, but showed no significant difference between high- and low-dose infections ($p > 0.05$) (**Figure 1C**, left panels), suggesting that the $CD27^{+}CD11b^{-}$ NK cell subset did not contribute to the overall difference in NK cell numbers observed in the lungs between high- and low-dose IAV infections. Similar to studies utilizing a single dose of IAV (32), the accumulation of all three NK subsets was also observed in the DLN of both high- and low-dose IAV-infected mice. NK cell subsets were evenly distributed within the DLN, and all NK cell subsets had a maximal accumulation at day 4 p.i. (**Figure 1C**, middle panels)—a kinetics which is in excellent agreement with those recently reported (31). Unlike what was observed in the lung and DLN, where all NK cell subset numbers increased within the tissues, only the $CD27^{-}CD11b^{+}$ NK cell subset had altered kinetics in the spleen during the course of both high- and low-dose IAV infection. The number of $CD27^{-}CD11b^{+}$ NK cells decreased in both infection doses (**Figure 1C**, right panels). Notably, the $CD27^{-}CD11b^{+}$ subset had the greatest increase in the lung, while it had the greatest decrease within the spleen, suggesting that NK cell accumulation within the lung during IAV infection may be due, in part, to recruitment of this NK cell subset from the spleen.

As expected, based on our prior studies (26), despite the differing accumulation level of NK cells during high- and low-dose infections, we observed no differences in IAV virus titers in the lungs on day 2, 4, 6, or 8 p.i. (Data Sheet S1 in Supplementary Material). Further, when we depleted NK cells from BALB.CT6 mice (BALB/c mice that express NK1.1), we did not observe changes in overall morbidity and mortality (Data Sheet S1 in Supplementary Material). This suggests that in the absence of NK cells, or when infection conditions lead to differing magnitudes of NK cell accumulation within lungs (**Figure 1**), other immune cell populations may compensate to control virus loads and dictate the outcome of the infection.

NK Cell Accumulation in the Lung During IAV Infection Is Not due to Apoptosis or Proliferation

Since we observed differential trafficking of NK cells during high- and low-dose IAV infections, we next sought to determine the mechanism(s) regulating this trafficking. The difference in accumulation within the lung between high- and low-dose IAV infections could be due to at least three potential mechanisms: differential levels of (1) apoptosis, (2) proliferation, and/or (3) recruitment. When we examined the level of apoptosis of NK cells *via* active caspase 3/7 and 7-AAD staining, we observed that less than 0.5% of the NK cells were undergoing apoptosis (i.e., caspase $3/7^{+}7\text{–}AAD^{+}$) in either the high- or low-dose IAV-infected lungs on day 4 p.i. (not shown). This suggested that differential apoptosis likely does not explain the differing accumulation of NK cells in the lungs after high- and low-dose IAV. Previous studies have examined NK cell proliferation during IAV infection and have shown that the main site of NK cell proliferation is the bone marrow, and only a small percentage of NK cells within the lung incorporate BrdU (33, 34). However, these studies did not directly differentiate between NK cells proliferating within the lung and NK cells proliferating in the periphery before being recruited to the lung. Further, it remains unknown as to what effect the initial dose of IAV has on NK cell proliferation. To quantify NK proliferation specifically within the lung, we used a CFSE followed by BrdU dual-labeling method that we have previously described (27) (**Figure 2A**). This labeling technique utilizes i.n. CFSE administration to tag cells within the lungs followed by a subsequent BrdU labeling to identify cells that have proliferated within the local lung environment. We observed NK cell proliferation in the lung during both high- and low-dose infections, which peaked at day 4 p.i. When comparing high- to low-IAV infection doses, we observed significantly more NK cells proliferating within the lungs of high-dose-infected mice at day 6 p.i. (**Figure 2B**). While viral dose affected the overall number of proliferating NK cells, similar frequencies of proliferating NK cells were observed between the two virus doses (**Figure 2C**), suggesting that the difference observed in the number of proliferating NK cells was reflective of the difference in the total number of NK cells recruited to the lungs, rather than a direct effect of the initial dose of virus on NK cell proliferation itself (**Figures 1** and **2C**). While our results demonstrate lung-specific NK cell proliferation for the first time, it should be noted that the proportion of NK cells proliferating within the lung is very low when compared to IAV-specific T cell proliferation at these same time points (**Figure 2A**) (27). At the peak of NK cell proliferation, approximately 2% of NK cells within the lung were found to be $CFSE^{+}BrdU^{+}$, while at the same time point, 55–75% of IAV-tetramer^{+} CD8 T cells (27) and 7–8% of all $CD3\varepsilon^{+}$ T cells (**Figure 2A**) within the lung are $CFSE^{+}BrdU^{+}$. Finally, although we observed NK cell proliferation within IAV-infected lungs, it is clear that the number of proliferating NK cells and the associated cell expansions could not account for the total increase in NK cell numbers observed within the lung. These data suggest that NK cell recruitment from the periphery into the lung is the main contributor to NK cell accumulation observed in both high- and low-dose IAV-infected lungs.

NK Cells Are Recruited to the Lung and DLN *via* Chemokine Receptors

As NK cell proliferation within the lung could not account for the total NK cell accumulation observed, we next determined if NK cells were recruited to the lung during IAV infection *via* chemokine receptors. Chemokine receptors are G protein-coupled receptors whose activation is required for gradient-directed recruitment of cells into tissues. To determine if chemokine receptors were necessary for NK cell accumulation within the IAV-infected lung, chemokine receptor activation was blocked by administering PT, an inhibitor of G protein-coupled receptors (35), to mice infected with either a high or a low dose of IAV. PT significantly decreased the number of NK cells in the lungs of both groups of IAV-infected mice (**Figure 3A**). Further, blocking chemokine receptor activation did not selectively inhibit the recruitment of a specific subset

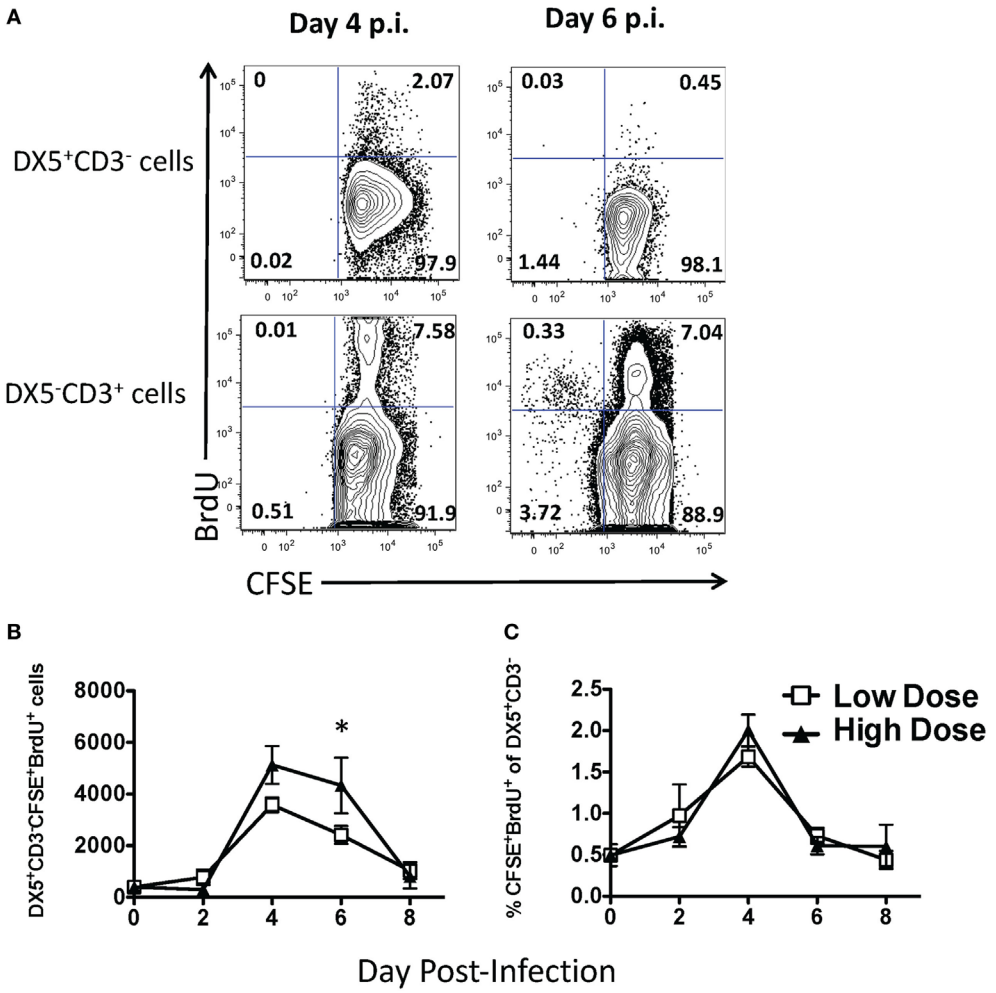

FIGURE 2 | Small frequency of natural killer (NK) cells proliferate in the lung during low- and high-dose influenza A virus (IAV) infections. BALB/c mice were infected with either a high- or low-dose IAV infection and administered carboxyfluorescein succinimidyl ester (CFSE) and bromodeoxyuridine (BrdU) intranasally on the day of analysis, as described in Section "Materials and Methods." **(A)** Representative flow plots for NK cells ($DX5^+CD3\varepsilon^-$ gated) and T cells ($DX5^-CD3\varepsilon^+$ gated) in the lungs of day 4 or 6 low-IAV dose-infected mice. The **(B)** number and **(C)** frequency of proliferating NK cells (i.e., the percentage of all $DX5^+CD3\varepsilon^-$ cells that were also $CFSE^+BrdU^+$) was determined by flow cytometry. The *p*-values comparing high- vs. low-dose IAV-infected animals are shown. $^*p < 0.05$ (two-tailed unpaired Student's *t*-test). Data are pooled from three high-dose experiments and two low-dose experiments, with five mice per group, per time point.

of NK cells, as there was no change in the subset distribution between PBS- and PT-treated mice (**Figure 3B**). Notably, while PBS-treated mice infected with a high dose of IAV had significantly more NK cells in the lungs at day 6 p.i. than low-dose-infected mice (**Figures 1** and **3A**), there was no difference in the number of NK cells between high- and low-dose infections in PT-treated mice, suggesting that the difference in NK cell accumulation in the lung between high- and low-dose infections in non-PT treated mice is likely due to enhanced recruitment *via* chemokine receptors. No difference was observed in NK cell numbers within the DLN or spleens between PT- and PBS-treated mice, suggesting that the difference in NK cell accumulation observed within the lung was due to a decreased trafficking, rather than a toxic effect of PT treatment (**Figure 3C**).

To determine a more specific role for chemokine receptors on NK cells in NK cell trafficking during IAV infection, NK cells from CD45 congenic mice were incubated with PT ($CD45.1^-CD45.2^+$) or PBS ($CD45.1^+CD45.2^+$) prior to adoptive transfer into IAV-infected hosts ($CD45.1^+CD45.2^-$) (**Figure 4A**). We observed a significant reduction in PT-treated NK cell trafficking to the lung compared to PBS-treated donor NK cells in high-dose IAV-infected mice at day 4 p.i. and in low-dose IAV-infected mice at day 6 p.i. (i.e., lower numerical ratio as a result of a reduced recruitment of PT-treated donor cells compared to PBS-treated NK cells, **Figure 4B**). In contrast to our findings *in vivo* in PT-treated mice, *in vitro* PT-treated NK cell trafficking to the DLN was significantly reduced compared to PBS-treated NK cells in both high- and low-dose IAV-infected mice on days 4 and 6 p.i. (**Figure 4B**). The reduced trafficking of PT-treated NK cells into the DLN at day 6 p.i. (**Figure 4B**) suggests that while the overall number of NK cells in the DLN is decreasing at that time, there is still a detectable influx of NK cells and that trafficking into the

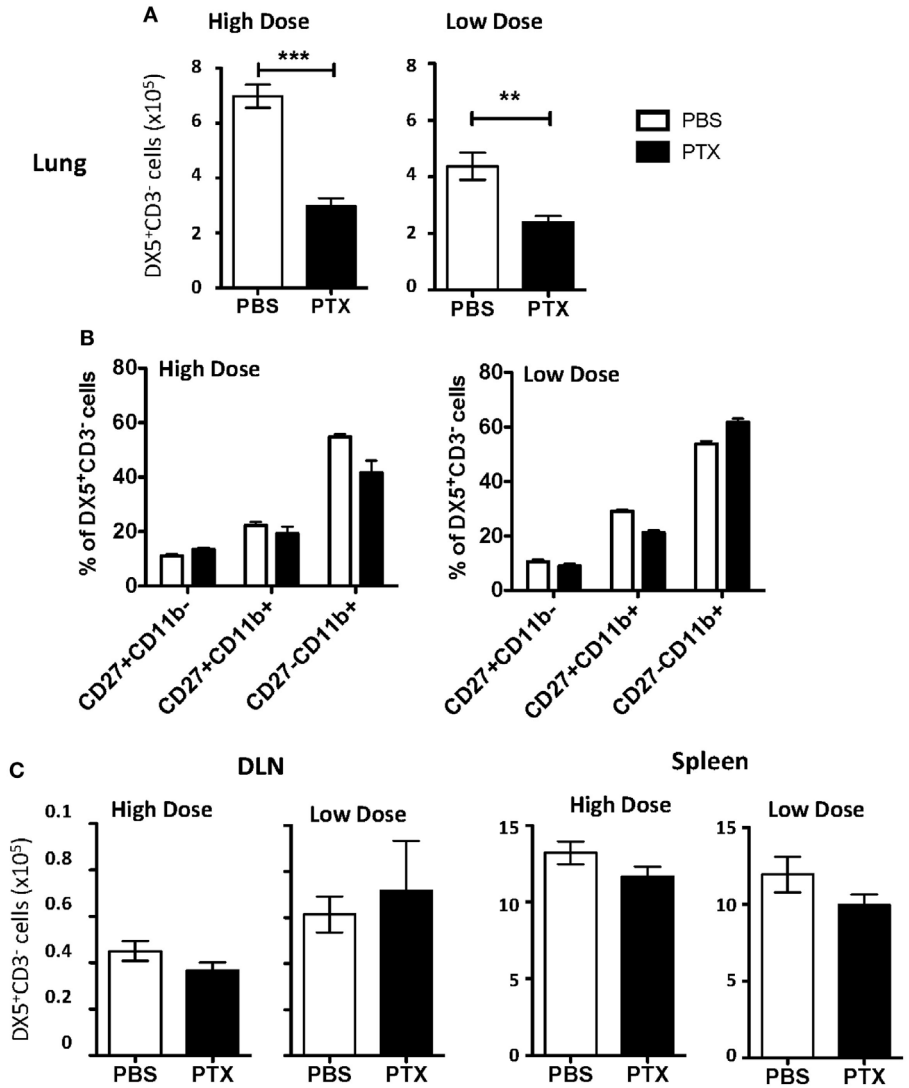

FIGURE 3 | Blockade of chemokine receptor-mediated recruitment results in decreased natural killer (NK) cell accumulation in the influenza A virus (IAV)-infected lung. BALB/c mice were infected with either a high or a low dose of IAV and administered a 500 ng/200 µl intraperitoneal injection of pertussis toxin (PT) or 200 µL phosphate buffered saline (PBS) on days 2, 3, 4, and 5 post infection (p.i.). On day 6 p.i., lungs **(A)**, draining lymph node (DLN) (per node) **(C)**, and spleens **(C)** were harvested from high-dose-infected mice or low-dose-infected mice, and the number and frequency of NK cells ($DX5^+CD3\varepsilon^-$ gated) were determined by flow cytometry. **(B)** NK cells ($DX5^+CD3\varepsilon^-$ gated) in the lung were further characterized by CD27 and CD11b expression to determine changes in functional NK subset distribution. Shown are the *p*-values comparing PT-treated mice to PBS-treated mice. $^{**}p < 0.005$, $^{***}p < 0.001$ (two-tailed unpaired Student's *t*-test). Data are pooled from three high-dose experiments and two low-dose experiments, with five mice per group.

DLN at later time points is mediated by chemokine receptors. No difference in NK cell trafficking to the spleen was observed (**Figure 4B**). These data further support a role for chemokine receptor-mediated trafficking of NK cells to the lungs and DLN during IAV infection.

CXCR3 Expression on NK Cells Increases NK Cell Recruitment to the Lungs and DLN of IAV-Infected Mice

Since our results implicate chemokine receptors in NK cell accumulation in the lung, we determined which chemokine receptors were used for NK cell trafficking to the IAV-infected lung. CXCR3 is an important chemokine receptor for NK cell homing in homeostasis (20), and the receptor is used by CD8 T cells to migrate into the IAV-infected lung (23). Therefore, to determine if CXCR3 was necessary for NK cell recruitment to the IAV-infected lung, we again utilized a competitive trafficking assay. Donor congenic WT $CXCR3^{+/+}$ ($CD45.1^+CD45.2^+$) and $CXCR3^{-/-}$ ($CD45.1^-CD45.2^+$) NK cells were transferred into IAV-infected $CXCR3^{+/+}$ hosts ($CD45.1^+CD45.2^-$) (**Figure 5A**). There was a significant decrease in $CXCR3^{-/-}$ NK cell trafficking compared to donor WT NK cell trafficking (i.e., KO:WT ratio <1) to the lung and DLN in high-dose-infected mice and to the lungs

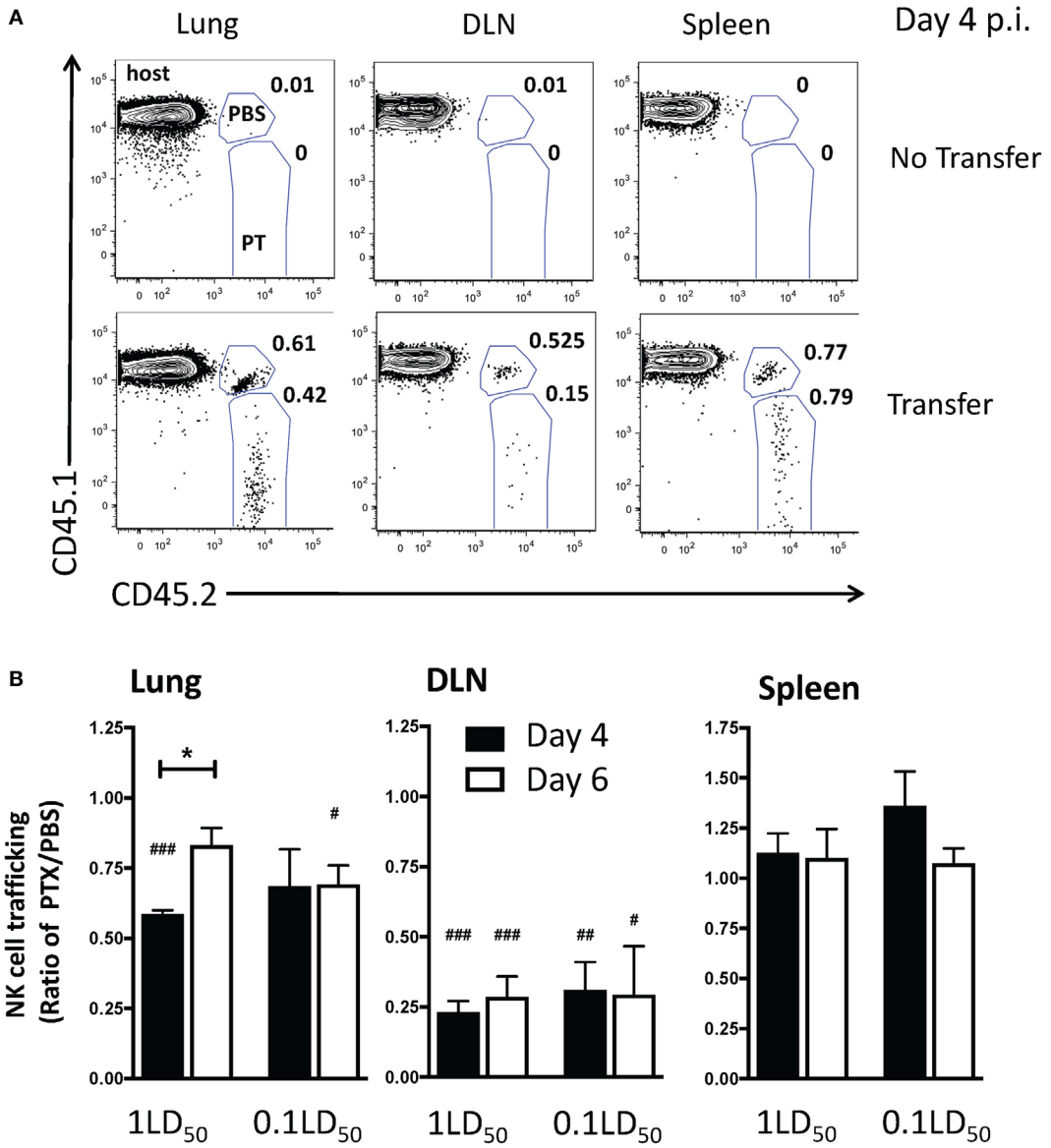

FIGURE 4 | Inhibition of chemokine receptor signaling in natural killer (NK) cells results in a decreased accumulation in the lungs and draining lymph nodes (DLNs) of influenza A virus (IAV)-infected mice. C57Bl/6 (CD45.1) host mice were infected with a high- or low-dose of IAV. 1×10^5 donor C57Bl/6 NK cells treated with pertussis toxin (PT) ($CD45.2^+$) and 1×10^5 C57Bl/6 NK cells treated with phosphate buffered saline (PBS) ($CD45.1^+CD45.2^+$) were transferred into infected host mice for 16–18 h before analysis (i.e., transferred on day 3 p.i. for day 4 analysis, or day 5 p.i. for day 6 analysis). A group of non-NK cell transferred mice was used as a control. **(A)** Representative flow plots for NK cells ($NK1.1^+CD3\varepsilon^-$) on day 4 p.i. in high-dose IAV lungs, DLN, and spleen showing host ($CD45.1^+CD45.2^-$), PBS-treated donor ($CD45.1^+CD45.2^+$), and PT-treated donor ($CD45.1^-CD45.2^+$) NK cells. **(B)** the normalized ratio of PT-treated NK cells to PBS-treated control NK cells in the lungs, DLN, and spleen of infected recipients is shown. Note: equal trafficking between PBS- and PT-treated NK cells = 1, an increased PT-treated NK cell trafficking >1, and a decreased PT-treated NK cell trafficking <1. *p*-values comparing PT-treated NK cell trafficking with PBS-treated cells within the same group are shown. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ (two-tailed paired Student's *t*-test). $^{*}p < 0.05$ represents comparison of the PT/PBS ratios between the indicated groups. High- and low-dose experiments were performed once with four mice per group.

of low-dose-infected mice (**Figure 5B**). Notably, the ratio of recruitment of donor $CXCR3^{-/-}$ to $CXCR3^{+/+}$ NK cell was also significantly reduced in high-dose compared to low-dose IAV-infected mice at day 6 p.i. (**Figure 5B**), suggesting that CXCR3 expression on NK cells could be more important for recruitment to the lung in high-dose IAV infections. Therefore, the amount of CXCR3 ligands, CXCL9, CXCL10, and CXCL11, was quantified in the lungs of high- and low-dose-infected mice (**Figure 6**) to determine if differences in ligand expression correlated with the differences observed in the competitive trafficking assay (**Figure 5B**). All CXCR3 ligands were significantly increased in the lungs of high-dose compared to low-dose-infected BALB/c mice at day 2 p.i., while only CXCL11 was expressed in higher amounts from days 2 to 6 p.i. (**Figure 6A**). Similarly, CXCL9

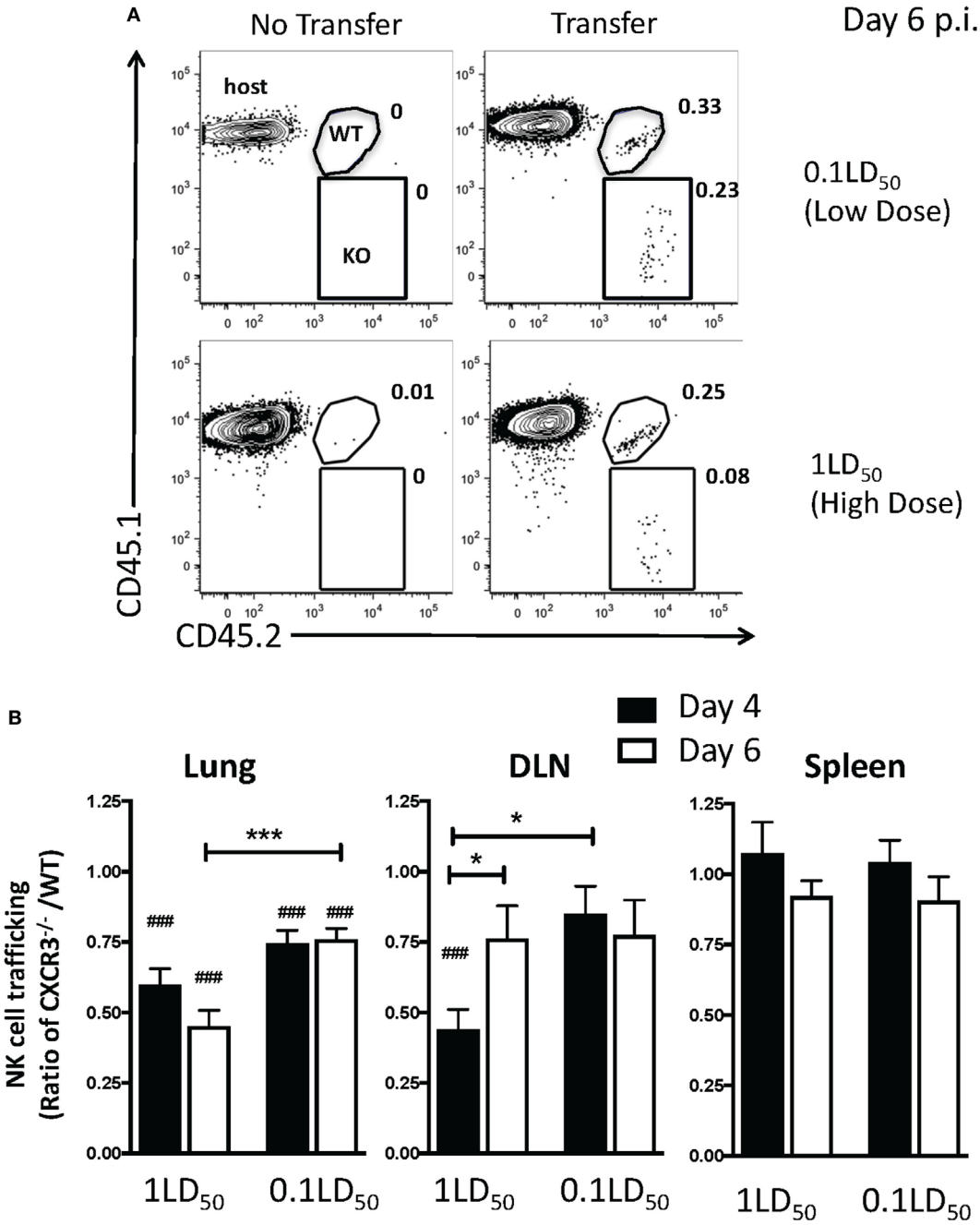

FIGURE 5 | CXCR3 expression by natural killer (NK) cells increases NK cell trafficking to the lung and draining lymph node (DLN) during influenza A virus (IAV) infection. C57Bl/6 (CD45.1) host mice were infected with a high or a low dose of IAV. 5×10^5 donor WT C57Bl/6 (CD45.1/CD45.2) and 5×10^5 CXCR3$^{-/-}$ (CD45.2) NK cells were transferred into infected host mice for 16–18 h before analysis. **(A)** Representative flow plots for NK cells (NK1.1$^+$CD3ε$^-$) on day 6 post infection (p.i.) in high- or low-dose IAV lungs showing host (CD45.1$^+$CD45.2$^-$), WT donor (CD45.1$^+$CD45.2$^+$), and CXCR3$^{-/-}$ donor (CD45.1$^-$CD45.2$^+$) NK cells. **(B)** The normalized ratio of CXCR3$^{-/-}$ NK cells to WT NK cells in the lungs, DLN, and spleen of infected recipients is shown. Note: equal trafficking between WT and CXCR3$^{-/-}$ NK cells = 1. *p*-values comparing WT to CXCR3$^{-/-}$ NK cell trafficking (two-tailed paired Student's *t*-test) within the same group are shown. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, and comparing, as indicated, the CXCR3$^{-/-}$ NK cells:WT NK cell ratio between high- and low-dose IAV-infected mice or by time post infection. $^{*}p < 0.05$, $^{***}p < 0.001$. Data are pooled from two high-dose experiments and three low-dose experiments, with five mice per group.

and CXCL10 were significantly greater in the lungs of high-dose vs. low-dose-infected C57Bl/6 mice at both days 2 and 4 p.i. (**Figure 6B**). The increased amount of CXCR3 ligand found in the lungs of mice receiving a high-dose of IAV correlated with the reduced CXCR3$^{-/-}$ NK cell trafficking that was observed in high-dose IAV-infected mice (**Figure 5B**).

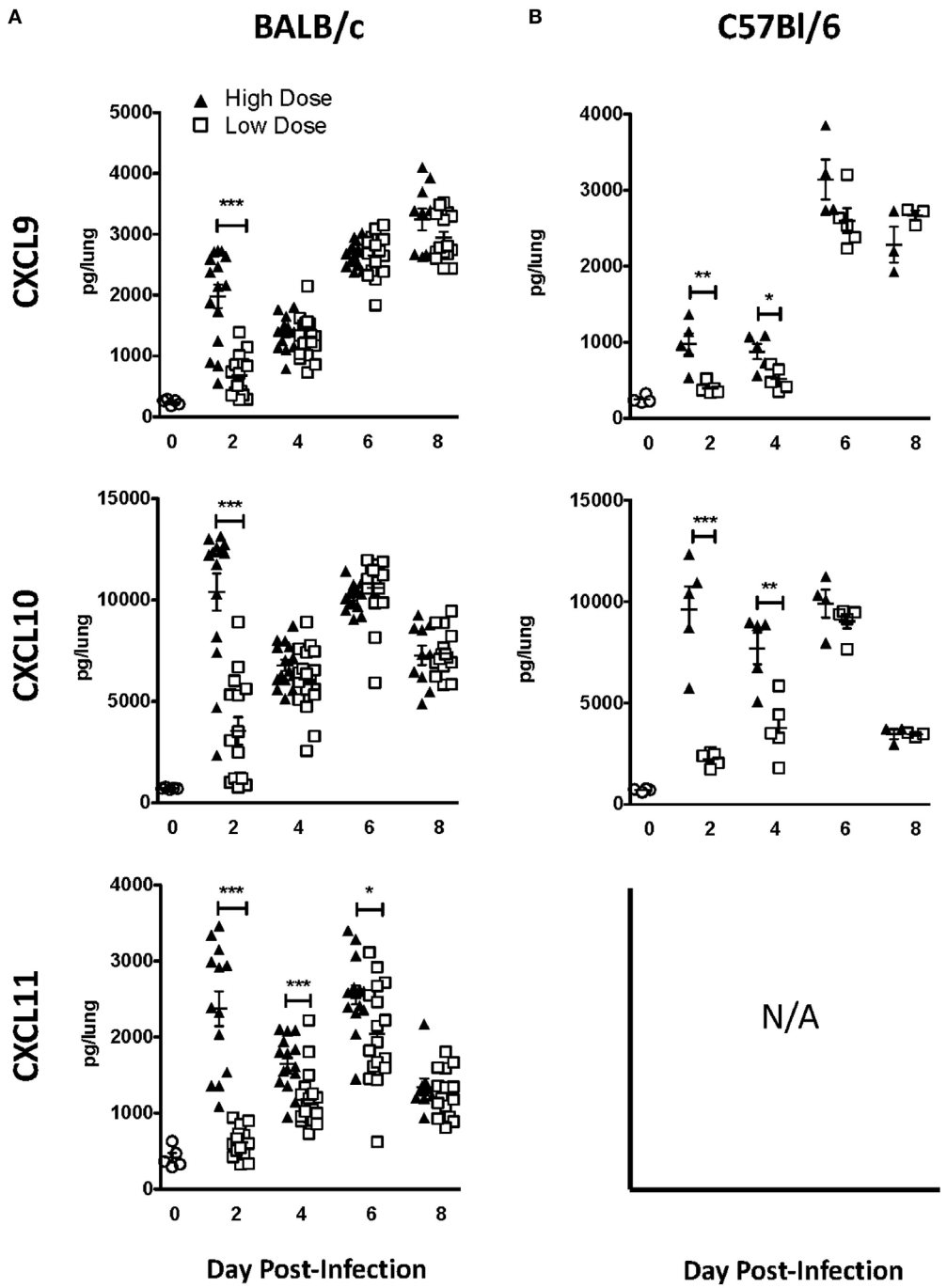

FIGURE 6 | CXCR3 ligands are upregulated in the lungs during influenza A virus (IAV) infection and expressed at higher levels in the lungs of high-dose-infected mice. **(A)** BALB/c and **(B)** C57Bl/6 mice were infected with either a high- or low-dose IAV. Lungs were homogenized, and the levels of CXCL9, CXCL10, and CXCL11 [not expressed (N/A) in C57Bl/6 mice] were quantified by ELISA. *p*-values comparing chemokine levels in high- vs. low-dose IAV-infected mice are shown. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$ (two-tailed unpaired Student's *t*-test). BALB/c data are pooled from two separate experiments with at least five mice per group. C57Bl/6 data are representative of one experiment with at least three mice per group.

CCR5 Localizes NK Cells to IAV-Infected Epithelium

While our above studies and others suggest that CXCR3 aids in NK cell recruitment to both the naïve- and IAV-infected lung (20), it is not known if or how NK cells are localized to infected epithelial cells. Similar to NK cells, CXCR3 expression on CD8 T cells increases their recruitment to the lung during IAV infection (23). However, once in the lungs, CCR5 is required for

memory CD8 T cell localization to the IAV-infected airway (22). Ligands for CCR5 (CCL3, CCL4, and CCL5) are upregulated in the lung of both BALB/c and C57Bl/6 mice [**Figure 7A** (36, 37)]. Interestingly, while similar trends in CXCR3 ligands were observed between BALB/c and C57Bl/6 mice, the pattern of relative CCL5 expression appeared to be inverted at later time points. While CCL5 was significantly upregulated at day 2 in high-dose IAV-infected lungs in both strains, CCL5 continued to have a higher expression in high-dose IAV-infected lungs in C57Bl/6 mice, while CCL5 expression was significantly higher in low-dose IAV-infected BALB/c mice (**Figure 7A**). Despite the difference in expression pattern, CCL5 was significantly upregulated in the lungs compared to naïve mice on days 2–8 during high-dose IAV infection and on days 4–8 during low-dose IAV infections for both mouse strains (**Figure 7A**, $p < 0.05$, student's unpaired *t*-test). Therefore, we hypothesized that CCR5 expression on NK cells would be required for localization to the infected epithelium. Previously, it had been shown that IAV-infected CCR5$^{-/-}$ mice have less NK cell accumulation in the lung than IAV-infected WT mice (15). However, this defect in NK cell recruitment could be due to a requirement for CCR5 in NK cell recruitment to the IAV-infected lung, or due to the known proliferative defects of CCR5$^{-/-}$ NK cells, which results in decreased NK cell numbers in the periphery of naïve mice (15). To more directly test if CCR5 was required for NK cell accumulation in the IAV-infected lung, CCR5$^{-/-}$ NK cells (CD45.1^{-}CD45.2^{+}) and WT NK cells (CD45.1^{+}CD45.2^{+}) were adoptively transferred into high-dose IAV-infected WT C57Bl/6 recipients (CD45.1^{+}CD45.2^{-}) (**Figure 7B**). A significant decrease in the accumulation of CCR5$^{-/-}$ NK cells compared to WT

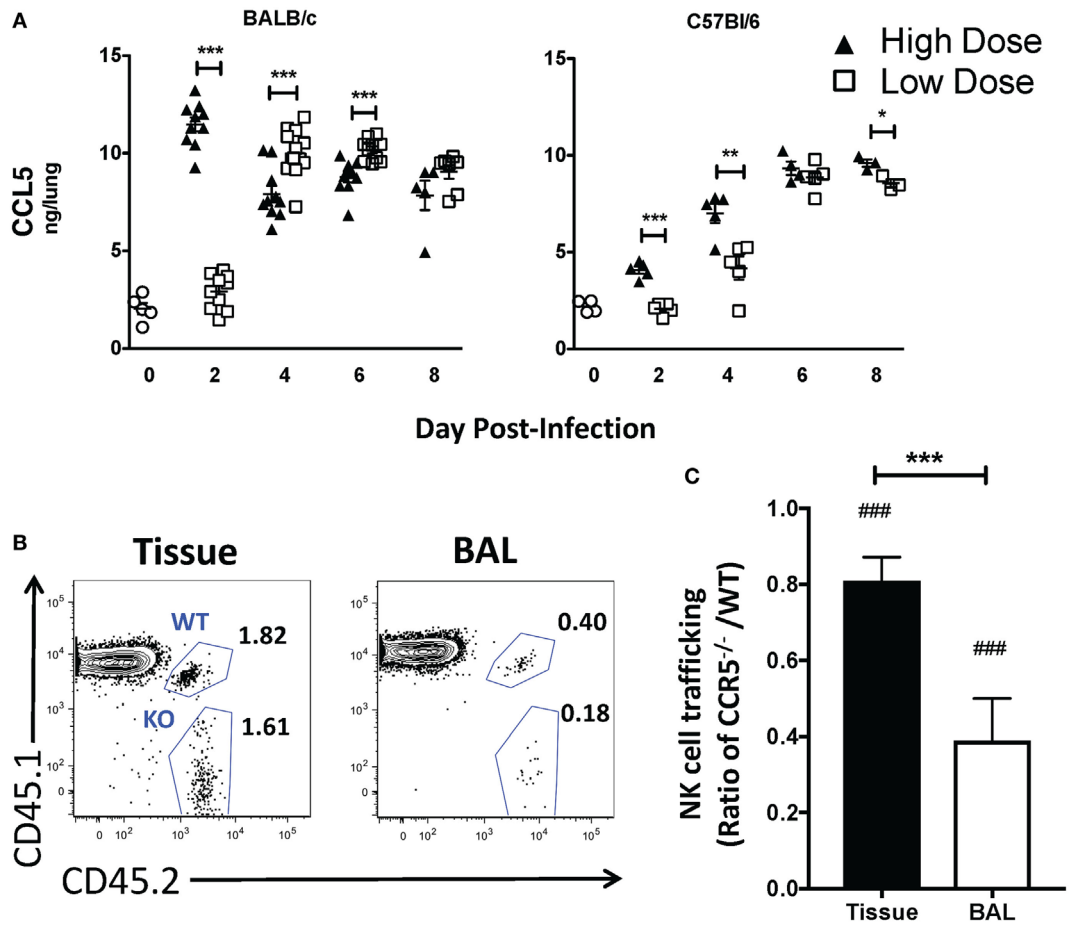

FIGURE 7 | CCL5 is upregulated in the lungs during high- and low-dose influenza A virus (IAV) infections. **(A)** BALB/c (left) and C57Bl/6 (right) mice were infected with high- and low-dose A/PR/8/34. Lungs were harvested at indicated time points, and CCL5 in supernatants was quantified by ELISA. Uninfected mice were used as naïve controls. *p*-values comparing CCL5 levels in high- vs. low-dose IAV-infected mice are shown. Data were obtained from samples as in **Figure 6**. **(B,C)** C57Bl/6 (CD45.1) host mice were infected with a high dose of IAV. 3 × 10^5 donor WT C57Bl/6 (CD45.1/CD45.2) and 3 × 10^5 CCR5$^{-/-}$ (CD45.2) natural killer (NK) cells were transferred into infected host mice for 16–18 h before analysis. **(B)** Representative flow plots for NK cells (NK1.1^{+}CD3ε^{-}) in the bronchoalveolar lavage (BAL) vs. lung tissue showing host (CD45.1^{+}CD45.2^{-}), WT donor (CD45.1^{+}CD45.2^{+}), and CCR5$^{-/-}$ donor (CD45.1^{-}CD45.2^{+}) NK cells. **(C)** Shown is the normalized ratio of CCR5$^{-/-}$ NK cells to WT NK cells in the lungs or BAL fluid of infected recipients. Data are from one experiment with six mice per group. Note: equal trafficking between WT and CCR5$^{-/-}$ NK cells = 1. *p*-values comparing WT to CCR5$^{-/-}$ NK cell trafficking (two-tailed paired Student's *t*-test) within the same group are shown. $^{###}p < 0.001$ and comparing the ratio of CCR5$^{-/-}$/WT NK cell trafficking in the BAL vs. lung tissue. $^{***}p < 0.001$.

NK cells in the lung tissue (i.e., ratio <1, **Figure 7C**) was observed at day 6 p.i. To further determine if CCR5 was used for NK cell localization within the IAV-infected lungs, CCR5$^{-/-}$ and WT NK cell accumulation within the bronchoalveolar lavage fluid was determined. The ability of CCR5$^{-/-}$ NK cells to traffic to the IAV-infected airway was significantly reduced compared to WT NK cells (**Figure 7C**), and the ability of CCR5$^{-/-}$ NK cells to traffic to the airway was significantly reduced compared to lung tissue, suggesting that while CCR5 may aid in NK cell recruitment to the lung tissue, it is also important for NK cell localization to the infected epithelium.

DISCUSSION

Our results show that NK cells are actively recruited to the lung and lung DLN during IAV infection, consistent with previous studies (5). However, herein, we show preferential organ-specific recruitment (31, 38, 39) that is dependent on the dose of infection. Recruitment to the lung is increased in high-dose IAV infections, and accumulation of NK cells within the DLN is increased in low-dose IAV infections. Importantly, studies have shown disparate roles for NK cells in immunity against IAV, with NK cells playing a protective role in lower-dose IAV infections and contributing to lethal pathology in lethal dose infections (5, 8, 9, 12, 13). While the results presented herein argue for differential NK cell recruitment and trafficking based on the IAV infection dose, they also suggest that such differences may not alter pulmonary virus titers from days 2 to 8 p.i. Furthermore, our results show that the loss of NK cells prior to both high- and low-dose IAV infections does not alter IAV morbidity or mortality. However, at this time, it is difficult to interpret the direct contribution of NK cells to the overall virus control and pathology as compensatory changes in other immune cell populations that contribute to these aspects occur in the absence of NK cells or during different infection doses. For example, our preliminary studies suggest that IAV-specific CD4 T cell responses may be increased in the absence of NK cells (not shown). Furthermore, our prior studies have demonstrated reduced levels of IAV-specific CD8 T cells in the lungs of $1LD_{50}$ dose vs. 0.1 LD_{50} dose IAV-infected mice (26). Therefore, future studies determining if the magnitude of the NK cell response within the lungs or DLN is a significant contributor to pathology or virus control in both high- and low-dose IAV infections will also need to assess the modulation of other immune cell populations critical to IAV immunity and pathology at the same time.

Herein, we show for the first time that there is a lung-specific NK cell proliferation during IAV infection by labeling pulmonary cells with CFSE in addition to intranasal BrdU administration. Our results are consistent with other studies that administered BrdU systemically (33, 34) and *in vitro* NK cell: alveolar macrophage co-cultures (10). Further, we show that NK cell proliferation within the lung is not dependent on IAV virus titers, as the same frequency of NK cells proliferated within the lung during both high- and low-dose IAV infections. Our findings that NK cell proliferation within the lung does not account for the total accumulation of NK cells and that PT treatment decreases NK cell accumulation within the lung suggest that NK cell accumulation in the lung is largely due to a directed recruitment rather than a local proliferation.

CXCR3 is important for NK cell trafficking to the lung both in homeostasis and in bacterial infection (20, 21). Herein, we show that CXCR3 also plays a role in recruiting NK cells to the lung during IAV infection, as CXCR3$^{-/-}$ NK cells did not traffic to the lung as efficiently as WT NK cells. The difference between WT and CXCR3$^{-/-}$ NK cell trafficking was more pronounced in mice infected with a high-dose IAV infection, and CXCR3 ligands were significantly higher in the lungs of high-dose compared to low-dose IAV-infected mice. Whether CXCR3 plays a more dominant role in NK cell recruitment during high-dose IAV infections or is solely responsible for the increased accumulation of NK cells within high-dose IAV-infected lungs remains to be determined. Importantly, although CXCR3$^{-/-}$ NK cells had significantly reduced recruitment compared to CXCR3$^{+/+}$ NK cells, CXCR3$^{-/-}$ NK cells did accumulate in IAV-infected lungs. This suggests that the recruitment of NK cells to the lung during IAV infection may be multifactorial, most likely utilizing several chemokine receptors. Indeed, we have shown herein that CCR5 is also used for NK cell recruitment to the IAV-infected lung (**Figure 7C**). CCR5$^{-/-}$ NK cells had impaired accumulation to the IAV-infected lung compared to WT NK cells; however, the decrease in CCR5$^{-/-}$ NK cell accumulation was not as significant as the decrease in CXCR3$^{-/-}$ NK cells accumulation when compared to WT NK cells (**Figures 5B** and **7C**). In addition to CXCR3 and CCR5, CCR2 is important for NK cell trafficking during IAV infection. While others have shown that CCR2 on NK cells does not affect accumulation within the IAV-infected lung tissue, it is used by NK cells to traffic to the IAV-infected airways (19). It is possible that CCR2, among other chemokine receptors, is able to compensate for the loss of CXCR3 or CCR5 in NK cell migration to the IAV-infected lung.

Our experiments utilizing PT treatment to inhibit NK cell trafficking resulted in opposing findings within the DLN, where NK cell accumulation was not affected in PT-treated mice, but was significantly inhibited when NK cells were treated with PT *in vitro*. Chemokine receptor signaling is required for both cell recruitment to organs and cell egress from organs. CXCR3 has been shown to increase NK cell recruitment to inflammation DLNs, while S1P5 and homeostatic chemokines are utilized for NK cell egress from lymph nodes (31, 32, 40, 41). By treating mice with PT for 4 days *in vivo* prior to analysis, we likely uncoupled these two processes, and it is not possible to determine if the lack of difference observed in NK cell numbers in the DLN between *in vivo* PT and PBS-treated mice is due to changes in recruitment to or egress from the lymph node. As the adoptive transfer model examines NK cell recruitment for 16–18 h after transfer, we are more likely to be observing NK cell recruitment to the DLN, rather than recruitment and egress. While the number of NK cells in the DLN begins to decrease by day 6 p.i. (**Figure 1B**), NK cell recruitment to the DLN could still be occurring at day 5 p.i., when PBS- and PT-treated NK cells were adoptively transferred to IAV-infected hosts. Finally, NK cells in *in vivo* PT- and PBS-treated mice had been exposed to inflammation for 5 days prior to analysis, while *in vitro* PBS- and PT-treated NK cells were adoptively transferred into

IAV-infected hosts and exposed to inflammation for 16–18 h before analysis. It is possible that this difference in inflammation exposure results in temporal differences in NK cell trafficking. Curiously, a recent study has also evaluated the role of CXCR3 in the trafficking of NK cells to the DLN during influenza infection and found that despite the expression of CXCR3 predominantly on the immature $CD27^{+}CD11b^{-}$ subset, the accumulation of $CXCR3^{-/-}$ NK cells in the DLN was largely unaffected (31). This result is consistent with our observations during low-dose IAV infection (**Figure 5**). However, during high-dose infection, our results suggest a greater importance for CXCR3 in NK cell recruitment to the DLN, as $CXCR3^{-/-}$ NK cell recruitment was reduced (**Figure 5B**). Altogether, these results suggest that a multifactorial model of chemokine receptor directed recruitment into the DLN where CXCR3 may play a more prominent role during high inflammatory or high pathogenic infection conditions.

Overall, our data suggest that the severity of IAV infection dictates the localization and number of NK cells in lymphoid tissue and the site of infection and that this localization is partially dependent on CXCR3 and CCR5 and differential expression of CXCR3 and CCR5 ligands.

ETHICS STATEMENT

All procedures were approved by the University of Iowa Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

LC and EH conducted the CCR5 adoptive transfer experiments. LC conducted the trafficking kinetics, BrdU, PTX, CXCR3 adoptive transfer, and ELISA experiments, and these experiments are part of her dissertation (42). ZZ conducted the viral titer experiments. LC, EH, JH, ZZ, and KL contributed to data analysis and interpretation. JH and KL contributed to the conception of the work. LC and EH wrote the manuscript with substantial input from JH and KL. All the authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00781/full#supplementary-material.

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Diverse Epitope Specificity, Immunodominance Hierarchy, and Functional Avidity of Effector CD4 T Cells Established During Priming Is Maintained in Lung After Influenza A Virus Infection

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One of the major contributions to protective immunity to influenza viruses that is provided by virus-specific CD4 T cells is delivery of effector function to the infected lung. However, there is little known about the selection and breadth of viral epitope-specific CD4 T cells that home to the lung after their initial priming. In this study, using a mouse model of influenza A infection and an unbiased method of epitope identification, the viral epitope-specific CD4 T cells elicited after infection were identified and quantified. We found that a very diverse specificity of CD4 T cells is primed by infection, including epitopes from hemagglutinin, neuraminidase, matrix protein, nucleoprotein, and non-structural protein-1. Using peptide-specific cytokine EliSpots, the diversity and immunodominance hierarchies established in the lung-draining lymph node were compared with specificities of CD4 T cells that home to the lung. Our studies revealed that CD4 T cells of all epitope specificities identified in peripheral lymphoid tissue home back to the lung and that most of these lung-homing cells are localized within the tissue rather than the pulmonary vasculature. There is a striking shift of CD4 T cell functionality that enriches for IFN-γ production as cells are primed in the lymph node, enter the lung vasculature, and finally establish residency in the tissue, but with no apparent shifts in their functional avidity. We conclude that CD4 T cells of broad viral epitope specificity are recruited into the lung after influenza infection, where they then have the opportunity to encounter infected or antigen-bearing antigen-presenting cells.

Keywords: CD4 T cells, influenza, lung, immunodominance, epitopes, antigen presenting cells, allophycoerythrin-conjugated

INTRODUCTION

The fate and function of CD4 T cells elicited by infection with influenza virus, although extensively studied by many groups [reviewed in Ref. (1–8)], are still incompletely understood. It is clear that some cells remain in the draining lymph node (dLN) and participate in the germinal center response that is needed to elicit high affinity protective antibodies. CD4 T cells that remain in the dLN can

also provide help to virus-specific CD8 T cells, promoting their expansion as effector cells as well as their establishment and maintenance as memory cells. Other subsets of virus-specific CD4 T cells enter the recirculating pool and can be detected in peripheral blood and in the spleen [reviewed in Ref. (6–8)]. Finally, a major subset of virus infection primed CD4 T cells traffic to the lung where they can convey a number of effector functions including production of anti-viral cytokines such as IFN-γ, facilitation in the localization of infiltrating CD8 T cells in the airway (9), or direct cytotoxicity of antigen-bearing cells [reviewed in Ref. (10)].

Coincident with the diverse functions of CD4 T cells in immunity to influenza is their very broad epitope specificity. Studies in our laboratory using several different mouse strains have indicated that influenza infection elicits a broad array of distinct epitope specificities (~20 to >60, depending on the mouse strain) that are derived from many different influenza proteins (11–13). One question that has not yet been addressed is whether these diverse peptide specificities that are elicited by infection persist in the CD4 T cells that home to the lung after infection. For the most part, CD4 T cell trafficking to the lung after influenza infection has been studied either by using T cell receptor (TcR) transgenic CD4 T cells (14) or other methods such as tetramer staining that tracks single epitope specificities (15, 16) or by analyzing infiltrating CD4 T cells independently of their specificity (17).

We speculated that there might be several mechanisms that could contribute to restricted epitope specificity in the lung during respiratory infection relative to the dLN where priming occurs. First, recent data (18–20) suggest that the "strength of signal" delivered through the TcR can determine CD4 T cell fate between follicular helper cells (Tfh) and other types of effector cells, leading to the possibility that epitope-specific preferences might emerge soon after priming in the dLN. In fact, our laboratory recently showed that divergence between Tfh and non-Tfh cells quantified in CD4 T cells isolated from the dLN at the peak of the response differs among distinct peptide epitopes. These epitope-dependent preferences in CD4 T cell differentiation persist independently of whether the epitopes are introduced *via* infection, protein vaccination, or in a completely heterologous protein vector (21). Second, CD4 T cells that home to the lung after infection likely have the opportunity to interact with viral antigen bearing, class II positive antigen-presenting cells (APC) in the lung that are distinct from those in the lymph node (22, 23). Third, in the lung, glycoprotein viral antigen or influenza virion handling by lectin receptors (24, 25) or viral antigen abundance could lead to distinct virus epitope display than that presented in the dLN. All of these events could affect CD4 T cell specificity, effector function, as well as selectivity of the repertoire established in the CD4 T cell memory pool.

Because of the importance of this issue, in this study, we sought to empirically examine the CD4 T cell peptide specificity, drawn from the endogenous, polyclonal CD4 T cell repertoire that homes to the lung after influenza infection. Using a mouse model of influenza A H1N1 infection and an unbiased method to identify CD4 T cell epitopes elicited by influenza infection, we compared the diversity of influenza-specific CD4 T cells and immunodominance hierarchies within the lung with that established in the priming lymph node. We also examined the distribution of CD4 T cells within pulmonary vasculature and lung tissue and their cytokine potential and the avidity of their T cell receptors. Our studies revealed that most of the antigen-specific pulmonary CD4 T cells are localized to the tissue as compared to the vasculature and that the extensive degree of epitope-specific diversity observed in the dLN is maintained in the lung after infection.

MATERIALS AND METHODS

Mice

A/JCr female mice were purchased from Charles River laboratories and were maintained in the specific pathogen-free facility at the University of Rochester according to the institutional guidelines. Mice were used at 2–6 months of age. Experiments typically involved cells from pooled tissues from six to eight mice unless otherwise noted.

Ethics Statement

All mice were maintained in a specific-pathogen free facility at the University of Rochester Medical Center according to the institutional guidelines. All animal protocols used in this study adhere to the AAALAC International, the Animal Welfare Act, and the PHS Guide and were approved by the University of Rochester Committee on Animal Resources, Animal Welfare Assurance Number A3291-01. The protocol under which these studies were conducted was originally approved March 4, 2006 (protocol no 2006-030) and has been reviewed and re-approved every 36 months with the most recent review and approval January 23, 2018.

Peptides

17-mer peptides overlapping by 11 amino acids to encompass the entire sequence of the HA and NA proteins from the H1N1 strain of influenza virus A/New Caledonia/20/99, the NS1 sequence from A/New York/444/2001, and the NP and M1 from A/New York/348/2003 were obtained from BEI Resources, ATCC. The internal proteins for influenza are generally conserved between the virus strains A/New Caledonia/20/99, A/New York/348/2003 and A/New York/444/2001. The peptides were reconstituted at 10 mM in PBS, with or without added dimethyl sulfoxide, to increase solubility of hydrophobic peptides, and 1 mM dithiothreitol, for cysteine containing peptides. Working stocks (1 mM) were prepared in complete DMEM, filter sterilized and stored at −20°C, as were concentrated stocks. **Table 1** provides the sequences and nomenclature for the peptides used in these studies.

Influenza Infections and Tissue Harvest

A/New Caledonia/20/99 virus was produced as we have previously described (11). Mice were infected intranasally at 50,000 EID_{50} in 30 µl of phosphate buffered saline (PBS) after being anesthetized by intraperitoneal injection with tribromoethanol (Avertin; 250–300 µl per mouse, normalized to weight). Spleen, mediastinal

TABLE 1 | MHC-class II restricted epitopes (A/J).

Peptide	Peptide sequence
*HA_{120}	**120** EQLSSVSSFERFEIFPK **136**
*HA_{174}	**174** YPNLSKSYVNNKEKEVL **190**
*HA_{215}	**215** VSVVSSHYSRRFTPEIA **231**
*HA_{358}	**358** TGMVDGWYGYHHQNEQG **374**
*HA_{375}	**375** SGYAADQKSTQNAINGI **391**
*HA_{398}	**398** VIEKMNTQFTAVGKEFN **414**
***HA*$_{492}$**	***492** MESVKNGTYDYPKYSEE **508***
*NA_{279}	**279** CSCYPDTGTVMCVCRDN **295**
*NA_{291}	**291** VCRDNWHGSNRPWVSFN **307**
*NA_{351}	**351** YKYGNGVWIGRTKSNRL **367**
*NA_{381}	**381** TDTDSDFSVKQDVVAIT **397**
*NA_{405}	**405** SFVQHPELTGLDCIRP **420**
*NA_{416}	**416** DCIRPCFWVELVRGLPR **432**
NA_{422}	**422** FWVELVRGLPRENTTIW **438**
*NA_{446}	**446** FCGVNSDTANWSWPDGA **462**
*NA_{452}	**452** DTANWSWPDGAELPFTI **468**
NP_{49}	**49** LNDYEGRLIQNSLTIER **65**
*NP_{103}	**103** KWVRELVLYDKEEIRRI **119**
*NP_{127}	**127** DDATAGLTHIMIWHSNL **143**
*NP_{187}	**187** GTMVLELIRMIKRGIND **203**
*NP_{258}	**258** FLARSALILRGSVAHKS **274**
*NP_{342}	**342** RVSSFIRGTRVLPRGKL **358**
*NP_{408}	**408** TQPTFSVQRNLPFDKTT **424**
NP_{414}	**414** VQRNLPFDKTTIMAAFT **430**
*NP_{426}	**426** MAAFTGNTEGRTSDMRA **442**
*NP_{432}	**432** NTEGRTSDMRAEIIKMM **448**
*NP_{474}	**474** PIVPSFDMSNEGSYFFG **490**
*$M1_{37}$	**37** TDLEALMEWLKTRPILS **53**
*$M1_{103}$	**103** LKREITFHGAKEIALSY **119**
*$M1_{151}$	**151** CEQIADSQHKSHRQMVT **167**
*$M1_{187}$	**187** KAMEQMAGSSEQAAEAM **203**
*$M1_{228}$	**228** GLKNDLLENLQAYQKRM **244**
$NS1_{13}$	**13** CFLWHVRKQVADQDLGD **29**
$NS1_{90}$	**90** LTDMTIEEMSRDWFMLM **106**
$NS1_{144}$	**144** LTLLRAFTEEGAIVGEI **160**
$NS1_{174}$	**174** VKNAIGVLIGGLEWNDN **190**
$NS1_{192}$	**192** VRVSETLQRFAWRSSNE **208**
$NS1_{192}$	**192** VRVSETLQRFAWRSSNE **208**

Antigenic peptides from major viral proteins (HA, NA, NP, M1, and NS1) used to identify and quantify epitope specific CD4 T cells in different tissues.
Peptides used in pools (Figures 6** and **7**) to stimulate antigen- specific CD4 T cells followed by intracellular cytokine staining.*
The bold numbers represent the amino acid numbers of the N- and C- terminal amino acids of each peptide.

lymph nodes (mLN), and lungs were excised at 9–11 days postinfection. For cytokine EliSpots, tissues were pooled (five to eight mice) and used as the source of CD4 T cells. For some flow cytometry analyses, individual mice were sampled, as indicated in the figure legends. Lung tissue was minced and digested in cell culture media (RPMI 1640 supplemented with L-glutamine, 2.5% FBS, 10 mM HEPES) containing collagenase type II (1 mg/ml) and DNaseI (30 μg/ml) at 37°C for 30 min. Digested lung tissue and other lymphoid tissues were crushed, passed through a 40 μm mesh filter and rinsed using DMEM supplemented with 10% FBS. Samples were depleted of red blood cells (RBC) using ACK Lysis Buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2-EDTA in H_2O, pH 7.2). After washing, cells were depleted of B cells, CD8 T cells, and MHC class II positive cells by negative selection using MACS negative selection (Miltenyi Biotec, San Diego, CA, USA), according to the manufacturer instructions.

EliSpot Assays

EliSpot assays were performed as previously described (12, 26, 27). Briefly, 96-well filter plates (Millipore, Billerica, MA, USA) were coated with 2 μg/ml purified rat anti-mouse IL-2 or IFNγ (clone JES6-1A12, clone AN-18, respectively, BD Biosciences, San Jose, CA, USA) in PBS overnight at 4°C, washed with media to remove any unbound antibody and incubated with 100 μl media per well for 1 h to block non-specific binding. Isolated CD4 T cells (200,000 mLN cells per well, 350,000 splenocytes per well, or 10,000 lung cells per well) were co-cultured with 500,000 syngeneic spleen cells and peptide at a final concentration of 5 μM in a total volume of 200 μl for 18–20 h at 37°C and 5% CO_2. The cells were removed from the plates, and the plates were washed with wash buffer (1X PBS, 0.1% Tween-20). Biotinylated rat anti-mouse IL-2 or IFNγ (clone JES6-5H4, clone XMG1.2, respectively, BD Biosciences) was added at a concentration of 2 μg/ml, 50 μl/well, in wash buffer with 10% FBS and incubated at room temperature for 30 min. The plates were washed again and streptavidin-conjugated alkaline phosphatase (Jackson Immuno Research, West Grove, PA, USA) was added at a dilution of 1:1000 in wash buffer with 10% FBS, 50 μl well, and incubated for 30 min at room temperature. The plates were washed with wash buffer and developed using Vector Blue substrate kit III (Vector Laboratories, CA) prepared in 100 mM Tris, pH 8.2. After drying, quantification of spots was performed with an Immunospot reader series 5.2, using Immunospot software, version 5.1.

Statistical Treatment of Data

Least squares linear regression models were fit to epitope-specific counts from EliSpot assays using CD4 T cells isolated from dLN and lung. Plots of residuals vs. predicted values and normal scores were examined to check the assumptions of normally distributed errors with constant variance. Studentized residuals and Cook's distance were computed to detect potential outliers. Base library and ggplot2 package in R were used for analyses and data representation.

T Cell Hybridoma Assays

NA446 and NP127 specific T cell hybridomas (see **Table 1** for amino acid sequences and nomenclature used) were generated by fusion of TcR-negative, BW5147 lymphoma cells with peptide-activated T cells harvested from the mLN or lung isolated from New/Caledonia/20/99 virus-infected A/J mice 10 days postinfection. Selection for fused cells with HAT was followed subcloning by limiting dilution cloning such that each clone was derived from a single cell. T cell assays were performed as previously described where T cell hybridomas were co-cultured with naïve splenocytes and peptide at a range of doses (0.0001–100 μM to) for 18 h in flat bottom 96-well plates. IL-2 produced by the CD4 T cells was quantified using CTL.L and MTT assays as previously described (28).

Intravascular Labeling and Lung Processing

Allophycoerythrin-conjugated (fAPC) anti-mouse CD45 (30-F11, Tonbo) was prepared in sterile DPBS (3 μg/mouse)

and loaded into 0.5-cc insulin syringes. Mice were anesthetized using isoflurane *via* inhalation and 100 µl of antibody solution was delivered intravenously (IV) per animal via retro-orbital injection. 3 min following IV injection, blood was collected in tubes containing sodium heparin followed by intraperitoneal administration of a lethal dose of avertin. The lungs were perfused via intracardiac injection of the right ventricle with 5 ml ice-cold lung perfusion media (1× DPBS supplemented with 0.6 mM EDTA). Lung tissue was excised, dipped in HBSS and then digested as described above prior to further handling for intracellular cytokine staining (ICS) and flow cytometry. PBLs were isolated from whole blood using lymphocyte separation medium (Corning) according to the manufacturer's instructions.

Flow Cytometry

Single cell suspensions were stained with a viability dye, followed by incubation with purified rat anti-mouse CD16/CD32 (mouse BD Fc Block, clone 2.4G2) as previously described (22). Cells were then stained for 25 min at 4°C in the dark using antibodies targeting the following markers: CD69 (H1.2F3, Biolegend), CD4 (RM4-5, BD Biosciences), CD11a (2D7, BD Biosciences), and CD44 (IM7, Tonbo), (MEL-14, Biolegend). Cells were then washed and prepared for flow cytometry analysis. Data was acquired using a BD LSR-II instrument, configured with 488 (blue), 633 (red), 407 (violet), and 532 (green)-nm lasers. Data were analyzed using Flowjo software (FlowJo, LLC), version 10.

Intracellular Cytokine Staining

Cells derived from infected lungs were enriched for CD4 T cells using negative paramagnetic bead selection using MACS technology. CD4 T cells were then co-cultured in u-bottom 96 well plates (3×10^5 cells/well) with APC from the spleens of naïve, syngeneic donors (5×10^5 cells/well) with or without exogenous, influenza peptide pools, separated by the protein from which the peptides were derived (see **Table 1**). A cocktail of monensin and Brefeldin A was then spiked into the cultures and incubated for 6 more hours for a total culture time of 8 h. Plates were then stored at 4°C for 12 h, cells were washed, stained with fixable live/dead Aqua, washed, and stained as described above for CD44 (IM7, Tonbo) and CD4 (RM4-5, BD Biosciences). Following incubation at 4°C for 25 min, cells were fixed and permeabilized using the eBioscience FoxP3 transcription factor staining buffer set according to manufacturer's instructions. Intracellular proteins were then stained using the following cocktail of antibodies: IFN-γ (XMG1.2, BD Biosciences) and IL-2 (JES6-5H4, BD Biosciences), and incubated for 45 min at 4°C. Data were acquired using a BD LSR-II instrument, configured with 488 (blue), 633 (red), 407 (violet), and 532 (green)-nm lasers. Data were analyzed using Flowjo software (FlowJo, LLC), version 10. Combination Boolean gating was performed to select cytokine-responses (IFN-γ and IL-2) from single, live, antigen-experienced CD4 T cells. Frequencies for each possible cytokine pattern (2^2) were tabulated in FlowJo, exported as an Excel file, annotated, and then finally plotted in Prism GraphPad software, version 6.

RESULTS

Infection Elicits a Broad Distribution of Epitope Specific CD4 T Cells

These studies began with the goal of defining the epitope specificity in A/J (H-2^a) mice that express the two isotypic forms of class II molecules (I-A^k and I-E^k) because this offered the opportunity to sample many different epitope specificities drawn from the endogenous CD4 T cell repertoire. A peptide pooling matrix (29, 30), in conjunction with splenic CD4 T cells isolated day 9–11 postinfection, were employed in order to define the epitope specificity of CD4 T cells elicited by influenza A/New Caledonia/20/99 (H1N1) virus, as we have previously described (11–13). This method, involving cytokine EliSpot assays and overlapping peptide libraries encompassing the entire translated sequence of pathogen-derived proteins, allows an unbiased approach to identify CD4 T cells epitopes. CD4 T cells specific for five distinct viral proteins, hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), matrix 1 (M1), and nonstructural protein 1 (NS1) were chosen for these studies (See **Table 1**). These viral proteins are abundantly expressed after infection and have distinct sites of localization in the virion and infected cells. Thus, these viral proteins might be handled differently *in vivo* after infection. HA and NA are transmembrane glycoproteins, M1 is associated with the cytosolic/inner membrane of infected cells/virions respectively, NP is abundant in viral ribonuclear protein complexes infected cells and virions, while NS1 is absent in virions, but is abundantly produced in infected cells and can be found in the cytosol and nucleus in infected cells (31).

Figure 1 shows the results of replicate cytokine EliSpot assays, using IL-2 (Panel A) and IFN-γ (Panel B) to quantify peptide-reactive cells for the epitopes previously identified, including as many as is feasible, from many different influenza viral proteins. It is clear from these analyses that the epitope specificity of CD4 T cells is very broad and that CD4 T cells specific for immunodominant epitopes from HA, NP and NA could be readily identified, as well as subdominant and more minor epitope specificities from M1 and NS1. Although early studies suggested that NP and HA were the major source of epitopes recognized by CD4 T cells after infection (32), more recent studies by our laboratory have demonstrated that epitope specificity can be quite broad and depends on the selectivity imposed by MHC class II molecules (12). This analysis of the response of A/J mice after infection confirms this conclusion.

The Broad Epitope Specificity of CD4 T Cells Detectable in the Lung dLN Is Readily Detectable in the Site of Infection

After the primary response, a fraction of the responding CD4 T cells migrate to the respiratory tract. Early studies by Woodland *et al.* (32) showed that a CD4 T cell repertoire with diverse epitope specificity can be detected in the lung after infection. In the experiments here, we sought to perform side-by-side analyses of CD4 T cells present at the peak of the response in the lymph node with those cells that had migrated to the lung.

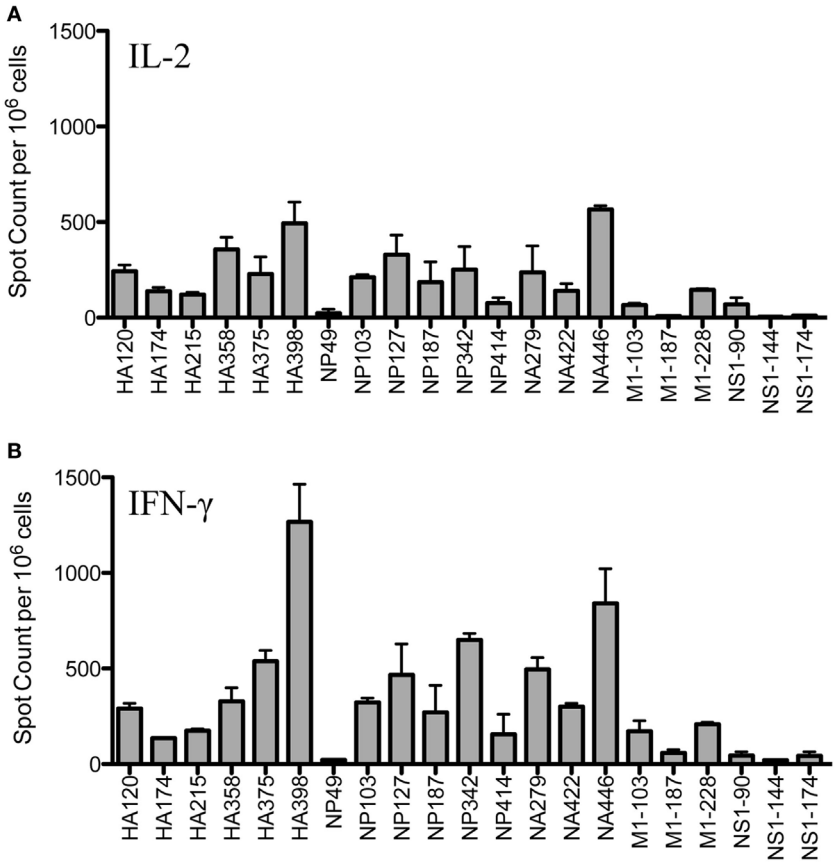

FIGURE 1 | Infection elicits CD4 T cells of broad epitope diversity. Female A/J mice (aged 2–6 months) were infected intranasally with 50,000 EID_{50} of A/New Caledonia/20/99 H1N1 virus, and the number of peptide-specific cytokine producing cells from the spleen were quantified. IL-2 cytokine EliSpots **(A)** are quantified in the top panel and IFN-γ producing cells **(B)** are shown in the bottom panel. Results are represented as cytokine-producing spots per million CD4 T cells and represent the average of two independent experiments with the range indicated.

The peptides validated by replicate assays shown in **Figure 1** were used to sample CD4 T cells from the lung-draining mLN and lung at days 9–11 after infection. **Figure 2** shows the results of these analyses, where mLN responses are shown in Panel A and lung-specific responses are shown Panel B. IL-2 (blue bars) and IFN-γ (green bars) producing cells were both sampled in peptide-stimulated cytokine EliSpots assays. It is clear from this side-by-side analysis that the CD4 T cell specificities primed in the lung-dLN can be readily detected in the lung. Both IL-2 and IFN-γ producers are detectable in the lung, although there is a striking shift in cytokine profile, with IFN-γ producing cells becoming much more prominently represented in CD4 T cells isolated from the lung relative to those from the LN (note the differences in scales used for cytokine producing cells in LN and lung). Even the minor epitope specificities, such those derived from NS1 and M1, are readily detectable in the lung. Thus, the broad peptide specificity in CD4 T cells primed in the lymph node persists in CD4 T cells isolated from the lung. We noted no striking gains or losses of CD4 T cell specificity when the dLN and lung are compared. **Figure 3** illustrates the diversity in lung homing CD4 T cells in a readily visualized manner using a pie diagram. Here, every peptide specificity that was tested is denoted by a different color with the width of the pie "slice" indicating the relative abundance of the epitope specificity, using either IL-2 or IFN-γ EliSpots as an indicator of the magnitude of the response to each peptide. The protein derivation of each of the peptides sampled is indicated by the outer arc in these diagrams. These studies show that each peptide specificity detected in the LN is represented in the lung independently of its protein origin. It is clear that our estimate of the immunodominance hierarchy is dependent on cytokine production by the virus-specific CD4 T cells. Alternative measures, such as tetramer staining, potentially detect of CD4 T cells that do not produce cytokine but are limited by their ability to detect only high affinity CD4 T cells, perhaps encompassing only 5–30% of the total antigen-specific CD4 T cell repertoire (33–35).

In order to visualize the absolute immunodominance of the peptide epitopes in the two different sites that were sampled after infection, the average number of cytokine producing cells (per million CD4 T cells) from three independent experiments was ranked in a two dimensional plot that allows the relative hierarchy to be readily visualized. These data, shown in **Figure 4**, indicate that epitope specificities that were dominant in the mLN, such as HA398, NP127, NA446, and NA279, remain

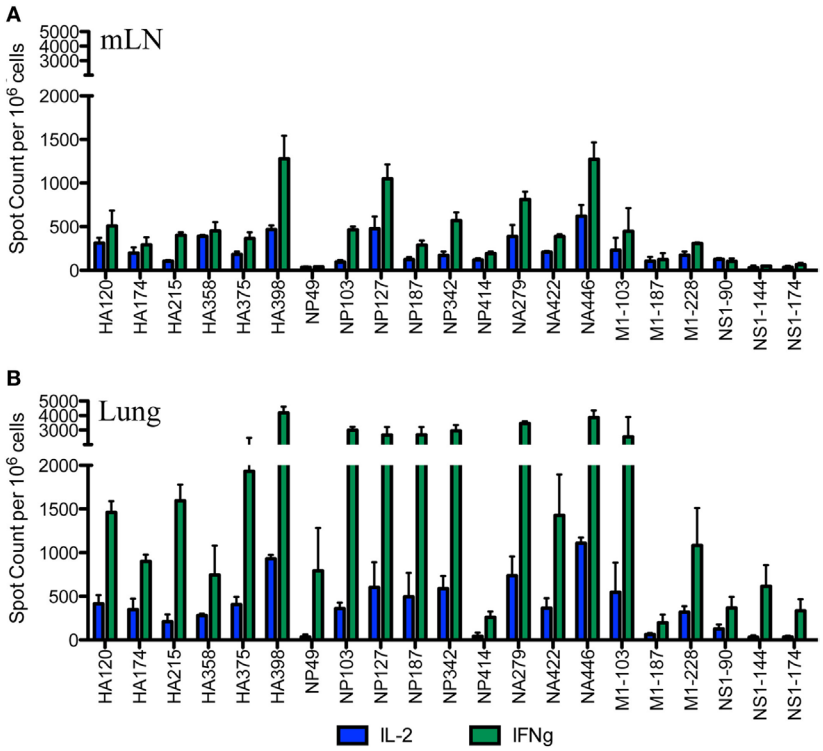

FIGURE 2 | Broad CD4 T cell specificity identified in the draining lymph node (dLN) is maintained at the site of infection. Female A/J mice were infected intranasally with 50,000 EID_{50} of A/New Caledonia/20/99 H1N1 virus. The number of virus peptide-specific IL-2 producing (blue) and IFN-γ producing (green) CD4 T cells were quantified at days 10–11 postinfection in CD4 T cells using cytokine EliSpots. Cells isolated from mediastinal lymph nodes (mLN) are indicated in **(A)** and from the lung in **(B)**. Results are represented as cytokine-producing spots per million CD4 T cells and represent mean ± SD of three independent experiments.

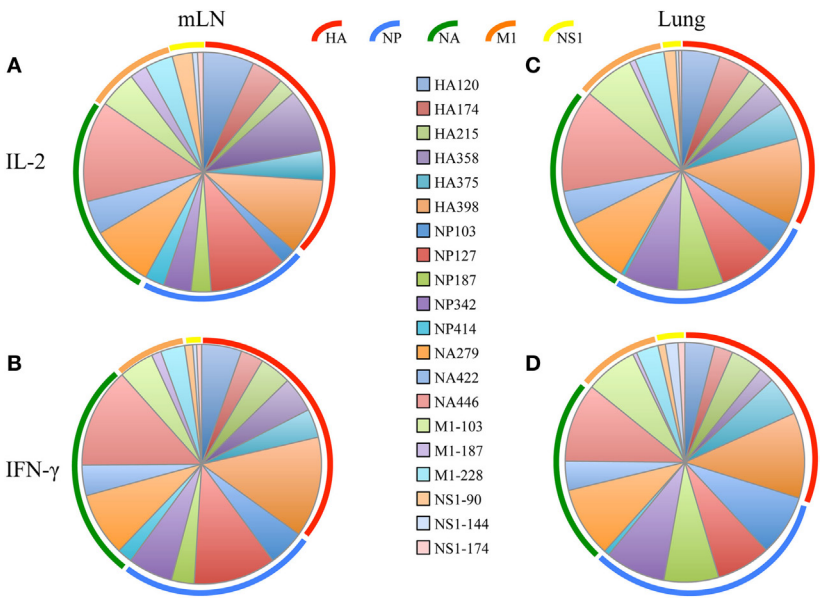

FIGURE 3 | Virus-specific CD4 T cell diversity in the draining lymph node (dLN) and lung. The number of peptide specific IL-2 producing CD4 T cells was quantified at day 10–11 postinfection by cytokine ELISPOT assay as indicated in **Figure 2**. The average percent response recruited by each peptide was calculated by the dividing the number of cytokine EliSpots elicited by the given peptide by the sum of the EliSpot responses elicited by all of the peptides tested. This percent response value is represented as a "slice" of the pie for mediastinal lymph nodes (mLN) **(A,B)** and Lung **(C,D)** for IL-2 **(A,C)** and IFN-γ **(B,D)**. The different color arcs indicate the source protein each epitope. Each individual epitope is indicated by a different colored slice of the pie and is colored for each peptide as indicated in the center panel. Results represent the average of the three independent experiments shown in **Figure 2** with the indicated SD.

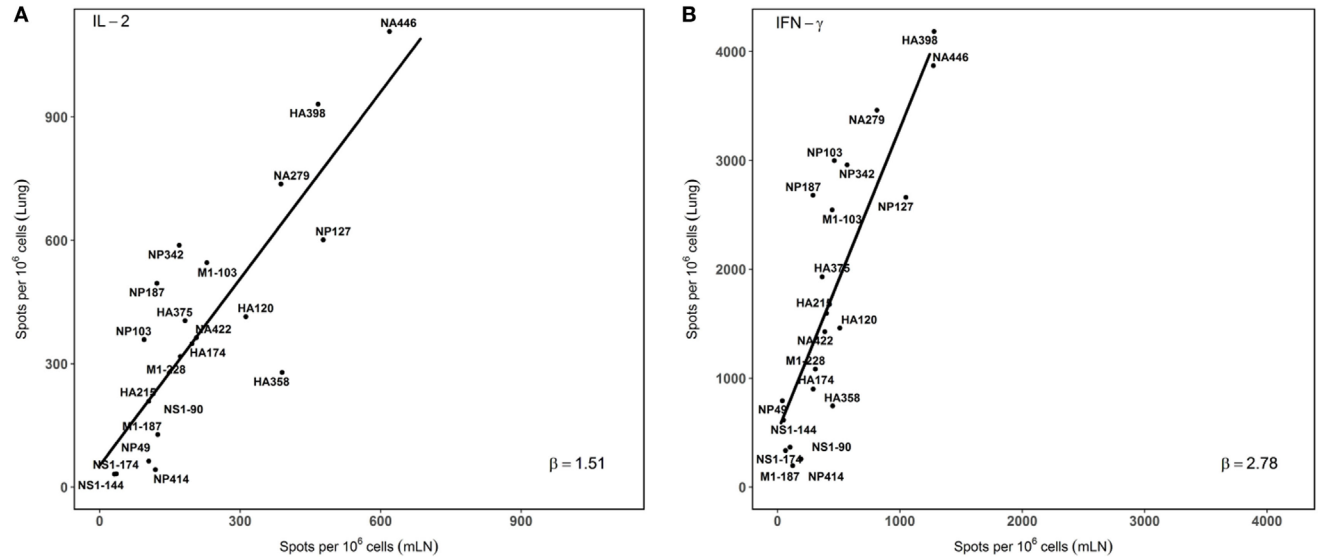

FIGURE 4 | Immunodominance hierarchy of epitope specific CD4 T cells isolated from draining lymph node (dLN) is preserved in the lung lung. IL-2 and IFN-γ Elispots were performed as described from cells isolated at day 10–11 postinfection. Individual values for IL-2 **(A)** or IFN-γ **(B)** EliSpots from three independent experiments from **Figure 2**, with the SD values indicated in **Figure 2**. The average values were tabulated and analyzed using linear regression models, using each data point. The slopes of the derived line ("β") were calculated and are indicated in each panel. Shown are the average values of spots per million CD4 T cells for each epitope data set tested. **(A)** beta (slope) = 1.51. $r^2 = 0.63$. p value < 0.001. **(B)**: beta (slope) = 2.78, $r^2 = 0.64$. p value < 0.001.

immunodominant in the lung, while subdominant epitopes, such as HA215 and NP342, are also subdominant in the lung. Minor epitope specificities detected in the dLN, such as NS190, although detectable in the lung, remain minor. The consistent immunodominance is evidenced by the general adherence to the straight line shown in panels A and B, for IL-2 and IFN-γ, respectively. We did note several epitope specificities (e.g., M1-103 and NP187) measured by both IL-2 and IFN-γ producing cells that were somewhat enriched in the lung relative to the dLN, but do not yet understand the basis for this enrichment. Interestingly, the slopes of the lines are distinct for IL-2 (1.51) *vs*. IFN-γ (2.78) again emphasizing the accumulation of IFN-γ-producing CD4 T cells in the lung, relative to what is detected in the draining LN, where the CD4 T cells are elicited after infection.

Compartmentalization of Cytokine-Producing Cells in the Lung following Infection

The previous results, suggesting that CD4 T cells of each specificity primed in the LN home to the lung after influenza infection, were unexpected because of the distinct types of APC in the lung that may have the potential to remodel the CD4 T cell repertoire. However, it is now understood that lung-localized lymphocytes can be situated into two discrete sites following infection: the vasculature and the tissue, the latter of which includes parenchyma, interstitium, and the airways (36–39). We considered the possibility that the virus-specific CD4 T cells that were extracted from the lung and quantified by peptide-induced cytokine EliSpots were localized to the vasculature and thus had not yet encountered pulmonary APC. Cells within the lung vasculature and the tissue can be distinguished by the accessibility to antibodies that are introduced into the bloodstream shortly before animal euthanasia (40, 41). Using this method, cells in the lung vasculature become labeled, whereas cells in the tissue are "protected" from this short-term introduction of antibody. To evaluate the location of the cytokine-producing cells quantified in the previous experiments, A/J mice infected 10 days previously were injected IV with an allophycoerythrin-conjugated (fAPC)-CD45 antibody that would label all hematopoietic cells. Mice were sacrificed 3 min later and lung homogenates were prepared, and the resulting cell population was analyzed by flow cytometry. **Figure 5A** (top panel) shows that 100% of the peripheral blood CD4 T cells stained positive directly *ex vivo*, assuring us that the IV-introduced CD45 was sufficient to saturate circulating cells. Within the cells isolated from the lung (**Figure 5A**, bottom panel) approximately 40% of the total CD4 T cells were accessible to the IV antibody (pseudocolored in red in this and future data sets), while a substantial proportion (60%) of the CD4 T cells were protected from labeling (pseudocolored in blue). We next evaluated the phenotype of the CD4 T cells distinguished by CD45 staining, using the markers associated with antigen experience (CD44) and with those associated with tissue residence [CD69 and CD11a (LFA-1)] (38, 42, 43). **Figure 5B** shows that the CD4 T cells in the vasculature were primarily composed of naïve cells, whereas cells in the protected tissue were greatly enriched for antigen experienced, $CD44^{hi}$ cells. **Figure 5C** shows a characterization of cells for the markers CD11a and CD69. The integrin CD11a,

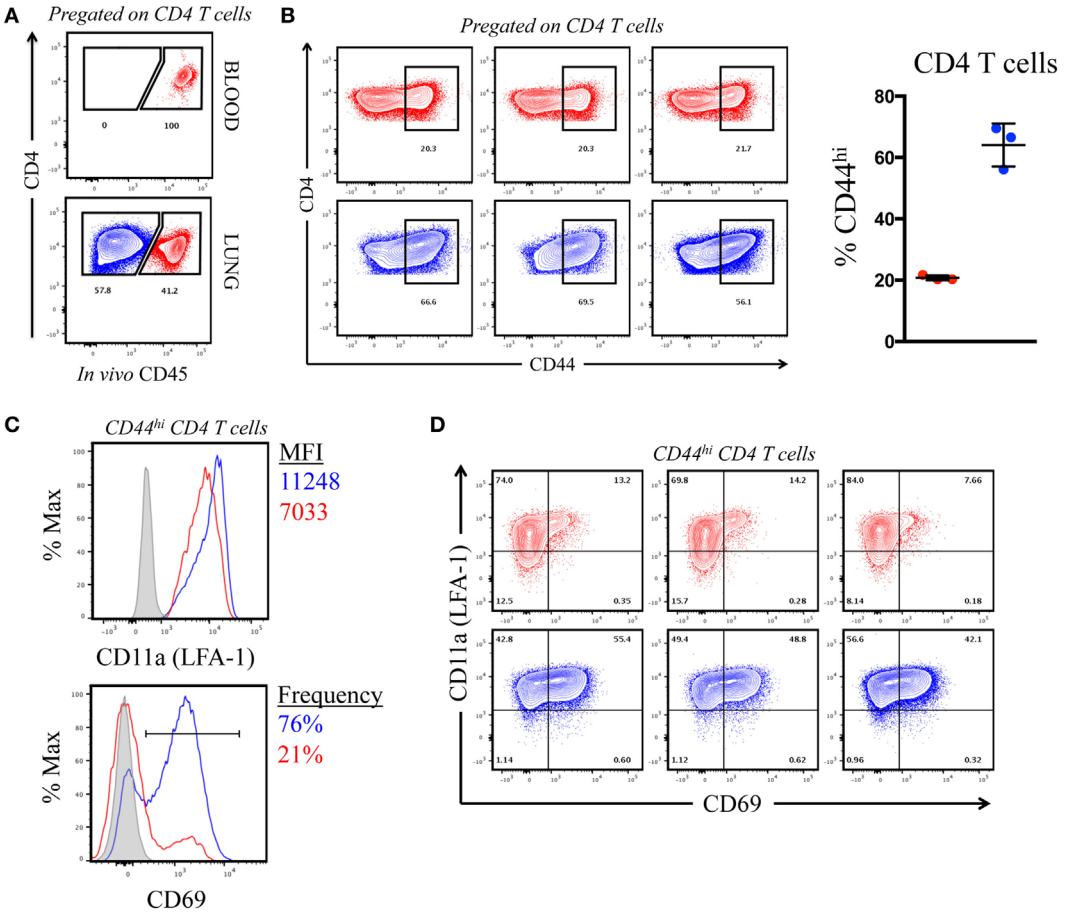

FIGURE 5 | Intravenously (IV) labeling reveals distinct compartmentalization and phenotype of pulmonary CD4 T cells following infection. **(A)** Representative IV CD45-fAPC staining profile from pre-gated, $CD4^+$ T cells in blood and lung following infection, where blue denotes "protected" cells and red denotes "labeled" cells. **(B)** Contour plots (CD4 *vs*. CD44) (pre-gated on $CD4^+$) and tabulated frequency of $CD44^{hi}$ CD4 T cells from three individual mice, shown at right. Symbols represent individual animals and error bars reflect standard deviation from the mean. **(C)** CD11a (LFA-1) MFI,% $CD69^+$ (from $CD44^{hi}$ CD4 T cells), and expression profile of CD69 *vs*. CD11a (LFA-1) on IV "labeled" and "protected" cells 10 dpi. Histograms reflect pooled responses from three animals and individual contour plots **(D)** reflect responses from the three individual animals.

important in T cell activation and for extravasation into tissue sites, has been shown to have increased cell surface density on tissue-localized cells, while CD69, a prototypical marker of tissue-resident T cells is known to enhance tissue localization in part through its reciprocal antagonism of the sphingosine 1 phosphate receptor (36, 44). **Figure 5C** shows the co-expression of these markers on $CD44^{hi}$ cells in the vasculature (in red) vs. the protected tissue (in blue). Among the CD44 bright cells, CD4 T cells in the tissue show increased cell surface density of CD11a (MFI = 11,000) compared to vascular localized (MFI = 7,000) and are greatly enriched for CD69 expression (76% vs. 21%, respectively). The data from individual animals, shown in **Figure 5D**, illustrate the reproducibility in the markers expressed on these distinct lung subsets. We conclude from these data that the IV labeling successfully distinguished CD4 T cells in the lung vasculature vs. the lung tissue, and that in this system, IV-protected cells have the markers associated with residence in the inflammatory microenvironment in the lung and thus the potential to contact a diverse set of lung-localized APC within the infected lung. However, this selective lung homing behavior and residency does not appear to restrict epitope specificity.

Viral Antigen-Specific Cytokine-Producing Cells Are Primarily Contained within the Lung Tissue

In order to determine the primary location of lung CD4 T cells whose epitope specificity was determined by cytokine EliSpots shown in **Figure 2**, mice were infected and sampled after infection after a brief IV treatment with fAPC-CD45 antibody. Peptide-induced ICS was then performed from the lung-isolated cells in conjunction with the fAPC-CD45 staining to distinguish CD4 T cells in vasculature and tissue. Peptide epitopes derived from the major viral proteins that serve as the source antigen (HA, NA, NP, and M1) were pooled based on their protein origin and co-cultured for 8 h with the cells isolated from the lung. Production of IFN-γ (**Figure 6A**) and IL-2 (**Figure 6B**) by the CD4 T cells was

quantified by ICS and flow cytometry, using CD44 cell surface density to identify antigen-experienced cells. In these figures, IV-labeled, vasculature-localized cells are indicated (in red) in the top panels and IV-protected tissue cells (indicated in blue) are shown in the bottom panels. The percentage of total cytokine-producing cells is indicated in the pie graphs below each flow cytometry profile. The results from these analyses indicated that 92–94% of the virus-specific IFN-γ production was produced by cells in the lung tissue, while 86–90% of the IL-2 producing cells were within the lung tissue. There was no notable difference in the distribution of cytokine-producing cells specific for different viral proteins. We conclude from these analyses that the vast majority of cells assayed by cytokine EliSpots were from CD4 T cells that had entered the lung tissue and do not simply represent circulating CD4 T cells trapped in the lung vasculature. These infiltrating, virus-specific CD4 T cells thus had an opportunity to survey any viral peptide-bearing APC within the tissue.

Comparison of the cytokine profiles within the cells localized to dLN, pulmonary vasculature and lung tissue were then analyzed in more detail (**Figure 7**). CD4 T cells specific for each protein-specific pool of peptides were quantified for IL-2 and IFN-γ and co-cytokine production by sequential gating strategies. **Figure 7A** shows the cytokine polyfunctionality in the dLN, where the cells are distributed almost equally between cells that produce only one cytokine: IL-2 only (turquoise), IFN-γ only (orange) and cells that produce both cytokines IFN-γ$^+$,

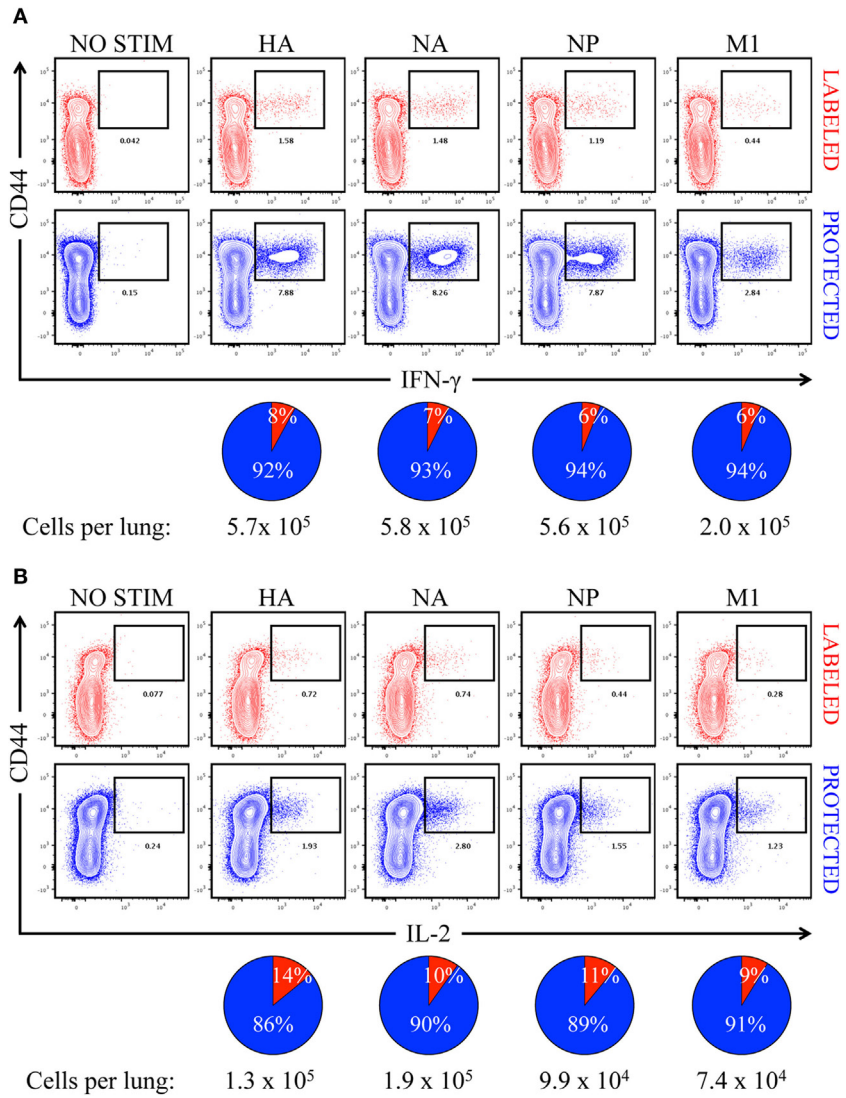

FIGURE 6 | Virus-specific, cytokine-producing CD4 T cells are enriched in "protected" niches in the lung following influenza virus infection. Contour plots illustrating antigen-specific reactivity to HA, NA, NP, and M1 proteins by intravenously (IV) "labeled" (red) and "protected" (blue) pulmonary CD44^{hi} CD4 T cells, as measured by **(A)** IFN-γ and **(B)** IL-2 production. Pools of peptides from each of four different viral proteins, indicated above each panel, were used for stimulation. The pie charts below each cytokine set reflect the fraction of cytokine-producing CD4 T cells in pulmonary vasculature *vs*. tissue (vasculature in red and tissue in blue), with the total number of responding cells indicated. The responses shown are from pooled samples from five animals.

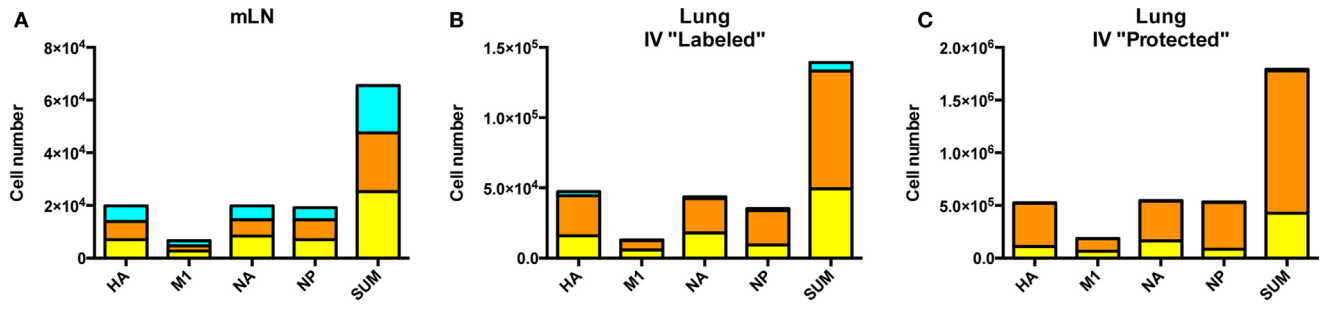

FIGURE 7 | Cytokine pattern of virus-specific CD4 T cells is distinct in lymph node and lung. Cytokine profile of antigen-reactive CD4 T cells in the draining lymph node (dLN) **(A)**, lung vasculature, **(B)** and lung tissue **(C)** identified by protein specificity. Cells producing IL-2 only are represented in turquoise, IFN-γ alone indicated in orange and cells producing both IL-2 and IFN-γ are indicated in yellow. Data reflects responses from samples pooled from five animals. Note the differences in scales for CD4 T cells from each tissue.

IL-2$^+$ (yellow). When the cells in the vasculature are analyzed (**Figure 7B**), there is an apparent commitment of lung homing cells to a Th1 IFN-γ producing population, with only a minor fraction of cells that exclusively produce IL-2. In the lung tissue (**Figure 7C**), the virus-specific cells are comprised primarily of IFN-γ producing cells, with most of the CD4 T cells producing only IFN-γ and very few cells producing only IL-2. Thus, there appears to be a readily detectable and progressive enrichment of cells toward IFN-γ producers from the site of CD4 T cell priming in the dLN, to when the cells traffic *via* circulation to the lung vasculature and finally establish residency in the lung tissue. All viral protein specificities appear to progress from the most diverse in cytokine potential within the lymph node, toward the most restricted cytokine-producing cells within the lung tissue. The vasculature-localized cells are intermediate between these extremes, containing some virus-specific cells producing only IL-2, a phenotype that is very poorly represented in the lung tissue. Overall, these data are consistent with the priming of CD4 T cells with diverse functional potential and for selection of a subset of these virus-specific CD4 T cells, enriched for IFN-γ potential for traffic to the lung after influenza infection.

Functional Avidity of Epitope-Specific CD4 T Cells Isolated From mLN and Lung

We next considered the possibility that, although the immunodominance hierarchy of CD4 T cells had not been altered from that in the LN to those that ultimately establish residence in the lung, the affinity of the TcR for its ligand may be distinct in CD4 T cells from the two compartments. There has been a number of recent studies suggesting that TcR signal strength, largely determined by the affinity and/or off rate of the TcR to its ligand, can influence the fate of CD4 T cells after priming (18–20). We sought to assess the functional avidity of virus-specific CD4 T cells isolated from lung vs. the dLN at the peak of the adaptive response, independently of any potential differences in signaling capacity or expression of co-stimulatory molecules, which can affect sensitivity to antigen or cytokine responses (45–47). Therefore, we established virus-specific CD4 T cell hybridomas. CD4 T cells isolated from the draining LN and lung were restimulated with antigenic peptides, and then T cell blasts were fused to the TcR-negative T cell lymphoma BW5147. Using the TcR negative BW5147 cell line as the fusion partner, the only source of T cell receptors are those expressed by the CD4 T cells from the individual cell which has entered into a fusion with the BW5147, thus allowing us to directly query the receptors expressed on the CD4 T cells from the host (48, 49). After selection, CD4 T cell hybridomas were derived and identified. This immortalization allows the T cell receptors to now be expressed on a "neutral" cellular background, allowing more objective comparative analyses of their relative avidity for antigen, thus will exclude the properties of freshly isolated CD4 T cells whose differentiation state or expression of co-stimulatory molecules can affect sensitivity to antigen.

The functional avidity of isolated T cell hybridoma clones that express distinct TcR V beta segments for two dominant epitopes were then compared using peptide dose-response curves, where TcR engagement was quantified by IL-2 production, which is the cytokine routinely used to assay T cell hybridomas. We (50) and others (51–54) have shown that hybridoma cells provide a very sensitive measure of functional TcR avidity. The results of these experiments are shown in **Figure 8** and **Table 2**. Individual clones of CD4 T cell hybridomas specific for NA446 (left) or NP127 (right) from each tissue were tested in peptide dose-response assays, and IL-2 was quantified using CTLL, an IL-2 dependent cell line. The peptide dose-response curves are shown from the hybridomas isolated from dLN (in red) or from lung (in black) are shown the top panels, with the average values shown in the bottom panels. **Table 2** provides that half maximal dose of peptide needed to activate the individual clones. This variability in functional avidity, as well as our studies indicating that many clones express distinct TcR Vbeta segments (not shown), indicates that the clones of hybridomas are not replicates of each other. Although there were notable differences in sensitivity to antigenic peptide by the individual CD4 T cell hybridomas, the ranges in "functional avidity" between CD4 T cells derived from dLN and lung were indistinguishable. We conclude from this that the CD4 T cell repertoire of T cells that home to the lung is not dramatically remodeled with regard to the functional avidities of the CD4 T cells isolated from the lung dLN.

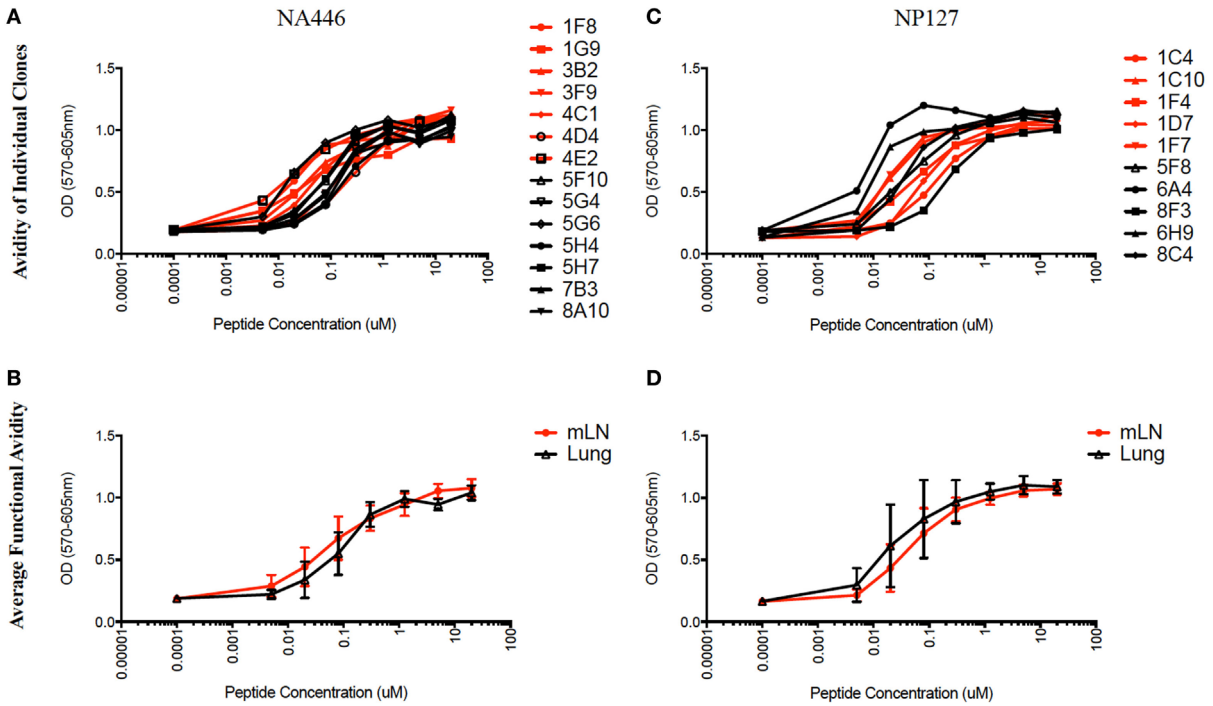

FIGURE 8 | Similar range in functional TcR avidity from CD4 T cells isolated from dLN and lung. NA (NA446) and NP (NP127) specific T cell hybridomas, generated from CD4 T cells isolated from the mLN and lungs of infected mice at day 10 postinfection were assayed for their ability to produce IL-2 in EliSpot when restimulated with the indicated antigenic peptides at a range of doses (0.0001–100 μM). **(A)** The left top panel shows the responses of clones specific for NA446 from the lymph node (black lines) and lung (red lines) and **(C)** the top right panel shows the response of clones specific for NP127 from the lymph node (black) and lung (red). **(B,D)** The bottom panels illustrate the average dose–response curves of the clones. IL-2 produced by the T cell hybridomas was quantified using CTL.L and MTT assays.

TABLE 2 | Relative affinities of T cell hybridoma clones.

Specificity	Tissue			
	mLN		Lung	
	Hybridoma	50% max concentration (μM)	Hybridoma	50% max concentration (μM)
NA446	4E2	0.02	5G6	0.025
	1F8	0.025	5F10	0.1
	3B2	0.055	5H7	0.1
	4C1	0.09	5G4	0.18
	1G9	0.1	7B3	0.2
	3F9	0.2	8A10	0.2
	4D4	0.4	5H4	0.3
NP127	1F7	0.02	6A4	0.0085
	1C10	0.025	6H9	0.015
	1F4	0.09	8C4	0.045
	1D7	0.15	5F8	0.07
	1C4	0.2	8F3	0.4

DISCUSSION

It is now clear that influenza-specific CD4 T cells have many diverse functions in the lung, some of which, such as cytotoxicity and cytokine production, likely involve contact with APC [reviewed in Ref. (55)]. In some systems, lung-homing CD4 T cells can also help in tissue-localized B cell responses (1, 56). In this study, we have evaluated whether the diverse CD4 T cell epitope specificity that is elicited in the lung dLN after influenza infection persists in the CD4 T cells that home to the lung. We were interested in this issue because recent data suggest that CD4 T cell fate can be influenced by the specific features of the peptide:class II-T cell receptor complex (18–20). These recent studies, coupled with our increasing appreciation of the multiplicity of functions that CD4 T cells carry out in the lung (42, 57) and the distinctive antigen bearing cells within the lung after infection (22, 58–60), raised the possibility that the CD4 T cell immunodominance hierarchy established in the dLN after infection might be remodeled as CD4 T cells are selected for lung homing during priming or alternatively, after establishment of residency in the lung tissue. Our studies revealed that when assayed by epitope-specific cytokine production, the epitope specificity of influenza-specific CD4 T cells is very broad in the dLN and that this diverse peptide specificity persists in cells that home to the lung. Also, the immunodominance hierarchies established in the dLN are maintained in lung CD4 T cells.

The factors that control CD4 T cell homing to the lung after influenza infection are not well defined. Recent studies have implicated upregulation of chemokine receptors such as

CXCR3 (61) and CCR4 (62) during priming as an important event in homing and establishing residency in the lung after respiratory pathogen infection. Upregulation of chemokine receptors such as CXCR3 has been observed with Th1 priming in the lymph node after infection with some respiratory pathogens (63–65) and has been suggested to play a role into recruitment in the lung after intranasal infection or vaccination (62). In the infection model studied here, we also observed a strong enrichment for IFN-γ-producing cells in the lung, for all epitopes examined, a result consistent with the Th1 CD4 T cell phenotype typical of influenza CD4 T cell immunity. Enrichment for IFN-γ in the cells that traffic to the lung suggests that the early priming events in the dLN delivered signals to the elicited CD4 T cells that promoted lung homing and IFN-γ production, features that appear to be functionally linked in most respiratory virus responses. The nature of the APC that are involved in initial of priming CD4 T cells in responses to infection or vaccination is not well understood, but may involve multiple subsets contacted sequentially (64, 66). The expression of co-factors that control trafficking of MHC class II, proteolysis of antigen, and editing of the final displayed epitopes on different APC (67, 68) have not been characterized in responses to respiratory infection. If there are specialized APC that promote homing of the responding CD4 T cells to the lung, our data suggest that these APC present the full diversity of virus-derived peptides, permitting all of the CD4 T cell specificities primed to be included in the subset of CD4 T cells destined for the lung. As of yet, there is little known about the TcR-mediated signaling events that might preferentially poise cells during priming of the naïve CD4 T cell repertoire to be selected to the CXCR3/Th1 pathway of differentiation that determines lung homing potential. Recent studies suggest that choices in the fate of CD4 T cells after priming (e.g., Tfh *vs.* non-Tfh "effector cells") can be strongly influenced by specific features of peptide:class II-TcR complexes described by their "signal strength" or functional avidity [reviewed in Ref. (19)]. However, the data accumulated thus far have sometimes been contradictory and, in any case, have not yet analyzed the priming events or APC that poise CD4 T cells elicited by respiratory infection to leave the dLN and migrate to the lung. The paucity of information is particularly notable for polyclonal CD4 T cells drawn from the endogenous response. In the studies reported here, when the TcR repertoire for CD4 T cells specific for two dominant influenza epitopes isolated from dLN and lung were examined here for "functional avidity" using peptide dose–response curves, although there were a range in sensitivity to antigenic peptides among individual clones, the range in the avidities was indistinguishable within CD4 T cells isolated from the two compartments, suggesting that CD4 cells selected for homing to the tissue were not atypical with regard to affinity from the range elicited during priming and that persist in the dLN at day 10–11 postinfection. Although it would be of interest to study the CD4 T cell repertoire at later time points, as immune memory is established, several factors preclude analyses of this for the purposes of this study. First, in a comprehensive survey of the immunodominance in CD4 T cell responses to influenza using the HLA-DR1 mouse strain, we found that the abundance of total influenza reactive CD4 T cells in the periphery at memory time points (e.g., day 30–60 postinfection) diminished to 5% of the levels detected at the peak of the response (day 8–10 postinfection). In this earlier study, we tracked more than 50 different influenza epitopes. Remarkably, although much lower in abundance, the CD4 T cell immunodominance hierarchy was stable (69). For the current study, when we have examined the abundance and distribution of CD4 T cells at an even modestly extended time (day 18 postinfection), the number of antigen-experienced CD4 T cells in the lung drops dramatically, to less than 15% of that seen at the peak. Even more important, using the IV labeling technique, we found that the total lung CD4 T cells are now composed equally of vasculature and tissue localized cells (data not shown). Because in the current study, we explicitly sought to examine cells within the unique microenvironment of the lung tissue after infection, analyzing CD4 T cell specificity at even just a few additional weeks postinfection would be complicated by the very large representation of CD4 cells from the lung vasculature.

The persistence in broad immunodominance, with little skewing or narrowing of specificity or functional avidity, raised the possibility that the cells detected from disrupted lung tissue were contained within the vasculature and had not yet entered the infected lung and contacted lung APC. However, our studies revealed that most of the cytokine-producing cells within the lung were within the lung tissue, rather than the vasculature, suggesting that the infiltrating cells likely have had the opportunity to encounter potential antigen bearing cells in the lung. There have been several recent studies that have suggested that viral antigens persist long after virus is cleared from the lungs (70, 71). Early studies included the demonstration that viral peptide:MHC class II complexes at levels sufficient to activate adoptively transferred CD4 T cells persist in the lung draining LN for at least 60 days postinfection (70). More recent studies indicated that viral RNA and viral protein can be detected primarily in radio-resistant non-hematopoietic cells in the lung tissue for more than 2 weeks after virus is cleared from the lung (70). Finally, contact of infiltrating T cells with lung APC after infection has been supported by recent reports in the literature (55, 72–76).

Whether influenza viral antigens are accessible in the form of peptide:class II complexes to infiltrating CD4 T cells and persists as the adaptive response progresses in the infection model studied here is not known. For influenza-specific MHC class I restricted T cells, Legge and coworkers provided evidence that recruited dendritic cells that persist in the lung serve as APC for newly primed infiltrating virus-specific CD8 T cells (74). Recent studies by Swain *et al.* of CD4 T cells primed in influenza infection suggest that late (e.g., day 5–7 postinfection) contact with antigen bearing cells is important in establishing long lived virus-specific CD4 T cell memory. In this study, tracking of epitope-specific cells and use of reporters that detect TcR engagement suggested that these later (day 5–7 postinfection) antigen-dependent interactions occur in the lung (77). Collectively, these studies suggest that influenza-specific CD4 T cells that home to the lung after

infection can encounter peptide:MHC class II complexes and such contact might affect survival and potential remodeling of the immunodominance hierarchy. Our recent studies performed early after infection with a Venus reporter virus suggests that a diverse array of both bone marrow derived and non-bone marrow derived, class II positive cells acquire viral proteins during infection and that many of these subsets express detectable viral peptide:class II complex (22). However, the anatomic distribution and persistence of these diverse antigen-bearing APC are not known at this time, nor their options for encounter with lung-infiltrating CD4 T cells after primary infection. We have not yet quantified the epitope display on MHC class II positive cells in the lung at later time points when CD4 T cells begin to infiltrate the lung (day 5–6 postinfection) or the presence long lived antigen depots in this infection system. Thus, we do not yet know whether the lung homing CD4 T cells identified here have contacted epitope-bearing APC in the lung. If APC-CD4 T cell antigen dependent contact has occurred, it seems not to have resulted in detectable remodeling of CD4 T cell repertoire that is established in the dLN. Despite these limitations in our studies thus far, the data presented here provide new insight into tissue-specific homing after influenza infection and demonstrate that CD4 T cells of broad viral epitope specificity elicited after H1N1 influenza infection are recruited into the lung, where they then have the opportunity to encounter infected and/or antigen bearing APC and deliver effector function.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the University of Rochester Medical Center. All animal protocols used in this study adhere to the AAALAC International, the Animal Welfare Act, and the PHS Guide and were approved by the University of Rochester Committee on Animal Resources, Animal Welfare Assurance Number A3291-01. The protocol under which these studies were conducted was originally approved March 4, 2006 (protocol no 2006-030) and has been reviewed and re-approved every 36 months with the most recent review and approval January 23, 2018.

AUTHOR CONTRIBUTIONS

KR, AD, AR, and ZK helped design, perform and interpret experiments, HY performed statistical treatment of data, AS assisted in the design and interpretation of experiments. All authors assisted in the preparation of the manuscript.

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REVIEW
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The CD8 T Cell Response to Respiratory Virus Infections

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Humans are highly susceptible to infection with respiratory viruses including respiratory syncytial virus (RSV), influenza virus, human metapneumovirus, rhinovirus, coronavirus, and parainfluenza virus. While some viruses simply cause symptoms of the common cold, many respiratory viruses induce severe bronchiolitis, pneumonia, and even death following infection. Despite the immense clinical burden, the majority of the most common pulmonary viruses lack long-lasting efficacious vaccines. Nearly all current vaccination strategies are designed to elicit broadly neutralizing antibodies, which prevent severe disease following a subsequent infection. However, the mucosal antibody response to many respiratory viruses is not long-lasting and declines with age. CD8 T cells are critical for mediating clearance following many acute viral infections in the lung. In addition, memory CD8 T cells are capable of providing protection against secondary infections. Therefore, the combined induction of virus-specific CD8 T cells and antibodies may provide optimal protective immunity. Herein, we review the current literature on CD8 T cell responses induced by respiratory virus infections. Additionally, we explore how this knowledge could be utilized in the development of future vaccines against respiratory viruses, with a special emphasis on RSV vaccination.

Keywords: CD8 T cell, memory T cell, respiratory virus, respiratory syncytial virus, influenza A virus, human metapneumovirus, rhinovirus, coronavirus

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INTRODUCTION

Given its continuous exposure to the outside environment, the respiratory mucosa is highly susceptible to viral infection. The human respiratory tract can be infected with a variety of pulmonary viruses, including respiratory syncytial virus (RSV), influenza virus, human metapneumovirus (HMPV), rhinovirus (RV), coronavirus (CoV), and parainfluenza virus (PIV) (1). The severity of disease associated with respiratory viral infection varies widely depending on the virus strain as well as the age and immune status of the infected individual. Symptoms can range from mild sinusitis or cold-like symptoms to more severe symptoms including bronchitis, pneumonia, and even death. RSV is the leading cause of severe lower respiratory tract infection in children under 5 years of age (2). RSV is commonly associated with severe lower respiratory tract symptoms including bronchiolitis, pneumonia, and bronchitis and is a significant cause of hospitalization and mortality in children, the elderly, and immunocompromised individuals (2–6). Similarly, PIV commonly infects children and is a major cause of croup, pneumonia, and bronchiolitis (7, 8). Seasonal influenza infections, most often of the influenza A virus (IAV) subtype, are responsible for 3–5 million cases of severe infection annually (9). Seasonal IAV infections also result in approximately 290,000–650,000 deaths per year, most commonly in either young children or elderly populations (9–11). However, infection with emerging pandemic IAV strains, such as the 2009 H1N1 pandemic strain, primarily induces severe disease and mortality in otherwise healthy adults younger than 65 years of age (12).

In contrast, respiratory infection with HMPV, RV, and CoV are most commonly associated with symptoms of the common cold (13–15). Two notable exceptions are severe acute respiratory syndrome (SARS) CoV and Middle East respiratory syndrome (MERS) CoV, which cause acute respiratory distress and mortality in infected individuals (16–18).

Despite their profound impact on human health, most common respiratory viruses lack an approved vaccine. The strategy employed most often in vaccine development is the induction of robust neutralizing antibody responses. However, the hallmark of many respiratory viral infections, including RSV, HMPV, and RV, is the ability for reinfections to occur frequently throughout life (19–21). This suggests that the antibody response to these respiratory viruses may wane over time. Indeed, despite a correlation between pre-existing nasal IgA and protection from reinfection, the development of long-lasting RSV- and RV-specific mucosal IgA responses was poor in infected adults (22, 23). Although IAV-specific neutralizing antibodies are elicited efficiently through either infection or vaccination, IAV vaccine formulations must be redeveloped annually to account for the rapid mutations of HA and NA genes in seasonal strains (24). Therefore, vaccinations that solely promote the induction of neutralizing antibodies may not be optimal in providing protection against many respiratory virus infections. The induction of cellular immune responses has thus far received little attention in respiratory virus vaccine development. CD8 T cells play a critical role in mediating viral clearance following many respiratory virus infections including RSV, IAV, and HMPV (25–27). In addition, recent murine studies utilizing CD8 T cell epitope-specific immunization strategies observed significantly reduced lung viral titers following IAV, RSV, or SARS challenges (28–30). Therefore, the induction of virus-specific CD8 T cell responses has the potential to improve upon the efficacy of current vaccination strategies. Here, we review the current literature on CD8 T cell responses following respiratory virus infections and discuss how this knowledge may best be utilized in the development of future vaccines.

CD8 T CELL RESPONSES TO ACUTE RESPIRATORY VIRUS INFECTIONS

Following an acute respiratory infection, dendritic cells (DCs) that have taken up viral antigen stimulate the activation of naive CD8 T cells in the lung draining lymph node to induce robust virus-specific CD8 T cell responses [reviewed in Ref. (31–33)]. Respiratory virus infection in mouse models results in an increase in the frequency and number of total and antigen-specific CD8 T cells in the lungs and airways. RSV-specific CD8 T cell responses typically reach peak numbers in the lung at approximately day 8 following an acute infection (34–37). The kinetics of virus-specific CD8 T cells are slightly more delayed following other respiratory virus infections with peaks occurring at approximately day 10 for IAV, days 10–14 for HMPV, and days 12–14 for pneumonia virus of mice (PVM), a model respiratory virus (38–42). The peak of the antigen-specific CD8 T cell response generally corresponds to lung viral clearance following RSV, IAV, HMPV, and PVM infections (36, 40–43). Human CD8 T cell kinetics following respiratory virus infections are less well known given the lack of identified CD8 T cell epitopes and the difficulty in obtaining respiratory tract samples from children following initial virus exposure. The frequency of total activated CD8 T cells in tracheal aspirates peaked at approximately 10 days after the onset of symptoms in children with RSV, IAV, RV, or CoV infections (44). RSV-specific CD8 T cells were detected in the tracheal aspirates of children; however, the evaluated epitopes were present at very low frequencies, comprising up to only 2% of the total CD8 T cell response (44). Following peak expansion, CD8 T cell contraction occurs and a memory population of virus-specific CD8 T cells remains within the lung. The majority of virus-specific CD8 T cells are located within the lung parenchyma, rather than the pulmonary vasculature, following localized respiratory infections in mice (37). Similarly, human RSV- and IAV-specific CD8 T cells were enriched within the lung compared to the blood (45). RSV-specific CD8 T cells in tracheal aspirates of children remain elevated during convalescence following a severe RSV infection, in contrast to murine studies (46). These studies suggest that CD8 T cell responses in the airways may be more prolonged following viral clearance in humans compared to mice.

Following respiratory virus infection, CD8 T cells become activated and develop the ability to produce inflammatory cytokines. Virus-specific CD8 T cells in the lung and airways of mice upregulate expression of markers associated with activation including CD11a, CD25, NKG2a, and CD44 as well as downregulate expression of the lymphoid homing receptor CD62L (34, 35, 47, 48). Activated CD8 T cells also acquire effector functions following viral infection. Virus-specific CD8 T cells rapidly produce cytokines including IFN-γ and TNF as well as degranulate, as measured by CD107a expression, following *ex vivo* peptide stimulation (35, 38, 41, 48). Human virus-specific CD8 T cells also acquire an activated phenotype and effector functions following a respiratory virus infection. CD8 T cells from the tracheal aspirates of children following RSV, RV, or CoV infections expressed elevated levels of the activation markers CD38 and HLA-DR and the proliferation marker Ki-67 (44). Expression of effector molecules such as granzyme B and perforin were also increased. Similarly, CD8 T cells from bronchiolar lavage (BAL) fluid samples exhibited increased expression of Ki-67, granzyme B, CD38, and HLA-DR following either experimental RSV infection of adults or severe, natural RSV infection of infants (46, 49). Additionally, human virus-specific CD8 T cells produce cytokines following respiratory virus infection, as peripheral blood CD8 T cells secreted IFN-γ, TNF, and IL-2 following stimulation with peptides derived from RSV, IAV, HMPV, or RV (49–53).

Following contraction, a subset of virus-specific CD8 T cells remain in the host to form a long-lasting memory population that provides protection against subsequent infection. CD8 T cell contraction to form long-term memory populations in the lung is regulated in part by inflammatory chemokine signaling (54). Mice deficient in either CXCR3 or CXCR3 and CCR5 exhibit a significant increase in the number of memory CD8 T cells following IAV infection, suggesting that chemokine

signaling through CXCR3 and CCR5 plays a critical role in T cell memory generation (54). Following respiratory viral infections in mice and humans, virus-specific CD8 T cells can be detected up to several months post-infection (47, 49, 55, 56). However, respiratory virus-specific memory CD8 T cell populations decline in magnitude with age in the peripheral blood (57). Interestingly, adult RSV-specific CD8 T cell responses are significantly reduced compared to IAV-specific CD8 T cell responses in the peripheral blood, suggesting that memory CD8 T cell responses to IAV in humans may be more stable than RSV (57). Memory CD8 T cells rapidly expand in the lung following a secondary respiratory virus infection in both mice and humans (35, 38, 39, 44, 49). The observed expansion is primarily due to the migration of circulating CD8 T cells into the lung and airways, rather than proliferation of resident cells (58). The expansion of virus-specific CD8 T cells in the lung and airways following infection corresponds with an increase in CXCR3- and CCR5-binding chemokines, supporting a role for chemokine-mediated migration of CD8 T cells following secondary infection (59). Indeed, CCR5 expression on memory CD8 T cells is required for their early recruitment into the airways after secondary infection, but not to the lung parenchyma (59). Following secondary expansion, memory CD8 T cells rapidly produce effector cytokines such as IFN-γ and TNF (30, 38, 60). Additionally, virus-specific memory CD8 T cells express high levels of CD11a and produce cytolytic molecules, such as granzyme B, after infection (61, 62). These effector functions of respiratory virus-specific memory CD8 T cells are critical for mediating viral clearance and protecting against infection, as discussed below.

Based on the expression of activation marker CD45RA and lymphoid homing receptor CCR7, human memory CD8 T cells have been broadly separated into four major subsets: (1) naive ($CD45RA^+CCR7^+$), (2) central memory (T_{CM}; $CD45RA^-CCR7^+$), (3) effector memory (T_{EM}; $CD45RA^-CCR7^-$), and (4) late effector memory (T_{EMRA}; $CD45RA^+CCR7^-$) (63). Due to their expression of CCR7, T_{CM} home primarily to secondary lymphoid organs, while T_{EM} migrate to peripheral tissues and rapidly exert effector functions. T_{EMRA} are a subset of T_{EM} cells that have re-expressed CD45RA. They exhibit reduced proliferative and functional capacity, and thus are considered to be terminally differentiated cells. Human virus-specific memory CD8 T cell populations are typically composed of a combination of T_{EM} and T_{EMRA} within the peripheral blood (44, 46, 50, 52, 55). Alternatively, RSV-specific memory CD8 T cells located in the airways in both adults and infants are primarily of T_{EM} phenotype and also express high levels of CD27, CD28, and CCR5 and low levels of CD62L (46, 49). Together, these studies indicate that T_{EM} CD8 T cells are dominant following respiratory virus infection in humans. Given the frequent exposure to viruses in the respiratory tract, T_{EM} cells may be critical for the rapid employment of CD8 T cell effector mechanisms following reinfection.

Recently, an additional population of memory CD8 T cells that persist within peripheral tissues has been identified, termed tissue-resident memory CD8 T cells (T_{RM}) (64). T_{RM} have been observed within several peripheral organs including the intestine, skin, female reproductive tract, and lung. T_{RM} generated following a respiratory virus infection represent a non-circulating population of memory CD8 T cells that are maintained within the lung parenchyma (65). Virus-specific T_{RM} are located along the wall of large airways and within pulmonary tissue surrounding bronchioles and alveoli (66, 67). Respiratory virus infection also induces T_{RM} within the airway lumen (68, 69). Airway T_{RM} downregulate CD11a expression and can be distinguished from recently trafficked CD8 T cells that express high levels of CD11a (70, 71). The localization of lung and airway T_{RM} following respiratory virus infection is distinctly different from that of T_{CM}, which traffic through the pulmonary vasculature and accumulate in the lung-draining lymph node (72, 73). Virus-specific T_{EM} are also differentially located from T_{RM} residing primarily in the pulmonary vasculature or within the lung tissue near blood vessels, spacially distinct from regions that contain T_{RM} (67, 73). Following either RSV or IAV infection in mice, lung and airway T_{RM} are induced and can be identified by their expression of the canonical resident memory markers CD69 and CD103, which promote their migration to and retention within the lung tissue (37, 65, 74–76). IAV- and RSV-specific T_{RM} are also generated in the lungs of mice that have been locally vaccinated via an intranasal route, but not mice that have been immunized systemically (30, 77–79). Importantly, IAV-specific T_{RM} expressing CD69 were also detected in human lung tissue sections but were absent from the spleen (65). Similarly, RSV-specific T_{RM} expressing both CD69 and CD103 were identified in the human BAL but were not present in the peripheral blood (49). Following secondary viral infection, T_{RM} expand prior to the recruitment of circulating memory CD8 T cell populations from the peripheral blood and rapidly produce IFN-γ (60, 66). Thus, T_{RM} provide a crucial first-line of defense for protecting the host from re-infection with a respiratory virus. However, in contrast to other memory CD8 T cell subsets that remain stable for long periods of time, IAV-specific T_{RM} exhibit limited longevity and enhanced apoptosis with time following infection (66, 80). The loss of IAV-specific T_{RM} corresponds to an increase in viral titers and weight loss following a heterosubtypic IAV infection (66, 80). Interestingly, infant mice generate fewer lung T_{RM} following IAV infection or vaccination and exhibit reduced heterosubtypic protection compared to mice initially infected as adults (79). Given the role of lung T_{RM} in providing protection against respiratory virus infections, identifying strategies to promote the generation of long-lived T_{RM} will be critical for future vaccines, particularly for infant populations.

MECHANISMS OF CD8 T CELL-MEDIATED VIRAL CLEARANCE

It has been well established that CD8 T cells are critical for viral clearance following an acute respiratory virus infection in mice. Adoptive transfer of CD8 T cell clones resulted in significantly reduced viral titers in the lung following RSV, IAV, or HMPV infections (25, 27, 81–84). Similarly, the transfer of either RSV- or IAV-immune splenic CD8 T cells accelerated viral clearance in the lung following infection (85–89).

Accordingly, RSV infection of mice depleted of CD8 T cells resulted in significantly increased lung viral titers at day 7 post-infection, although the virus was ultimately cleared by day 11 (26). In contrast, depletion of CD8 T cells alone did not alter clearance of HMPV (40). Instead, depletion of both CD4 and CD8 T cells together elevated lung virus titers at day 7 following infection with HMPV. Importantly, CD8 T cells have been shown to be sufficient to mediate viral clearance in the lung following acute respiratory infections (87, 88). Athymic nude mice, which lack T cells, fail to clear either RSV or IAV resulting in persistent infections (87, 90). However, the transfer of either RSV- or IAV-immune splenic CD8 T cells into athymic mice resulted in significantly reduced lung viral titers by day 15 and 21, respectively (87, 88). Together, these studies indicate that CD8 T cells play a critical role in mediating viral clearance following acute respiratory infections in mice.

Although studies are limited, a role for CD8 T cells in the elimination of respiratory viruses has also been established in humans. Early studies demonstrated that immunocompromised children with T cell defects experienced prolonged viral shedding following RSV, IAV, or PIV infections compared to immunologically normal children (3, 91, 92). Following bone marrow transplantation of an RSV-infected child with severe combined immunodeficiency, a marked reduction in nasal viral load was observed that correlated with an elevation of CD8 T cell counts (92). Recently, it has been demonstrated that the number of pre-existing virus-specific CD8 T cells in the airway of adults experimentally infected with RSV correlated with reduced overall viral load in the nasal cavity and bronchial brushings (49). In addition to pre-existing CD8 T cell numbers, CD8 T cell effector functions also correlate with reduced viral load. CD8 T cell target cell lysis activity measured by chromium-release assay correlated with a lack of viral shedding in the nasal washes of adults experimentally infected with H1N1 IAV (93). Additionally, individuals with the lowest frequencies of IFN-γ^+ CD8 T cells exhibited the highest viral titers following natural H7N9 IAV infection (94). These studies support the role of CD8 T cells in respiratory virus clearance in humans, consistent with the numerous murine studies.

CD8 T cells mediate viral clearance by utilizing a variety of effector mechanisms to induce the apoptosis of virus-infected cells (95). CD8 T cells can use direct cell–cell contact to eliminate target cells through the interactions of surface molecules such as Fas (CD95) and FasL (CD95L). Additionally, TRAIL expressed on CD8 T cells can interact with its receptors DR4 and/or DR5 to induce the destruction of infected cells. CD8 T cells can also secrete perforin and granzymes to cause membrane pore formation and induce apoptosis. Lastly, CD8 T cells produce inflammatory cytokines, such as IFN-γ and TNF, which may either directly or indirectly promote the cell death of virus-infected cells. While the exact mechanism utilized is unclear, many of these effector functions have been associated with CD8 T cell-mediated clearance of respiratory viruses. Fas/FasL interactions and the perforin pathway have been established as the primary mechanisms by which CD8 T cells eliminate infected cells following an IAV infection (96, 97). Studies utilizing TRAIL-deficient mice and antibody-mediated TRAIL blockade have also demonstrated a role for TRAIL in CD8 T cell-mediated clearance of IAV (98, 99). Similarly, Fas/FasL and perforin pathways have also been associated with virus elimination following RSV infection. Perforin-deficient and FasL-deficient *gld* mice exhibit significantly delayed viral clearance (100, 101). However, both perforin-deficient and *gld* mice achieve complete viral clearance by day 10 post-infection, suggesting that CD8 T cells compensate for those deficiencies through alternative mechanisms. One such mechanism is likely TNF production, as neutralization of TNF in perforin-deficient and *gld* mice significantly increased viral titers compared to IgG-treated controls (101). This is in contrast to studies following PVM and IAV infections, where viral clearance occurs independently of TNF (102, 103). IFN-γ does not appear to play a prominent role in CD8 T cell-mediated viral clearance, as both IFN-γ-deficient mice and mice that received IFN-γ-deficient CD8 T cells exhibit equivalent viral titers to wild-type mice following RSV, PVM, or IAV infections (101, 103–105). Together, these studies demonstrate that CD8 T cells use multiple complementary mechanisms to eliminate virally infected cells following a respiratory virus infection.

CD8 T CELLS PROTECT AGAINST A SECONDARY INFECTION

Given the ability of CD8 T cells to mediate viral clearance following an acute viral infection, it is no surprise that memory CD8 T cells also play a critical role in protecting against secondary respiratory virus infections. The adoptive transfer of airway IAV-specific memory CD8 T cells resulted in significantly reduced lung titers following IAV challenge compared to PBS transfer controls (60). Similarly, transfer of airway RSV-specific memory CD8 T cells reduced lung viral load and weight loss following subsequent RSV infection (76). These studies indicate that transferred memory CD8 T cells are capable of providing protection against secondary respiratory virus challenge.

Memory CD8 T cell-mediated protection against secondary infection has been shown more convincingly in mouse models through the use of vaccination strategies to generate virus-specific memory CD8 T cells. Recombinant baculovirus or murine cytomegalovirus (MCMV) vectors expressing the RSV M2 protein induced M2-specific CD8 T cells that mediated the reduction of lung viral titers following RSV challenge (78, 106). Whole protein vaccination with HMPV virus-like particles containing F and M proteins elicited HMPV-specific CD8 T cells that reduced viral titers in μMT mice, which lack antibodies (107). CD8 T cell epitope vaccines against either RSV or HMPV have also demonstrated CD8 T cell-mediated protection following challenge by reducing lung viral load and histopathology compared to unimmunized controls (108, 109). A similar strategy utilizing DC-peptide vaccination to generate pre-existing PVM-specific memory CD8 T cells also resulted in enhanced viral control following PVM infection (42). Recently, several studies have utilized DC-prime, recombinant *Listeria monocytogenes*- (DC-LM) or vaccinia virus-boost (DC-VV) vaccination protocols to generate a high frequency

of pre-existing antigen-specific memory CD8 T cells in the absence of virus-specific CD4 T cell memory and antibodies (28–30). Prime-boosted mice exhibited significantly reduced lung viral titers following RSV, IAV, or SARS infections compared to controls lacking virus-specific memory CD8 T cells. Additionally, memory CD8 T cells were able to reduce weight loss and mortality following lethal challenges with either IAV or SARS. Overall, these studies provide clear evidence that memory CD8 T cells provide protection against secondary respiratory virus infection by reducing viral titers.

The most well studied example of memory CD8 T cell-mediated protection from a secondary respiratory virus infection is heterosubtypic immunity to IAV subtypes. IAV-specific neutralizing antibody responses recognize the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), which vary between subtypes as a result of genetic reassortment, known as antigenic drift. However, the internal proteins of the virus are often conserved between IAV subtypes. Therefore, memory CD8 T cells that recognize epitopes within conserved viral proteins may be capable of providing cross-protection between IAV viruses of differing subtypes. Evidence of heterosubtypic immunity was first demonstrated by the protection of H1N1 IAV-immune mice from a lethal H2N2 IAV challenge without the induction of a neutralizing antibody response (110). Since then, a memory CD8 T cell-mediated role in accelerating clearance of a heterosubtypic IAV strain has been well-established in mouse, chicken and non-human primate models (66, 111–114). Recently, it has been demonstrated that T_{RM} are essential in providing cross-protection against secondary IAV infection with a heterosubtypic strain (60, 66, 80). Mice with $CD103^+$ T_{RM} in the lung exhibit more efficient viral clearance and reduced weight loss following heterosubtypic challenge than mice lacking a T_{RM} response (66). Importantly, the protection was provided solely by lung-resident memory CD8 T cells, as blocking the ability of recently proliferated T_{CM} cells from trafficking to the lung did not impact protection (66). Consistent with the limited lifespan of IAV-specific T_{RM}, heterosubtypic protection by memory CD8 T cells wanes over time, with a decline observed as early as 60 days following the initial infection (80, 111). Interestingly, systemic immunization with cognate antigen is capable of boosting the T_{RM} pool by expanding the circulating T_{EM} population that seeds the lungs (80). Therefore, it is possible that T_{RM}-mediated heterosubtypic protection could be re-established by vaccination after a waning of the protective T_{RM} population in the lung.

While protection in mouse models is well established, whether memory CD8 T cells play a critical role in protection following secondary respiratory infection in humans is currently unclear. Similar to studies in murine models, evidence for heterosubtypic immunity mediated by memory CD8 T cells has also been demonstrated in humans. Individuals lacking H1N1-specific neutralizing antibody titers exhibited an inverse correlation between memory CD8 T cell activity and viral shedding following their first exposure with H1N1 IAV (93). More recently, it was demonstrated that the frequencies of pre-existing cross-reactive memory CD8 T cells correlated with reduced symptoms, including fewer patients with fever, sore throat, and cough, following infection with the 2009 pandemic H1N1 IAV strain (50). Similarly, a correlation between pre-existing H3N2-specific memory CD8 T cells and reduced risk of viral shedding following 2009 pandemic H1N1 IAV infection was observed (115). Thus, memory CD8 T cell-mediated heterosubtypic protection is also likely to be critical in humans. Following experimental RSV infection in humans, the frequency of pre-existing RSV-specific memory CD8 T cells in the airways correlates with a reduction in both cumulative and lower respiratory tract symptom scores, suggesting a possible role for memory CD8 T cells in protection against RSV in humans (49). However, evidence has also been provided suggesting that memory CD8 T cells may not contribute to protection following respiratory virus infections in humans. Natural reinfection of infants with RSV did not result in a boosting of the CD8 T cell response (116). Similarly, the frequency of RSV-specific memory CD8 T cells in the peripheral blood of healthy adults is significantly reduced compared to IAV-specific memory CD8 T cells (57). Therefore, the extent to which memory CD8 T cells play a role in providing protection against RSV infection in humans remains unclear.

CD8 T CELL-MEDIATED IMMUNOPATHOLOGY FOLLOWING RESPIRATORY VIRUS INFECTION

Despite their beneficial role in mediating viral clearance and protecting against secondary infection, CD8 T cells have also been associated with the induction of immunopathology following respiratory virus infection. Although mice depleted of CD8 T cells exhibited elevated lung viral titers, weight loss and symptom illness scores were significantly reduced in CD8 T cell depleted mice following acute RSV infection (26). Similarly, the adoptive transfer of CD8 T cell lines exacerbated weight loss following an acute RSV infection, despite accelerating viral clearance (82–84). Similar reduction in disease severity was also demonstrated following either HMPV or PVM infection of CD8 T cell depleted mice or mice genetically deficient in CD8 T cells, respectively (40, 103). In addition to the induction of immunopathology following acute respiratory virus infections, we recently demonstrated that memory CD8 T cells also mediate severe immunopathology following secondary RSV infection (30). Large frequencies of systemic, pre-existing RSV-specific memory CD8 T cells generated through DC-LM immunization induced significant weight loss, pulmonary dysfunction, and mortality following RSV challenge, despite a significant reduction in lung viral titers. This result was in contrast to studies using similar immunization strategies to either IAV or SARS, in which memory CD8 T cells mediated protection against lethal viral challenge in the absence of immunopathology (28, 29). Interestingly, the immunopathology induced by RSV-specific memory CD8 T cells occurred only in the context of an RSV infection, as mice challenged with a recombinant IAV strain expressing an RSV-derived CD8 T cell epitope exhibited significantly reduced morbidity and were protected from mortality (30). This result is consistent with several studies that demonstrate CD8 T cells enhance viral clearance while preventing mortality following IAV infection (25, 81,

85, 87, 117). Together, these studies demonstrate a clear role for CD8 T cells in the development of immunopathology following primary and secondary infections with some respiratory virus infections, particularly RSV.

Antiviral mechanisms utilized by CD8 T cells to mediate viral clearance following respiratory virus infection also contribute to the development of immunopathology. Removal of the Fas/FasL pathway in *gld* mice resulted in significant amelioration of weight loss and symptom illness scores following RSV infection (101). Similarly, RSV-infected perforin-deficient mice exhibited prolonged weight loss and symptom illness scores compared to wild-type mice (100). TNF contributes substantially to immunopathology, as antibody-mediated depletion of TNF prior to either RSV or IAV infection significantly reduced weight loss (101, 102, 118). Additionally, mice that are IFN-γ-deficient, depleted of IFN-γ, or received an adoptive transfer of IFN-γ-deficient CD8 T cells prior to RSV infection lost significantly less weight than controls (89). CD8 T cell production of TNF and IFN-γ following PVM infection also induced pulmonary immunopathology by initiating a cytokine storm (119). In addition to causing disease in acute respiratory infections, IFN-γ produced by memory CD8 T cells mediated the severe and fatal immunopathology following RSV infection of DC-LM prime-boosted mice (30).

The role of CD8 T cells in the development of pathology following respiratory infections in humans remains unclear. The best evidence supporting a pathogenic role for CD8 T cells in humans infected with respiratory viruses comes from a study evaluating an RSV-infected severe-combined immunodeficiency patient after bone marrow transplantation (92). The patient exhibited increased CD8 T cell counts following bone marrow transplant, which corresponded to a sharp reduction in RSV nasal titers. However, the appearance of CD8 T cells also correlated with a marked increase in respiratory rate indicative of reduced pulmonary function. Also supporting a pathogenic role of CD8 T cells is the finding that children requiring mechanical ventilation due to severe RSV infection expressed significantly increased levels of activated granzymes and more CD8 T cells producing granzyme B compared to healthy controls (120). In contrast, a study of infants following either fatal IAV or RSV infections revealed a near absence of CD8 T cells from affected lung regions by immunohistochemical staining (121, 122). Similarly, infants with severe RSV infection exhibited an underexpression of genes related to CD8 T cells in the peripheral blood (123). In support of a protective, rather than pathogenic, role of CD8 T cells, correlations have been identified between increased CD8 T cell cytolytic activity and cytokine production with reduced symptom score, faster recovery, and fewer fatalities following H1N1 or H7N9 IAV infections (93, 94). Therefore, whether CD8 T cells play a primary role in mediating pathology versus protection following human respiratory virus infection remains controversial and is an important topic of future investigation.

REGULATION OF CD8 T CELL EFFECTOR FUNCTIONS

Given the potential of CD8 T cell effector functions to cause immunopathology following respiratory virus infection, the immune system has evolved critical regulatory mechanisms to prevent prolonged CD8 T cell effector activity following viral clearance. CD8 T cell effector functions, including production of IFN-γ and TNF, are suppressed in the lung following the resolution of IAV and RSV infections (124–127). One of the primary mechanisms utilized to limit the CD8 T cell response is through suppression by regulatory CD4 T cells (Tregs). Tregs accumulate in the lungs following either RSV or IAV infection peaking at approximately day 6 post-infection, prior to the peak of the CD8 T cell response (36, 128–130). Antibody-mediated depletion of $CD25^+$ Tregs prior to RSV infection resulted in exacerbated weight loss, pulmonary dysfunction, and lung inflammation (128). This enhanced illness corresponded to an increased frequency of RSV-specific CD8 T cells and elevated levels of IFN-γ and TNF protein in the lung (36, 128). Consistent with the Treg depletion studies, increasing RSV-specific Tregs prior to RSV infection using RSV peptide-immunization resulted in an amelioration of weight loss and a reduction in CD8 T cell numbers in the blood and spleen, but not the lung (131). Tregs also can suppress CD8 T cell effector functions following a secondary infection with IAV (130). Antibody-mediated $CD25^+$ Treg depletion prior to heterosubtypic IAV challenge resulted in enhanced inflammation and pulmonary dysfunction corresponding to an increase in CD8 T cell numbers and IFN-γ production. One mechanism through which Tregs may suppress CD8 T cell responses is through the production of the anti-inflammatory cytokine IL-10. $FoxP3^+$ Tregs secrete IL-10 following primary infection with RSV or IAV (130, 132–134). Infection of either IL-10-deficient mice or mice treated with IL-10 receptor blocking antibody resulted in increased numbers of either IFN-γ^+ or IFN-γ^+TNF^+ CD8 T cells, suggesting that IL-10 suppresses CD8 T cell effector functions following respiratory virus infection (132–134). Interestingly, IL-10 production by $FoxP3^-$ CD4 T cells and CD8 T cells following either RSV or IAV infection has also been reported, indicating that effector T cell responses may self-regulate their effector functions (132–135). Together, these studies demonstrate that Tregs and IL-10 production play a critical role in regulating CD8 T cells following primary and secondary respiratory virus infections to prevent immunopathology.

Interactions between inhibitory receptors on CD8 T cells with their ligands represents another important mechanism mediating the inhibition of CD8 T cell effector functions following infection. Regulation of CD8 T cells through the PD-1:PD-L1 pathway is a common inhibitory pathway utilized following respiratory virus infection. Expression of PD-1 on pulmonary CD8 T cells is upregulated following RSV, IAV, or HMPV infection in mice (41, 136, 137). Blockade of PD-L1 in primary RSV, IAV, or HMPV and secondary HMPV infections results in enhanced CD8 T cell effector functions, including IFN-γ, TNF, and granzyme B production (41, 136–138). CD8 T cell effector functions are also enhanced following either HMPV or IAV infections in PD-1-deficient mice (41). Importantly, the PD-1:PD-L1 pathway has also been associated with human CD8 T cell responses. Human CD8 T cells in the nasal cavity significantly upregulated PD-1 following RSV infection compared to CD8 T cells from the blood of either healthy or RSV-infected individuals (137). PD-1 and PD-L1 are also both upregulated in the lung

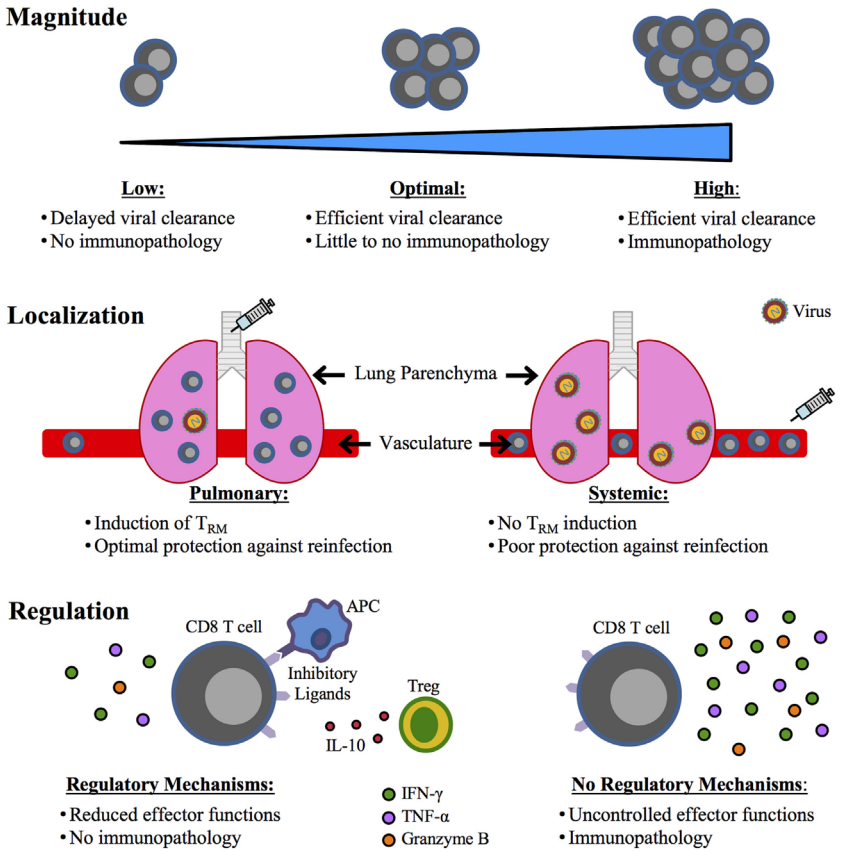

FIGURE 1 | Critical factors for an optimal CD8 T cell-mediated respiratory syncytial virus (RSV) vaccine. A future RSV vaccine designed to elicit a CD8 T cell response will require a balance between CD8 T cell-mediated protection and immunopathology, which may be achieved through the consideration of three important aspects: (1) magnitude, (2) localization, and (3) regulation. An optimal magnitude of the CD8 T cell response will be one that achieves efficient viral clearance in the absence of immunopathology. The vaccination route will be critical in determining the localization of the CD8 T cell response. A pulmonary route of vaccination will induce T_{RM} in the lung that provides superior protection compared to a systemic immunization that would likely not generate protective T_{RM}. Lastly, regulation of the CD8 T cell response generated through vaccination will be crucial, as uncontrolled effector functions, particularly IFN-γ production, can result in immunopathology.

tissue following severe infections with either RSV or the 2009 H1N1 IAV pandemic strain (41). *In vitro* human studies have demonstrated that PD-L1 is constitutively expressed on human airway and bronchial epithelial cells, but expression is significantly upregulated following either IAV or RSV infection (136, 139). Similar to *in vivo* mouse studies, *in vitro* PD-L1 blockade resulted in significantly increased CD8 T cell production of IFN-γ, IL-2, and granzyme B following RSV infection (139). Together, these studies demonstrate a critical role for PD-1 in the suppression of CD8 T cell-mediated immunopathology and cytokine production in both mice and humans. In the absence of PD-1 signaling following HMPV infection, CD8 T cell IFN-γ production remains impaired, suggesting the involvement of compensatory inhibitory pathways (140). Antigen-specific lung CD8 T cells express inhibitory receptors Tim-3, LAG-3, and 2B4 following HMPV infection and exhibit enhanced cytokine production following *in vitro* blockade of each receptor individually (140). *In vivo* blockade of LAG-3 partially restored CD8 T cell IFN-γ production in PD-1-deficient mice following HMPV infection (140). Tim-3 has also been demonstrated to be critical in suppressing CD8 T cell responses *in vivo*, as Tim-3 receptor (Galectin-9)-deficient mice exhibited significantly enhanced CD8 T cell responses following both primary and secondary IAV infections (141). Together, these studies indicate that multiple inhibitory receptor pathways are utilized following pulmonary virus infection to dampen the pathogenic CD8 T cell response and prevent immunopathology.

CONCLUDING DISCUSSION

Successful vaccinations against the majority of respiratory viruses remain elusive. The goal of most vaccination strategies is to induce robust virus-specific neutralizing antibody responses. However, the antibody response generated by infection with many respiratory infections, including RSV and RV, wanes over time. Therefore, neutralizing antibody responses as the sole mediator of a vaccine against most respiratory viruses may not provide long-term protection without yearly

vaccination. Vaccination strategies that include the induction of virus-specific CD8 T cell responses, either alone or in combination with humoral immunity, may be advantageous by providing many benefits associated with cellular immune responses. CD8 T cells are critical for the elimination of virus-infected cells, and viral clearance was prolonged in the absence of CD8 T cells following acute respiratory virus infections. Additionally, robust memory CD8 T cell responses efficiently reduced lung viral titers in the absence of neutralizing antibodies following RSV, IAV, or SARS secondary infections. An important property of CD8 T cells is that they often recognize conserved viral proteins, allowing for cross-protection between different virus strains. This is particularly important for heterosubtypic protection of IAV strains, as neutralizing antibodies are not capable of recognizing IAV strains of differing subtypes. Despite their benefits in mediating viral clearance and providing protection against secondary infections, memory CD8 T cell responses have been associated with the induction of immunopathology following respiratory virus infections. The same antiviral mechanisms employed by memory CD8 T cells to accelerate viral clearance also contribute to immunopathology, including the Fas/FasL pathway- and perforin-mediated cytolysis and IFN-γ and TNF cytokine secretion. Thus, the efficient elimination of respiratory viruses by memory CD8 T cells comes at a cost of disease for the host. CD8 T cell-mediated immunopathology appears to be virus-specific. Although high frequency, systemic, antigen-specific memory CD8 T cells induced severe disease and mortality following RSV infection, no pathology was observed using similar systems for IAV and SARS infections. Therefore, induction of memory CD8 T cells as the sole immune mediator may be particularly dangerous for an RSV vaccine, but significantly less so in either an IAV or a SARS vaccine.

To be able to include CD8 T cell responses within a future respiratory virus vaccine, it will be extremely important to determine how to balance CD8 T cell-mediated protection versus immunopathology following respiratory infection. For RSV in particular, three critical aspects to consider in this balance include magnitude, localization, and regulation of the RSV-specific CD8 T cell response (**Figure 1**). DC-LM immunization generated $M2_{82\text{-}90}$-specific CD8 T cells at a frequency of approximately 20% in the peripheral blood, but induced fatal immunopathology following RSV challenge (30). However, DC-prime only and TriVax immunizations generated a much lower frequency of total $M2_{82}$-specific CD8 T cells, and RSV induced significantly reduced disease in these mice (30, 109). Thus, identifying the optimal magnitude of RSV-specific CD8 T cells for protection in the absence of immunopathology is crucial. It is also clear from recent studies in mouse models that localization of RSV-specific CD8 T cells is a significant factor for both their efficacy of mediating viral clearance and their ability to induce immunopathology following infection. Intranasal immunization with MCMV-M generated T_{RM} within the lung tissue that accelerated viral clearance. In contrast, mice administered MCMV-M systemically did not generate T_{RM} and exhibited delayed viral clearance (78). Similar results were observed with local immunization with DC-IAV-$M2_{82}$ (30). $M2_{82}$-specific lung T_{RM} generated by pulmonary immunization did not induce immunopathology following RSV infection, in contrast to systemic DC-LM immunization, which resulted in severe pathology in the absence of T_{RM} cells. Thus, vaccination strategies against RSV will likely be the most effective when administered through a pulmonary route to generate T_{RM} that will provide protection within the lung following reinfection without inducing immunopathology. Lastly, identifying ways to regulate vaccine-generated CD8 T cell responses will likely reduce immunopathology following subsequent infection. IFN-γ produced by CD8 T cells was the primary mediator of immunopathology following RSV infection of DC-LM vaccinated mice (30). However, neutralization of IFN-γ had no effect on lung viral titers, suggesting that CD8 T cells utilize other antiviral mechanisms to mediate viral clearance in this system. Since CD8 T cells are able to reduce viral titers in the absence of IFN-γ, reducing the amount of IFN-γ produced by CD8 T cells would likely result in ameliorated disease following RSV infection. If vaccination strategies can identify mechanisms by which CD8 T cell cytokine production, particularly IFN-γ, can be attenuated without altering their ability to eliminate virus-infected cells, the pathology induced by CD8 T cells would likely also be decreased.

The development of a CD8 T cell-mediated vaccine should be pursued given the limitations of antibody responses to respiratory viruses. It is possible that the ideal vaccine for respiratory virus infections will include the induction of both virus-specific CD8 T cells and neutralizing antibodies. A vaccination approach combining both arms of the adaptive immune response may allow for optimal viral control in the absence of disease symptoms. However, before CD8 T cells can be developed further as a mediator of protective immunity, the balance between protection and pathology must be achieved. Future studies evaluating aspects of memory CD8 T cell magnitude, localization, and regulation will greatly assist in reaching this balance.

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REVIEW
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Past Life and Future Effects—How Heterologous Infections Alter Immunity to Influenza Viruses

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Influenza virus frequently mutates due to its error-prone polymerase. This feature contributes to influenza virus's ability to evade pre-existing immunity, leading to annual epidemics and periodic pandemics. T cell memory plays a key protective role in the face of an antigenically distinct influenza virus strain because T cell targets are often derived from conserved internal proteins, whereas humoral immunity targets are often sites of increased mutation rates that are tolerated by the virus. Most studies of influenza T cell memory are conducted in naive, specific pathogen free mice and do not account for repetitive influenza infection throughout a lifetime, sequential acute heterologous infections between influenza infections, or heterologous chronic co-infections. By contrast to these mouse models, humans often experience numerous influenza infections, encounter heterologous acute infections between influenza infections, and are infected with at least one chronic virus. In this review, we discuss recent advances in understanding the effects of heterologous infections on the establishment and maintenance of CD8+ T cell immunological memory. Understanding the various factors that affect immune memory can provide insights into the development of more effective vaccines and increase reproducibility of translational studies between animal models and clinical results.

Keywords: CD8+ T cells, influenza, heterologous, bystander, attrition, memory, cross-reactivity, chronic co-infection

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INTRODUCTION

Influenza Virus

In the United States, seasonal epidemics caused by influenza virus lead to 3.1 million hospitalized days, 31.4 million outpatient visits, and direct medical costs of $10.4 billion, on average (1). While vaccination against influenza virus has decreased morbidity and mortality, influenza virus is particularly efficient at evading the immune system, and more research is needed to improve vaccine efficacy. A key aspect of influenza virus biology, which confers higher pathogenicity and contributes to immune evasion, is its high rate of mutation due to the error-prone activity of its RNA polymerase, which lacks proofreading function. Accrual of point mutations over time, known as antigenic drift, can lead to antigenically distinct proteins that cannot be recognized by established protective immunity. Evasion of immune memory can also occur when more than one parental virus strain infects the same host and reassortment of various genome segments leads to viral progeny of a new subtype, a process known as antigenic shift. Indeed, the 2009 H1N1 pandemic was the result of reassortment between an Eurasian swine H1N1 and a triple reassortant swine H1N2, which contained gene segments from an avian virus, North American classical swine H1N1, and human seasonal H3N2 (2).

Influenza virus is a member of the Orthomyxovirus family. There are three classes: A, B, and C; which vary in their host, pathogenicity, and structure. The genome consists of 7–8 segments of negative-sense single stranded RNA, encapsulated in nucleoprotein (NP). At the end of each segment is a heterotrimer of three polymerase proteins: polymerase basic protein 1, polymerase basic protein 2, and polymerase acidic protein (PA). The genome is enclosed in a capsid, which is encapsulated in a host derived lipid bilayer envelope. Imbedded into the lipid envelope is the matrix 2 protein, and two spike proteins that are important for binding, fusion/entry, and egress from target cells—hemagglutinin (HA) and neuraminidase (NA). Influenza viruses are often subdivided and referred to by their HA and NA subtypes. Of the 18 HA and 11 NA subtypes currently known, only H1N1, H2N2, and H3N2 have caused a human pandemic. Currently circulating human influenza viruses include: influenza A virus H1N1, influenza A virus H3N2, and influenza B virus.

Control of influenza virus is ultimately achieved by the virus-specific adaptive immune response. CD4+ T cells aid in the activation of both B and CD8+ T cells, important for production of antibodies and clearance of virus infected cells, respectively. Antibodies produced by B cells serve non-neutralizing and neutralizing functions. HA and NA are major targets of neutralizing antibodies; however, these proteins are often sites of mutations, which may lead to antigenic drift over time. Conversely, CD8 T cells, also known as cytotoxic T lymphocytes (CTLs), target conserved regions of internal proteins, which are less prone to mutation due to fitness cost and/or potential for loss of function. By contrast to innate immunity, adaptive immunity is pathogen-specific and results in immunological memory. In cases of antigenically distinct subtypes, which are common for influenza virus, and often lead to pandemics, targeting of conserved internal proteins by memory CD8 T cells can lead to rapid and effective control of influenza virus. Indeed, numerous studies have shown the benefits of immunological memory during heterosubtypic influenza virus infection [recently reviewed in detail in Ref. (3)]. Given the importance of memory CD8 T cell responses in influenza virus infection, it is important to understand the various factors that can affect the establishment and/or maintenance of immunological memory in the CD8 T cell compartment, particularly with regard to heterologous infections, which commonly occur in humans. Here, we review the effects of heterologous acute sequential or chronic co-infection on recruitment of the CD8 T cell response and memory generation and maintenance during influenza virus infection.

CD8+ T Cell Immunity

T cells recognize peptides presented in the context of major histocompatibility complex (MHC) molecules located at the cell surface. The immunogenicity of a given epitope is dependent on many factors, including but not limited to: protein processing, protein affinity for MHC, frequency of epitope-specific T cells, and competition amongst other T cells for interactions with antigen presenting cells (APCs) (4). Immunogenic epitopes do not all stimulate the same magnitude of CD8 T cell response; rather, there is an immunodominance hierarchy, in which epitopes can be classified as dominant, codominant, or subdominant. Epitope immunodominance is not directly correlated with epitope abundance and appears at least partially dependent on the relative frequency of high avidity epitope-specific T cells, recruitment of CD8 T cell precursors, and the extent of precursor proliferation throughout the primary response (5, 6). In addition, an epitope's immunodominance can change upon secondary infection. For example, during primary infection with the H3N2 lab strain X31, $NP_{366–374}/D^b$ and $PA_{224–233}/D^b$ epitopes elicit a CD8 T cell response similar in size; however, upon secondary infection with the H1N1 lab strain PR8, NP-specific CD8 T cells become dominant (7). The observed change in immunodominance upon secondary infection is associated with increased epitope presentation of NP (presented by multiple APCs) vs. PA [presented by dendritic cells (DCs) only], which augments activation and expansion of NP-specific memory CD8 T cells.

The CD8 T cell receptor (TCR) is composed of an alpha and beta chain. Each chain is generated by a semi-random recombination mechanism known as V(D)J recombination. In humans, the alpha locus consists of 42 variables (V) and 61 joining (J) segments; the beta locus consists of 47 V, 2 diversity (D), and 13 J segments. Diversity in the TCR is a result of three factors: (1) Semi-random pairing of a single V, D, and J segment. (2) Recombining of Vα-Jα or Vβ-Dβ-Jβ results in random nucleotide insertions and deletions at junction sites. (3) Combinatorial diversity of an alpha chain and a beta chain. The TCR generation process has the potential to generate 10^{15}–10^{61} unique receptors (8–10). However, the size of the peripheral TCR repertoire in humans is estimated at ~10^6–10^8, and with many sequences overlapping between individuals, despite the enormous potential repertoire diversity (11–14). This is due, at least in part, to the preferential use of particular VDJ segments and positive-negative selection of T cells in the thymus, before entry into the periphery. The pool of T cells capable of recognizing a specific epitope is referred to as the epitope-specific T cell repertoire and comprised of various unique TCRs. On average, the size of an epitope-specific repertoire consists of 50–500 naive T cells (6, 15, 16).

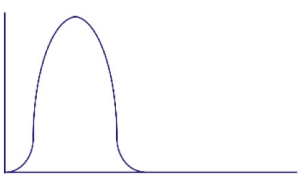
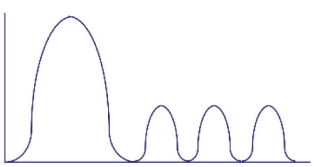
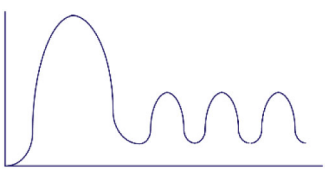
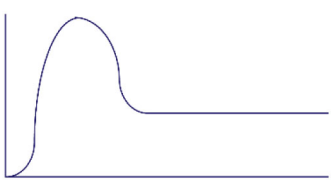
Three signals contribute to the priming of a CD8 T cell: (1) Recognition of cognate antigen *via* interaction of TCR: peptide:MHC. (2) Interaction with activating co-stimulatory molecules. (3) Cytokines in the surrounding microenvironment. If the accumulation of these signals exceeds the threshold of activation, a T cell will be recruited into the T cell response and begin to proliferate. The T cell response occurs in three general phases: activation and expansion, contraction, and memory. Following activation, T cells undergo extensive division, replicating every 6–8 h and expanding up to 10^4–10^5 fold (17). Differentiation of CD8 T cells involves acquisition of effector functions, such as production of anti-viral IFN-γ, pro-survival IL-2, and cytolytic enzymes. Generally, the contraction phase begins following control of pathogen growth, during which 90–95% of activated T cells die *via* apoptosis by 2–3 weeks post peak expansion (17). The remaining CD8 T cells will further differentiate into various memory populations. There are three broad types of memory CD8 T cells commonly recognized: central memory T cells, T_{CM} ($CD44^{hi}$ $CD62L^+$ $CCR7^+$ $CD127^+$ $CD69^-$ $CD103^-$), circulate through secondary lymphoid tissues *via* the blood and lymph. Effector memory T cells, T_{EM} ($CD44^{hi}$

CD62L$^-$ CCR7$^-$ CD127$^+$ CD69$^-$ CD103$^-$), migrate throughout the periphery. Resident memory T cells, T_{RM} (CD44^{hi} CD62L$^-$ CCR7$^-$ CD11a$^+$ CD69$^+$ CD103$^+$), remain in tissues and do not recirculate *via* the bloodstream. Memory CD8 T cells undergo epigenetic modifications that lead to a transcriptionally poised state, conferring rapid recall of effector function upon reencounter of a pathogen (18).

Given the high rate of mutations in influenza virus and potential for evasion of population immunity, it is imperative to understand how to optimize memory CD8 T cell responses, especially in the face of a new influenza subtype, during which CTL responses against conserved epitopes could play a key role in controlling infection. Most studies to date are conducted in specific pathogen free mice, in controlled environments, and do not take into account repetitive influenza infection throughout a lifetime, sequential acute heterologous infection between influenza infections, or co-infection with chronic heterologous infections. This is particularly important because humans may encounter numerous heterologous acute infections between influenza infections and the average adult is estimated to harbor ~8–12 chronic infections (19). Indeed, recent work has shown that mice infected with sequential heterologous infections, both acute and chronic, have immune responses to vaccination that are more human-like as compared with naive, specific pathogen free mice (20). Furthermore, in a study of influenza vaccine responses in humans, young CMV+ subjects had higher antibody titers and a generally activated immune system compared with young CMV-subjects (21). These data suggest infection history plays a role in shaping our response to immune challenge and may, at least in part, provide insight into the discrepancy between vaccination efficacies in the laboratory vs. in the clinic.

There are two general categories of heterologous infections—acute and chronic. It is important to note that in addition to acute infections, there are three distinct types of chronic infection that are often referred to interchangeably, but actually represent different scenarios for the immune system and conclusions from one category cannot be generally applied to another (**Table 1**). For this review, we will use the following definitions: (1) Acute, such as influenza virus infection, wherein T cells are transiently exposed to viral antigen and the virus is eventually cleared from the host (22–24). (2) Latent chronic, such as Epstein–Barr virus (EBV), where there are periodic phases of latency (no viral replication) and reactivation (production of infectious virus), during which T cells rest is exposed to antigen, respectively (25–27). (3) Smoldering chronic, such as Cytomegalovirus, wherein there is ongoing subclinical, low-level viral replication and T cells are continually exposed to antigen, with little rest (27–29). (4) Persistent chronic, such as Hepatitis C virus, where there is a continuous high-level of viral replication (viremia) and thus constant T cell stimulation with no periods of rest (30–34). In this review, when appropriate, sections will be divided into "Acute, Sequential" and "Chronic Co-infection."

TABLE 1 | Types of heterologous infections [modified from Ref. (17)].

Infection type	Category	Example	Characteristics	Antigen burden	Reference
Acute	–	Influenza virus	Eventual clearance of pathogen and transient exposure of T cells to antigen		(22–24)
Chronic	Latent	Epstein–Barr virus and Herpes simplex virus	Chronic infection with periodic reactivation and periods of T cell exposure and rest		(25–27)
	Smoldering	Cytomegalovirus	Chronic infection with low-level ongoing viral replication and infrequent T cell rest		(27–29)
	Persistent	Hepatitis C virus and HIV/AIDs	Chronic infection with high viral replication (viremia) and no T cell rest, constant exposure to high levels of antigen		(30–34)

EARLY KINETICS AND BYSTANDER ACTIVATION

Most studies are conducted in naive, specific pathogen free mice; however, humans encounter daily immune challenges that may impact pre-existing immunological memory and control of subsequent infections (homologous or heterologous). Indeed, prior infection with influenza virus protects mice from respiratory syncytial virus (RSV) induced eosinophilia and weight loss (35). Protection can be conferred *via* transfer of splenocytes from influenza virus-exposed animals, and is thought to be mediated by non-specific bystander activation, cross-reactive T cells, immunological imprinting (and skewing toward Th1 response), and/or structural remodeling after the first infection (35). This study highlights the impact infection history may have on the control and disease severity of a sequential heterologous infection.

Memory CD8 T cells are transcriptionally poised for rapid recall of effector function upon reencounter of a pathogen, and are capable of responding to 1/50th of the peptide concentration necessary for naive T cell stimulation (18, 36). Cytokine stimulation alone is even sufficient to induce memory CD8 T cell activation and cytokine production. Activation of T cells in the presence of an inflammatory microenvironment, but in the absence of cognate antigen, is termed bystander activation. Studies have shown bystander T cell proliferation can be induced by viruses, type I IFN, cytokines, and polyI:C (37, 38). Cytokines, including but not limited to, IFNα/β, IFN-γ, IL-12, IL-15, IL-7, and IL-18 have been shown to have unique and synergistic effects on bystander activation of T cells (39, 40). Furthermore, the extent of bystander activation depends on the infection dose and subsequent level of inflammation (41). Recent work in a primary influenza infection model shows early expansion of highly activated non-specific (bystander) memory CD8 T cells, which are CD25 negative, a component of the IL-2 receptor and a molecule that is up-regulated upon TCR stimulation, but are NKG2D positive, an activating receptor expressed on NK- and T-cells, and are restricted to the site of infection (42). Importantly, blockade of NKG2D resulted in increased influenza viral titers, suggesting a role for NKG2D in viral control (42). Similar results have been observed in a mouse model of *Listeria monocytogenes* (LM) infection, where bystander-activated memory CD8 T cells mediated early clearance of infection, in a NKG2D-dependent manner (43). Additional studies have shown NKG2D can act as a co-stimulatory molecule for CD8 T cells, augment cytotoxicity, and is sufficient to rescue unhelped memory CD8 T cells (44, 45). These results suggest a role for bystander-activated memory CD8 T cells in initial pathogen control.

Acute, Sequential

An acute, sequential heterologous infection model of primary Sendai virus and secondary influenza virus infection, and the reverse sequence of infection, show early recruitment of non-specific memory CD8 T cells into the lung (46). Specifically, there is a 4–5 fold increase in Sendai virus specific CD8 T cells at day 4 after secondary influenza infection and a fourfold increase in influenza-specific CD8 T cells at day 4 after secondary Sendai virus infection. Similar results were observed in other respiratory infection models—there is 4.4 and 1.7 fold increase in murine herpesvirus 68 (MHV68)—and Vaccinia virus (VV)-specific CD8 T cells, respectively, at day 3 after secondary Sendai virus infection (46). Furthermore, in the primary Sendai and secondary influenza virus infection model, bystander Sendai-specific CD8 T cells exhibit a transiently altered phenotype at day 3–4 (coinciding with the peak of their presence in the lung), are recruited *via* circulating memory CD8 T cells, and recruitment is independent of proliferation, although a portion of non-specific cells do proliferate (46).

Chronic Co-Infection

Epstein–Barr virus is a member of the gamma-herpesvirus family, and its seroprevalence in humans approaches 80–90% in adults (19). Studies using MHV68, a natural mouse pathogen that is closely related to human gamma herpesviruses, suggest latent MHV68 co-infection confers protection during challenge with influenza virus (47). Co-infected mice show enhanced survival, enhanced viral clearance at early time points, decreased lung injury, increased recruitment of activated CD4 and CD8 T cells at early and later time points, enhanced activation of alveolar macrophages, and augmented levels of anti-viral IFN-γ in response to influenza virus infection (47). Similar results were observed in a co-infection model of murine cytomegalovirus (MCMV) and influenza virus, wherein decreased influenza virus titers and increased numbers of influenza-specific CD8 T cells were observed in early (5 weeks) and established (12 weeks), but not long-standing (9 months), MCMV latently infected mice (21). Human cytomegalovirus (HCMV), a beta-herpesvirus family member, is also a significant human pathogen that infects approximately 50% of adults, with seroprevalence increasing up to 90% with age (48, 49). HCMV and EBV infections are largely subclinical and well tolerated; however, they are associated with significant increases in memory CD8 T cells over time, termed memory inflation. A study sought to determine whether unrelated virus specific memory CD8 T cells were activated in the immune response to acute, heterologous infections in humans (50). In peripheral blood mononuclear cells from patients at the onset of acute hepatitis B virus (HBV) infection, antigen-specific CD8 T cells within the total activated (CD38+ HLA-DR+) CD8 T cell compartment ranged (when detectable) from: 43 to 89% for HBV, 5.5 to 20% for HCMV, 22 to 41% for EBV, but only 0 to 2% for IAV (influenza A virus), as determined by pentamer binding. In addition, 54, 4.9, 8, and 0% of HBV-, HCMV-, EBV-, and IAV-specific CD8 T cells were proliferating, respectively. These results suggest that CD8 T cells specific for chronic pathogens may be preferentially activated during acute, heterologous infections. Moreover, acute infection with dengue, adenovirus, and influenza virus also induced activation of HCMV- and/or EBV-specific CD8 T cells (50). In one influenza case, approximately 25% of the onset activated CD8 T cells were HCMV-specific, but influenza-specific CD8 T cells could not be detected until day 5 (50). The study also found IL-15, a cytokine important for maintenance of memory T cells and often produced during acute viral infection, selectively activates HCMV- and EBV-specific, but not influenza-specific, CD8 T cells, is sufficient for spontaneous IFN-γ production, and enhances anti-viral cytokine production in conjunction with TCR stimulation (50).

These studies demonstrate that early recruitment of non-specific CD8 T cells is a common feature of respiratory infections, including influenza virus infection; however, analysis of viral load and illness outcome measures were not always included, and additional studies are needed to determine the extent to which bystander-activated CD8 T cells can contribute to the early immune response and control of a pathogen and/or the risk of immunopathology due to excessive T cell responses. It is likely that these effects are specific to the infection model studied.

CROSS-REACTIVITY AND THE T CELL REPERTOIRE

It is estimated that a single TCR can recognize up to 10^6–10^7 foreign nonamer peptides and $\geq 10^8$ 11-mers (51). Degeneracy of the TCR repertoire facilitates heterologous immunity *via* cross-reactive T cells primed during primary infection and activated during a secondary, unrelated infection. This may be advantageous in defense against influenza infection where T cells primed from a previously circulating influenza virus strain may respond to a novel, antigenically distinct strain.

Acute, Sequential

With respect to influenza virus infection, there are two types of acute, sequential heterologous infection scenarios. First, heterosubtypic influenza immunity refers to the effect of pre-existing immunity to influenza virus strain 1 on the immune response to a secondary infection with influenza virus strain 2. Studies in mice show priming with H9N2 or H1N1 confers protection against challenge with H7N9; however, CD8 T cell immunodominance hierarchies, weight loss, and viral clearance varied by the priming influenza virus strain (52). Although the diversity of the CD8 TCR repertoire and presence of cross-reactive CD8 T cells was not specifically tested, this study highlights the significance infection history may have on cell mediated immunity and illness outcome.

The second scenario is the effect of pre-existing immunity against an acute, non-influenza virus infection on the quality or magnitude of the immune response to influenza virus infection, and *vice versa* (discussed more in the Section "Maintenance of Memory and Attrition"). The effects of heterologous infections on influenza immunity (either establishment or maintenance of memory) have not been sufficiently studied; however, studies with acute lymphocytic choriomeningitis virus (LCMV), VV, and Pichinde virus (PV) infections in the mouse model show that heterologous infection can induce activation of putatively cross-reactive CD8 T cells and alter LCMV-specific T cell immunodominance (53). However, the sequence of infection is important and heterologous protective immunity is not necessarily reciprocal, i.e., LCMV confers protection against VV, but VV does not protect against LCMV (53). Additional work in the acute LCMV–PV model shows PV-immune mice infected with LCMV exhibit an altered immunodominance hierarchy, such that the immunodominant epitope is NP_{205}, a normally subdominant epitope with high sequence similarity between the two viruses (six out of eight amino acids) (54). Alterations in immunodominance and the CD8 TCR repertoire may change the pool of memory CD8 T cells, and thus could impact secondary immune responses. Given that humans may encounter various infections between influenza infections, it is imperative to understand how heterologous infections may alter influenza-specific immunity and subsequently illness outcome.

Chronic Co-Infection

Altered activation of APCs (signal 2) and cytokine levels (signal 3) as a result of chronic co-infection may decrease the threshold of activation for inclusion into the T cell response, allowing for recruitment of lower avidity and/or cross-reactive T cells that may otherwise not be included in the response (**Figure 1**). Indeed, studies in humans show the naive T cell pool in chronic hepatitis C virus (cHCV)-infected subjects has more biased Vβ segment usage and decreased expression of CD5, a known T cell co-inhibitory receptor (55). Upon low dose anti-CD3 and anti-CD28 stimulation, compared with healthy donors, naive T cells from cHCV patients showed increased ERK phosphorylation, higher frequency of CD25 and CD69 expression, and activation induced cell death (55). In addition, work by Che et al. shows acute LCMV immune mice infected with MCMV exhibit increased MCMV viral titers and enhanced immunopathology. Conversely, prior MCMV infection conferred protection against acute LCMV infection *via* augmented CD8 T cell responses against a normally subdominant LCMV epitope, $L_{2062–2069}$, mediated *via* cross-reactivity with a MCMV epitope, $M57_{727–734}$ (56). Furthermore, studies show chronic infection with LCMV (IFN inducing), *Toxoplasma gondii* (*T. gondii*, IL-12 inducing), or *Heligmosomoides polygyrus* (*H. polygyrus*, Th2 inducing) lead to impaired development of immune memory and protective immunity (57). This study also found that there is a distinct transcriptional profile between HCMV-specific (A02*01 $pp65_{495}$, NLVPMVATV and B07*02 $pp65_{417}$, TPRVTGGGAM) memory CD8 T cells from healthy vs. persistent HCV infected humans (57). Gene set enrichment analysis shows the gene expression profile of HCMV-specific memory CD8 T cells in healthy donors and also found enriched in memory OT-I cells from naive mice, but not in chronic LCMV-infected mice (57). Collectively, these data demonstrate that chronic infections may: (1) Alter the basal status of naive CD8 T cells, such that they are hyperactivated upon stimulation. (2) Enhance CD8 T cell responses *via* inclusion of cross-reactive clones. (3) Alter the transcriptional profile of memory CD8 T cells. These results underscore the importance of studies which explore the extent to which this impacts influenza cell mediated immunity, whether the results are collectively beneficial or detrimental to the host, and to what degree the results depend on the infection model and/or sequence of infection.

A study of HCV-specific T cell responses confirmed cross-reactivity between epitopes from two unrelated viruses can occur in humans. Specifically, approximately 60% of HCV negative, healthy controls have functionally cytotoxic memory (CD45RO+) CD8 T cells specific for an immunodominant HCV epitope, A02*01 $NS3_{1073}$ (CVNGVCWTV) (58). The $NS3_{1073}$ epitope shares seven of the nine amino acids with the influenza virus A02*01 NA_{231} epitope (CVNGSCFTV), with conserved residues at positions key in binding to HLA A02*01. Indeed, HCV negative controls with NS3-specific CD8 T cells showed functional responses to NA_{231}

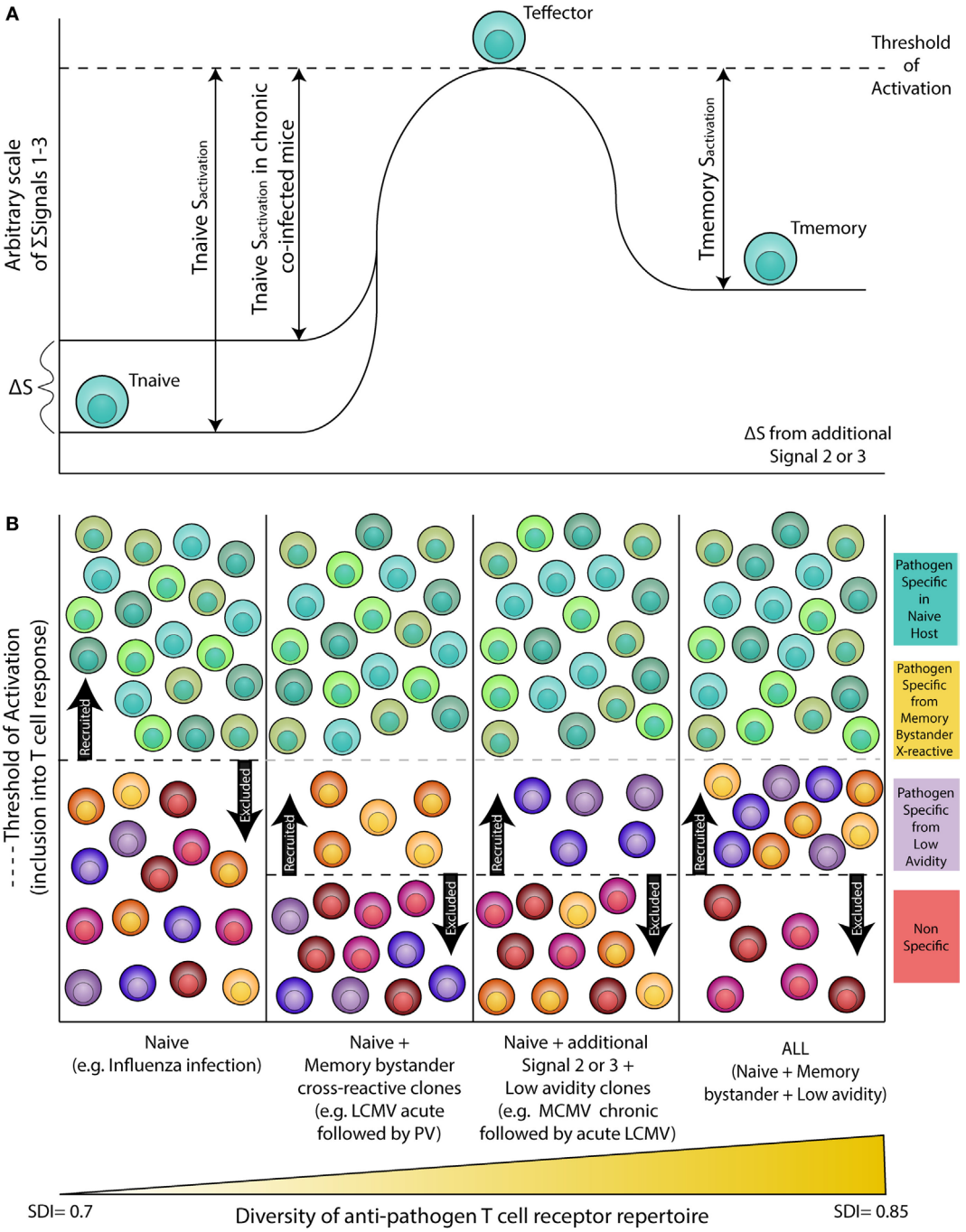


FIGURE 1 | Altered threshold of CD8 T cell activation during latent and/or smoldering chronic co-infection or from memory bystander activation. **(A)** Three signals contribute to the activation and recruitment of naive CD8 T cells into a specific anti-pathogen response: (1) the T cell receptor (TCR), (2) co-stimulation, and (3) cytokines in the microenvironment. If the sum of these signals exceeds the threshold of activation (Tnaive $s_{activation}$), the naive CD8 T cell will be recruited into the immune response. Chronic co-infection is known to augment (ΔS) basal cytokine levels and activation status of antigen presenting cells, thereby decreasing the signal required by naive CD8 T cells to exceed the threshold of activation (Tnaive $s_{activation}$ in chronic co-infected mice). In addition, memory CD8 T cells are transcriptionally poised for rapid recall of effector function upon reencounter of a pathogen, thus the amount of signal required for inclusion of a memory cell (Tmemory $s_{activation}$) is smaller than that of a naive T cell. **(B)** Most infectious disease studies are conducted in a naive host (far left panel), in which challenge with a pathogen will result in recruitment of pathogen-specific CD8 T cells from the naive T cell pool (blue-green T cells). In previously infected hosts, memory T cells have a lower threshold of activation and can contribute unique cross-reactive clones (yellow-orange T cells, center left panel). In addition, chronic co-infected hosts have altered signals 2 and 3 that permit the recruitment of lower avidity clones (purple T cells, center right panel). Addition of memory bystander and lower avidity clones increases the diversity of the anti-pathogen CD8 TCR repertoire. Most humans have at least one chronic infection and encounter multiple infections throughout a lifetime, thus a combination of all three scenarios (far right panel) more accurately depicts what occurs in life, and represents the most diverse population of pathogen-specific CD8 T cells.

and A02*01 $M1_{58}$ (a known immunodominant influenza epitope), suggesting prior influenza virus exposure (58). Furthermore, NS3-specific T cells could be induced by influenza infection in A02*01 transduced mice (58). Although the effects on control of influenza infection are not clear, it is possible that HCV infection will lead to a more narrow anti-influenza T cell response, due to expansion of HCV-specific CD8 T cells (both non cross-reactive and cross-reactive) in an attempt to limit HCV replication, and will ultimately result in poor influenza illness outcome.

Cross-reactive T cells against an influenza and heterologous virus epitope were also observed in EBV-associated infectious mononucleosis (IM) patients, where two out of eight patients had tetramer-defined cross-reactive CD8 T cells specific for EBV A02*01 $BMLF_{280–288}$ (GLCTLVAML) and IAV A02*01 $M1_{58–66}$ (GILGFVFTL), despite only 33% sequence homology (59). This result is further supported by sequencing of the CDR3β regions of Vβ17+ cells (from M1-specific cell lines), which shows that diversity of Jβ segment usage in the influenza M1-specific Vβ17+ TCR repertoire changed throughout IM disease progression (59). Furthermore, follow up studies show while cross-reactive and non cross-reactive BMLF-specific T cells utilize the Vβ14 segment, sequencing of the CDR3β loop of the cross-reactive clones showed 64% of them were not previously observed in the non cross-reactive repertoires (60). Analysis of TCR-α chain segment usage shows cross-reactive and non cross-reactive repertoires utilize Vα15, but cross-reactive repertoires utilize unique Jα families (60). In addition, BMLF-M1 cross-reactive T cells utilize a greater number of Vβ segments, as compared with non cross-reactive M1 or BMLF-specific T cells (60). These results have two important implications: (1) Cross-reactive T cells can increase TCR repertoire diversity through inclusion of unique TCRs that would not be seen in single epitope-specific repertoires. (2) Compared with analysis of segment usage alone, analysis of CDR regions provides more insight to the number of unique clones and diversity of the TCR repertoire. It is important to note that segments of interest when comparing usage between cross-reactive and non cross-reactive repertoires were dependent on the individual, with some segments common across individuals (possibly reflective of "public" clones), while others were unique to individuals (possibly reflective of "private" clones) (60). This study also utilized computer simulations of cross-reactive responses and the results suggest that cross-reactive responses between structurally similar epitopes, termed "near cross-reactive" responses, will lead to a more narrow TCR repertoire, whereas cross-reactive responses between structurally divergent epitopes, termed "far cross-reactive" responses, would lead to a broad TCR repertoire (60).

Reciprocal Effects of Influenza Infection

Numerous studies have shown that heterologous infections can impact influenza immunity and/or illness outcome, but the effect of influenza infection on the control of heterologous infection has not been sufficiently studied, and the extent to which these scenarios are dependent on cell mediated immunity is not clear. We previously mentioned prior infection with influenza virus protects mice from RSV induced eosinophilia and weight loss, and is thought to be mediated by non-specific bystander activation, cross-reactive T cells, immunological imprinting (and skewing toward Th1 response), and/or structural remodeling after the influenza infection (35). However, the aforementioned IM study observed BMLF-M1 cross-reactive T cells were enriched in severe IM cases, suggesting the magnitude of the anti-EBV CD8 T cell response is associated with disease severity (59). Indeed, later work in a study of acute infectious mononucleosis (AIM) shows IAV-M1 ($R^2 = 0.4$), EBV-BMLF ($R^2 = 0.3$), and cross-reactive IAV-M1 + EBV-BMLF ($R^2 = 0.6$) CD8 T cells are the only tetramer positive populations which directly correlate with AIM disease severity and are predictive of severe AIM in a relative-risk analysis (61). Other tetramer positive populations analyzed include CMV-pp65, EBV-BLRF1, cross-reactive IAV-M1 + EBV-BLRF1, and cross-reactive EBV-BLRF1 + EBV-BMLF. These results suggest influenza infection history and the frequency of influenza-EBV cross-reactive CD8 T cells in the influenza memory T cell pool may alter anti-EBV cell mediated immune responses during acute infection and subsequent illness outcome (61).

Taken together, these data demonstrate heterosubtypic influenza infections, acute sequential heterologous infections and chronic co-infections can alter anti-influenza memory CD8 T cell responses, with respect to kinetics, magnitude, quality, and repertoire diversity. Alterations in signals 2–3 of T cell priming may alter the threshold of activation and subsequently the pool of CD8 T cells included in the anti-influenza response. If this leads to a more diverse TCR repertoire, it could be beneficial in the face of a novel influenza virus strain; conversely, if it results in a narrow repertoire, it may lead to variant escape. Further studies are needed to assess the consequences of common heterologous infections on influenza virus immune memory; does cross-reactivity narrow or diversify the TCR repertoire, and to what extent are the effects context/infection dependent? In addition, what are the reciprocal effects of influenza infection on control of heterologous infections?

MAINTENANCE OF MEMORY AND ATTRITION

It is well established that the ability to mount an effective immune response declines with age, and annual surveillance of influenza infections show the elderly (>65 years old) are at risk for severe disease from influenza infection (62, 63). Immunosenescence is associated with poor immune responses and is a collective term used to describe various changes in the immune system that occur over time, such as thymic involution, dysregulated innate immune responses, inverted CD4/CD8 T cell ratios, decreased naive T cells coupled with increased memory T cells, and decreased diversity in the TCR repertoire (64–67). Alterations in naive/memory T cell frequencies, often observed in the elderly, are partially due to exposure to numerous pathogens throughout a lifetime, decreased thymic output, and the large expansion of CMV-specific CD8 T cells, termed "memory inflation"(68–70).

Altered immunological memory as a result of pathogen exposure throughout a lifetime coupled with memory inflation from chronic infections including CMV raise the concern that

heterologous infections could consume the limited space in the memory compartment, "crowding out" protective memory responses to influenza virus. However, recent studies show the CD8 T cell compartment can grow in size upon immunological experience (71). In this study, mice were infected with LCMV, followed by three heterologous prime-boost vaccines against vesicular stomatitis virus: New Jersey strain, recombinant VV expressing VSV nucleoprotein, and Indiana strain. The memory CD8 T cell population specific for the N protein of VSV induced by this vaccination strategy was equivalent in size to the entire memory CD8 T cell population in control mice (71). Furthermore, the total number of CD8 T cells increased following sequential vaccination, highlighting the importance of analyzing and reporting both cell number and frequency, as a decreased frequency may be the result of increased cell numbers of other CD8 T cells (71). Importantly, moderate attrition occurred in non cross-reactive, P14 LCMV-specific CD8 T cells in various tissues, ranging from 25.6 up to 33.4%. However, no attrition was observed in LCMV-specific CD8 T cells when mice were sequentially infected with VV, LM (intracellular bacteria), or *Plasmodium yoelii* (parasite) (71). These results suggest the CD8 T cell compartment size is flexible, though this flexibility appears to depend on specific features of the infecting pathogens, such as induced innate immune profiles, that we do not currently fully understand; however, with additional investigation and careful vaccine design, the magnitude of memory CD8 T cell attrition may be reduced following repeated heterologous challenge.

Two models have been suggested for memory T cell attrition: (1) Passive competition, in which new memory T cells compete with pre-existing memory T cells for space in limited survival niches and (2) Active deletion, wherein some mechanism, such as early type I IFN, induces apoptosis of pre-existing memory T cells, to make room for newly arising memory T cells (72). Attrition of T cells during the early phase of an acute immune response is mediated, at least in part, through type I IFN (IFN-α/β), followed by activation of initiator caspase 8 and effector caspase 3, ultimately leading to apoptosis (73, 74). Additional studies in mice show loss of T cells during early infection is age dependent, such that aged mice are less susceptible to T cell attrition mediated by type I IFN due to decreased expression of caspase 3, as compared with young mice (75). This is significant because thymic output decreases as age increases; a lower apoptotic potential of memory T cells in an aged host would minimize loss of this population when limited naive cells are available to replace them.

LCMV, PV, VV, and MCMV studies in mice demonstrate heterologous infections have prospective and/or retrospective effects on immune responses and memory: prospectively, prior infection with Virus A can lead to beneficial or detrimental effects during sequential infection with Virus B, and reciprocal (Virus B → Virus A) effects are not necessarily equal (53, 76). Retrospectively, infection with Virus B in a Virus A-immune host leads to the loss of bystander-activated T cells, including Virus A-specific memory T cells (74, 76–78). Studies of influenza and MHV68 show reciprocal effects in a challenge and vaccination model (79). Compared with an influenza only control group, mice infected with influenza virus (PR8 then X31) followed by MHV68 exhibit decreased frequencies and numbers of influenza (NP) specific memory CD8 T cells in the spleen, peripheral blood, lung, and bone marrow; however, no difference was observed in the mediastinal lymph node (MLN), cervical lymph node (CLN), or liver at day 100 (79). Compared with MHV68 only mice, mice infected with influenza followed by MHV68 showed decreased numbers of total MHV68 (p79)-specific memory T cells (sum of all anatomical locations tested); however, this is likely driven by a difference in the liver at day 100, because no difference was observed in the spleen, peripheral blood, MLN, CLN, bronchoalveolar lavage, lung, or bone marrow (79). Moreover, vaccination for MHV68 followed by influenza infection resulted in a higher number of influenza-specific CD8 T cells at day 14, but a lower number at day 200 (79). In each scenario, the reduction of antigen-specific T cells was approximately twofold or less, and likely would not result in a loss of protection following secondary challenge with influenza virus or challenge post priming for MHV68 (79). Latent MHV68 infection actually confers protection against influenza infection (described in more detail in the Section "Early Kinetics and Bystander Activation") (47).

These studies utilized MHV68, a murine model for chronic EBV infection in humans; however, most attrition studies are conducted in acute, sequential infection models. Given the high prevalence of chronic infections in humans, it is important to consider how they may alter observations of T cell attrition, and how this may vary by the category of chronic infection (latent, smoldering, or persistent). A study of PV, LCMV strain Armstrong (acute), and LCMV clone 13 (persistent chronic) sought to examine these differences and found more profound attrition of PV-specific memory T cells in chronic (clone 13) sequential vs. acute (Armstrong) sequential infection (80). Importantly, $CD44^{hi}$ memory CD8 T cells and non cross-reactive T cells were more susceptible to attrition (80). One possible explanation for the differences observed between acute and chronic LCMV, and important factors to consider when comparing chronic infection models, is the duration of antigen burden and the magnitude of subsequently induced pro-inflammatory cytokines. Studies in mouse models show out-of-sequence signal 3, such as the strong cytokine stimulatory conditions induced by sepsis and systemic immunotherapy, can lead to transient immunosuppression of T cells which is mediated, at least in part, through increased expression of suppressor of cytokine signaling, likely as a means to prevent extensive immunopathology from hyperactivation or autoimmunity (81, 82). For influenza infection, detrimental effects are likely to arise from persistent chronic co-infection, such as chronic LCMV; whereas smoldering MCMV- and latent MHV68-influenza co-infection models have shown enhanced CD8 T cell responses and improved illness outcome (21, 47).

Earlier studies have also suggested that memory T cells are maintained through cross-reactive stimulation, and recent work further supports this hypothesis (83, 84). In a mouse model of LM (wild type or recombinant expressing OVA) followed by *Mycobacterium bovis* (BCG, wild type or recombinant expressing OVA), mice infected with LM then BCG showed significant reduction in LM-specific CD4+ and CD8+ T cells; however, attrition did not occur in mice infected with LM-OVA followed by BCG-OVA (85). These data show heterologous bacterial, sequential infections also lead to T cell attrition, but attrition

can be prevented when T cells cross-react across pathogens (85). Utilizing PV and LCMV acute, sequential infection models, Brehm et al. (discussed in more detail in the Section "Cross-Reactivity and the T Cell Repertoire") has also demonstrated that cross-reactive NP_{205}-specific T cells are preferentially maintained at a higher frequency as compared with non cross-reactive T cells, whether LCMV is given to PV-immune mice or PV is given to LCMV-immune mice (54).

These data have shown that attrition of memory T cells is a common phenomenon in a variety of viral and bacterial infection models. In addition, non cross-reactive clones are more susceptible to attrition, whereas cross-reactive clones are maintained. Thus, it is possible that analysis utilizing known immunodominant epitopes may not account for increases in frequency and number of cross-reactive T cells specific for normally subdominant responses not analyzed. To provide evidence for this possibility, more studies are needed across a broader range of specificities, such as all activated CD8 T cells rather than just tetramer-specific cells, coupled with a more detailed analysis of paired αβ TCR sequences to look for the expansion of cross-reactive clones. In-depth analysis of TCR sequences can address additional questions, such as how different is the TCR repertoire pre and post attrition? Does attrition result in fewer numbers of each clone or complete loss of specific clones? If the latter, is there a selective mechanism, such as T cell phenotype (T_{CM} vs. T_{EM}), perhaps with varying transcription of genes involved in the apoptotic process, or divergent vs. canonical TCRs (with respect to epitope and pathogen)? In addition, to what extent do observations depend on features of the infection model, such as a restricted site of infection vs. systemic, Th1 vs. Th2 bias, low vs. high pathogenicity, and the extent to which CD8 T cells contribute to pathogen clearance? The answers to each of these questions have important implications for control of influenza virus infection and the development of prophylactic methods. For example, if attrition preferentially results in the loss of divergent TCRs or complete loss of specific clones, this may lead to influenza virus escape variants due to decreased diversity in the influenza-specific CD8 TCR repertoire. Understanding the factors that affect memory T cell attrition can be utilized in the development of more effective influenza vaccines that minimize the loss of pre-existing memory CD8 T cell populations, such as choosing adjuvants which limit type I IFN production or skew Th1/Th2 ratios to preferential levels.

RESIDENT MEMORY

Resident memory T cells (T_{RM}) reside in nonlymphoid tissues and serve as the first line of defense upon secondary infection. Histological examination of uninflamed human lung, counting CD3 positive cells in the lung parenchyma, suggests there are approximately 1×10^{10} resident T cells (86). Human influenza-specific lung CD8+ T_{RM} cells exhibit high proliferative capacity, are polyfunctional, and have a diverse paired TCR repertoire, likely a key attribute to prevent viral escape variants (87). Indeed, a comparison of human CD8+ T_{EM} in the blood and lung CD8+ T_{RM}^{CD103+} cells show distinct chemokine and adhesion molecule profiles, reflective of their corresponding localization (88). For example, lung T_{RM}^{CD103+} cells were enriched for CXCR3, CXCR6, and CCR5, but expressed low levels of CX3CR1, a chemokine receptor that mediates migration from circulation. Without *in vitro* stimulation, lung T_{RM}^{CD103+} cells also expressed higher mRNA, but lower protein, levels of effector molecules, such as granzyme B, IFN-γ, and TNF (88). Conversely, blood T_{EM} had higher granzyme B levels, despite lower mRNA levels. Lung T_{RM}^{CD103+} cells also expressed higher mRNA levels of chemokines and inhibitory molecules at a resting state, and expressed higher levels of IFN-γ upon stimulation with phorbol ester PMA and ionomycin (88). These results show lung T_{RM} are transcriptionally poised to mediate rapid effector responses and recruitment of additional leukocytes, while expression of co-inhibitory molecules may represent a means to prevent excessive immune responses and subsequent immunopathology (88).

Characteristic markers to identify T_{RM} cells in the epithelium include: (1) αE(CD103)β7 integrin, which interacts with E-cadherin in the epithelia and mediates retention in the lung and (2) C-type lectin CD69, an activation marker associated with recent antigen exposure, but also up-regulated in response to cytokines, such as type I IFN and TNF-α (89–91). It is important to note, not all T_{RM} express these markers and a comparison of T_{RM} at three anatomically distinct sites (lung, skin, gut) shows expression of 37 commonly up- or down-regulated genes, but 25–127 transcripts unique to T_{RM} from a given location (92, 93). An estimate of CD103+ CD8+ αβ T cells suggests this population comprises approximately 1/3 of the total T cells in the human lung and is primarily located above the basement membrane of small airways (94). Turner et al. has shown anti-influenza CD4+ and CD8+ T_{RM} cells localize to distinct niches in the lungs near airways and bronchovascular bundles, and were maintained independently of circulating and lymphoid T cell reservoirs (95). Analysis of human T_{RM} cells also shows compartmentalization depending on the site of viral infection; specifically, influenza-specific CD8 T cells were enriched in lung T_{RM} vs. the spleen, whereas similar frequencies of CMV-specific CD8 T cells were found in the lung and spleen (95). An independent study in humans also observed selective localization of antigen-specific CD8 T cells. The lungs were enriched for influenza-specific and RSV-specific CD8 T cells compared with blood, but CMV and EBV-specific CD8 T cells were equally distributed between both locations (96).

T_{RM} utilize a variety of methods to enhance the immune response and improve illness outcome, including, but not limited to: upregulation of adhesion molecules important for leukocyte migration, maturation of DCs, activation of NK cells, and rapid upregulation of broadly active anti-pathogen genes (97–100). In addition, lung T_{RM} have enhanced survival during influenza infection due to higher expression of anti-viral IFITM3, which confers protection against viral infection (101). Their location at the epithelial layer (which is the initial site of influenza virus infection) and rapid effector function make T_{RM} a key population in the initial control of influenza virus replication after secondary infection. Indeed, heterosubtypic influenza challenge models and studies of vaccines which induce lung CD8+ T_{RM} demonstrate the protective effects of this population during influenza infection; these results highlight the importance of understanding the

various factors which may affect the generation and maintenance of T_{RM} (102–107). To date, studies in influenza infection models have demonstrated the importance of four general factors in the generation and maintenance of T_{RM}: cytokines, co-stimulation, APC differentiation, and antigen (presence and avidity). Importantly, the extent to which a factor impacts T_{RM} may vary by anatomical location, and requires further investigation.

CD4+ T cells can enhance activation and differentiation of CD8+ T cells through cytokine production (Signal 3), and licensing of DCs, leading to enhanced expression of co-stimulatory molecules (e.g., 4-1BBL, Signal 2) which engage cognate receptors on CD8 T cells (e.g., 4-1BB). Indeed, studies of primary influenza infection in 4-1BB$^{+/+}$ and 4-1BB$^{-/-}$ mixed bone marrow chimeras show 4-1BB$^{-/-}$ CD8+ T cells have an impaired ability to develop into lung T_{RM}, indicating a role for this co-stimulatory pathway in the generation and/or maintenance of T_{RM} (108). Furthermore, studies in a CD4 depleted mouse model of influenza virus challenge show IFN-γ+ CD4+ T cells are important for the development of CD8+ T_{RM} (109). Unhelped CD8+ T cells have decreased T_{RM}, impaired ability to confer heterosubtypic protection, and show enhanced Tbet expression (109). Overexpression of Tbet in CD8 T cells abrogates transforming growth factor-beta (TGF-β) induced expression of CD103, *via* binding at the first intron in the *Itgae* (CD103) locus and blocking a putative Smad3 binding site (109). Importantly, these observations may also reflect differences in DC licensing, as CD4+ T cell depleted mice will also lack CD4 T cell:DC interactions. Indeed, previous work in a HSV mouse model has shown that unhelped DCs exhibit decreased proliferation and expression of IL-2R, IL-7R, and anti-apoptotic Bcl-2 (110). Moreover, anti-HSV CD8 T cell responses in MHC II knockout and/or CD4 T cell antibody depleted mice were impaired at peak primary (day 7 post infection) and memory (90–110 days post priming) time points (110).

In addition to activation and co-stimulatory molecule expression, human and mouse models have suggested DC phenotype is an important factor in the activation and differentiation of CD8 T cells. Human blood DCs can be divided into three categories based on cell surface marker expression; CD303+ (also known as BDCA-2) expressed on plasmacytoid DCs (pDCs), CD1c (also known as BDCA-1) expressed on most circulatory DCs, and CD141 expressed on a smaller subset (111). Studies of influenza vaccine responses in human tissues and humanized mice show both human CD141+ CD1c- and CD1c+ DCs are capable of activating and inducing expansion of influenza-specific memory CD8+ T cells; however, CD1c+ DCs have an enhanced capability to induce differentiation of CD103+ CD8+ T cells which express effector molecules, such as granzyme B, and are retained in the epithelium (111). Expansion of CD103+ CD8+ T cells by CD1c+ DCs was TGF-β dependent—a cytokine with a well established role in the regulation of CD103 expression on memory T cells (91, 93, 111). Mouse challenge models of influenza have also shown a differential capacity of respiratory dendritic cell (RDC) subsets to activate CD8 T cells during influenza infection and further support the human results. Specifically, CD103+ RDCs (CD103+ MHCII^{hi} CD11b$^{neg\text{-}hi}$) and CD11b^{hi} RDCs (CD103- MHCII^{hi} CD11b$^{med\text{-}hi}$) exhibit higher antigen uptake, increased expression of co-stimulatory and antigen presenting molecules (CD1d and MHC II), and decreased expression of inhibitory molecules (B7-H1) as compared with moRDCs (CD103- MHCII$^{neg\text{-}med}$ CD11b^{hi}) and pDCs (B220+ Gr-1 + MHCII^{lo}) (112). Furthermore, although CD103+ and CD11b^{hi} RDCs similarly activate CD4+ T cells in terms of proliferation and cytokine production, CD103+ DCs induced more robust activation of CD8 T cells, leading to increased proliferation and production of effector molecules (e.g., granzyme B and IFN-γ) (112). Additional studies support differential activation of CD8 T cells by CD103+ RDCs and CD11b^{hi} RDCs, as determined by homing/migration patterns, proliferation, and the expression of activation markers, effector molecules, and transcription factors important for T cell fate (113).

The respiratory tract can be divided into two sections; the upper respiratory tract (URT) is comprised of the nose, mouth, and pharynx, whereas the lower respiratory tract (LRT) includes the trachea, bronchi, and lungs. Studies by Pizzolla et al. show distinct requirements between T_{RM} in the upper vs. LRT. T_{RM} in the URT can develop independently of local cognate antigen and TGF-β, exhibit increased longevity over time, and are sufficient to prevent dissemination of influenza infection from the URT to the LRT, thereby protecting against severe disease (104). These results suggest the factors that affect the generation and/or maintenance of T_{RM} can vary by location. In addition, comparison of lung CD8+ T_{RM} against two immunodominant influenza epitopes, $NP_{366–374}/D^b$ and $PA_{224–233}/D^b$ shows distinct transcriptional profiles, suggesting a role for specific pMHC:TCR interaction parameters, such as avidity, in the differentiation of T_{RM}, in addition to presence of cognate antigen (114).

After the resolution of infection, lung T_{RM} wane over time and this loss is associated with impaired control of heterosubtypic influenza virus challenge. Recent work by Slütter et al. shows loss of lung-resident T_{RM} is due to apoptosis rather than migration, and T_{RM} maintenance in the short term depends on immigration of circulating CD8+ memory T cells. In addition, T_{EM} are precursors of lung T_{RM}, boosting of T_{EM} results in increased frequency of T_{RM} in lung, and TNF-α is important for recruitment and conversion of memory CD8 T cells to T_{RM} phenotype (115). Furthermore, late circulating memory CD8 T cells (>100 days post infection) have an inherently decreased capacity to form lung T_{RM}, as compared with early circulating memory cells (20–30 days post infection); this is reflected by differences in transcriptional profiles (115). Compared with early memory, late memory CD8 T cells have differential expression of three T_{RM} master regulators (Eomes, Blimp-1, and Hobit) and decreased expression of various genes important for T cell migration (115).

Heterologous infections have been shown to alter epitope immunodominance, CD4+ T cell Th1/Th2 ratio, cytokine levels (e.g., TNF-α), and augment expression of antigen presentation and co-stimulatory molecule expression on APCs (35, 47, 54, 116). Collectively, these data suggest sequential acute and chronic co-infection may alter factors known to play a key role in the establishment and maintenance of T_{RM}, and underscore the importance of studies which examine these relationships. Increased numbers or diversity of lung CD8+ T_{RM} could result in more rapid control of influenza virus and improved illness outcome, or excessive responses and immunopathology. However, vaccination studies suggest the former is more likely, and transcriptional analysis of T_{RM} suggests they have augmented expression of co-inhibitory

TABLE 2 | Overview of studies showing heterologous acute sequential or chronic co-infection can alter influenza virus immunity.

Infection type	Species	Priming strain (vaccine or infection)	Secondary strain (vaccine or infection)	Experimental design	Disease outcome	Effects on magnitude or quality of immune response	Reference
Acute, sequential (heterosubtypic)	C57BL/6	Influenza virus (H9N2 and H1N1)	Influenza virus (H7N9)	Mice were primed with 10^4 $TCID_{50}$ of H9N2 or 10^2 $TCID_{50}$ of H1N1 intranasally, and challenged with H7N9 intranasally at 10–12 weeks post priming	Mice primed with H9N2 or H1N1 showed increased survival, enhanced viral clearance, and decreased weight loss compared with naive mice	Prior infection with H9N2 or H1N1 leads to early and robust CD8 T cell responses during secondary infection with an antigenically distinct influenza virus, H7N9. Importantly, the magnitude of the priming-virus memory CD8 T cells was the best correlate of protection against H7N9 challenge. In addition, the degree of conferred protection (i.e., viral clearance, weight loss profile, and survival) and immunodominance of CD8 T cell responses varied by the priming-virus strain.	(52)
Acute, sequential	BALB/c	Influenza virus (X31 and H3N2)	Respiratory syncytial virus (RSV, A2 strain); recombinant Vaccinia virus (VV) expressing RSV attachment protein (rVV-G) or control β-galactosidase (rVV-β-gal)	Mice were infected with 3×10^6 PFU human RSV, 50 hemagglutinin (HA) units of X31, or HEp-2 lysate intranasally at day 0. Three to five weeks later, they were infected with 3×10^6 PFU rVV-G or rVV-β-gal *via* scarification, and 14 days later they were challenged with 3×10^6 PFU human RSV intranasally	Mice previously infected (Flu-G-RSV or RSV-G-RSV) exhibit decreased eosinophilia and weight loss (compared with Hep-2-G-RSV)	Flu-G-RSV mice had decreased TNF-α and IL-4 cytokine levels. In addition, 16.9 ± 2.7% of CD8 T cells recruited into the lung (post RSV infection) bound influenza tetramer, and 39.4 ± 3.8% expressed IFN-γ. Transfer of splenocytes at 21 or 149 days post influenza virus infection, followed by rVV-G and RSV challenge 14 days later also resulted in decreased eosinophilia	(35)
Acute, sequential	C57BL/6, B6.PI-*Thy1*^{a}/Cy (Thy1.1) and B6.SJL*ptprc*^{a}*pep3*^{b}/BoyJ (CD45.1)	Sendai virus (enders strain)	Influenza virus (X31 and H3N2)	Mice were infected with 250 EID_{50} Sendai virus and challenged 30–35 days later with 300 EID_{50} X31. For reverse order, mice were infected with 300 EID_{50} of X31 and challenged with 250 EID_{50} of Sendai virus 30–35 days post flu infection	Requires further investigation	Early infiltration and ~5× increase in cell number of Sendai virus specific CD8 T cells into the lungs of flu infected mice (day 4 post flu). Flu specific [nucleoprotein (NP) and polymerase acidic protein (PA)] CD8 T cell responses were unaltered, and early recruitment of memory cells was from migration of cells from other anatomical sites. When the sequence of infection was reversed, early infiltration and ~4× increase of flu specific memory CD8s occurred at day 4 post Sendai virus infection	(46)
Acute, sequential	Human	Influenza virus	Acute Epstein–Barr virus (EBV)	Influenza A virus-immune patients with acute EBV infection were recruited from the Umass Student Health Services. Age ranged from 18 to 23 years old. Acute EBV infection was confirmed using a monospot test and detection of anti-EBV capsid IgM in patient sera. Healthy volunteers were recruited from UMass Medical School. Age ranged from 24 to 50 years old	See Ref. (61)	Identified cross-reactive CD8 T cells specific for influenza A virus $M1_{58}$ and EBV-$BMLF1_{280}$, despite only 33% sequence homology	(59)

(*Continued*)

TABLE 2 | Continued

Infection type	Species	Priming strain (vaccine or infection)	Secondary strain (vaccine or infection)	Experimental design	Disease outcome	Effects on magnitude or quality of immune response	Reference
Acute, sequential	Human	Influenza virus	Acute EBV	Influenza A virus-immune patients with acute EBV infection were recruited from the Umass Student Health Services. Age ranged from 18 to 23 years old. Acute EBV infection was confirmed using a monospot test and detection of anti-EBV capsid IgM in patient sera. Healthy volunteers were recruited from UMass Medical School. Age ranged from 42 to 50 years old	See Ref. (61)	Cross-reactive M1 and BMLF-specific CD8 T cells utilize unique clones not found in single M1 or BMLF-specific CD8 T cell pools. Computer simulation suggests the effects of cross-reactivity on T cell receptor (TCR) repertoire diversity depends on the degree of similarity between epitopes. If epitopes are structurally similar, termed "near cross-reactive," responses will lead to a more narrow TCR repertoire, whereas cross-reactive responses between structurally divergent epitopes, termed "far cross-reactive," will lead to a broad TCR repertoire	(60)
Acute, sequential	Human	Influenza virus	Acute EBV	College students with symptoms of acute infectious mononucleosis (AIM) were recruited. Age ranged from 18 to 30 years old. Acute EBV infection was confirmed by a monospot test and the detection of anti-EBV capsid IgM in patient serum. Healthy, EBV-seropositive donors, age >18 years old, were used as controls	CD8 T cells cross-reactive for influenza and EBV epitopes may contribute to AIM disease severity by augmenting CD8 T cell responses.	IAV-M1+/EBV-BMLF+ double positive CD8 T cells had the strongest correlation with AIM disease severity and predict severe AIM in a relative-risk analysis. Single IAV-M1 and EBV-BMLF each had weaker associations and no other tetramer + population tested (2 two from CMV and EBV) were correlated with AIM severity	(61)
Acute, sequential and chronic co-infection	C57BL/6	Influenza virus	Acute and chronic murine herpesvirus 68 (MHV68)	Mice were primed with $10^{7.9}$ EID_{50} PR8 intraperitoneally, challenged with $10^{6.5}$ EID_{50} X31 intranasally, and later were or were not infected with 10^4 PFU of MHV68 intranasally. In another study, mice were infected intranasally with MHV68, boosted intraperitoneally with 5×10^7 PFU of recombinant VV expressing MHV86 p56 peptide AGPHNDMEI (Vacc-p56), and then were or were not challenged with X31 intranasally. Each infection was delivered 6 weeks apart	Requires further investigation	Co-infected mice (PR8-X31-MHV68) show attrition of influenza (NP) and MHV68 (p79)-specific memory CD8 T cells compared with their respective single infected counterparts at day 100. The presence and degree of attrition varies by anatomical site in both cases. In addition, mice primed with MHV68 then sequentially infected with influenza virus (MHV68-vacc-p56-X31) exhibit higher numbers of influenza-specific CD8 T cells at day 14, but a lower number at day 200	(79)
Chronic co-infection	BALB/c	MHV68 (WUMS strain)	Influenza virus (PR8 and H1N1)	Mice were infected with 4×10^4 PFU MHV68 or PBS (mock-infected) and 28, 60, or 120 days later were challenged with 1×10^4 PFU PR8	Latent MHV68 infection confers protection against influenza virus challenge, as determined by improved survival, enhanced influenza viral clearance, and decreased lung injury	Co-infected mice exhibit increased levels of IFN-γ, TNF-α, and IL-12p40, but decreased levels of neutrophil chemokines CXCL1 (KC) and CXCL2 (MIP-2α). Co-infected mice also had increased numbers of CD69+ CD4+ (day 0 and 4) and CD8+ T (day 0, 4, and 6) cells in the lung, decreased neutrophils (day 8), and enhanced activation of alveolar macrophages. Adoptive transfer of macrophages from co-infected mice was sufficient to confer protection against influenza virus challenge	(47)

(*Continued*)

TABLE 2 | Continued

Infection type	Species	Priming strain (vaccine or infection)	Secondary strain (vaccine or infection)	Experimental design	Disease outcome	Effects on magnitude or quality of immune response	Reference
Chronic co-infection	C57BL/6 and IFN-γ KO	Cytomegalovirus (Smith)	Influenza virus (X31 and H3N2)	Mice were infected with 4×10^4 PFU of murine cytomegalovirus (MCMV) intraperitoneally. Co-infected mice were also infected with 1×10^6 EID_{50} of X31 at 5 weeks (early latency), 12 weeks (established latency), or 9 months (long-standing latency) post MCMV infection	MCMV co-infection confers protection against influenza virus challenge, but protection wanes with time and is not observed in long-standing latent MCMV infection	Mice co-infected with influenza virus at 5 or 12 weeks post MCMV infection exhibit higher influenza-specific CD8 T cell responses against three immunodominant influenza epitopes (polymerase basic protein 1, PA, and NP) and decreased influenza virus titers	(21)
Chronic co-infection	Human	Cytomegalovirus	Fluzone vaccine (each dose contains 15 μg of HA from H1N1, H3N2, and B strains)	Ninety-one healthy donors were enrolled at the Stanford-LPCH Vaccine Program in fall of 2008 (89 completed the study). The validation cohort consisted of 77 individuals who returned in fall of 2009, plus 37 subjects vaccinated in another study between 2010 and 2011 flu seasons	Young CMV seropositive subjects had higher antibody response to the Fluzone vaccine at 28 days and 1 year post vaccination, as compared with young, CMV seronegative subjects. However, no difference was observed in the elderly, based on CMV serostatus	Young CMV+ subjects have a broadly activated immune system compared with their CMV- counterparts. This is reflected by augmented expression of genes important for immune activation (e.g., antigen processing and presentation, NK cell cytotoxicity), increased levels of IL-13, IFN-γ, and CD8+ pSTAT1/3 in response to IL-6 stimulation. This study also found, elderly CMV+ subjects showed lower responses to IL-6, compared with young CMV+	(21)
Chronic co-infection	Human	Human cytomegalovirus (HCMV) and/or EBV	Influenza virus	Samples were collected from 50 patients [20 hepatitis B virus (HBV), 12 influenza, 12 dengue, 3 adenovirus, 3 fevers with unknown etiology] and 5 healthy volunteers attending clinics in Singapore or Italy. Diagnosis was confirmed utilizing appropriate methods for the infection within 5 days of selection. For example, influenza infections were confirmed with isolation of influenza A virus from nasal swabs	Requires further investigation	Acute infection with influenza, HBV, dengue, and adenovirus induce activation (CD38+ HLA-DR+) of HCMV- and EBV-specific CD8 T cells. In one influenza patient, 1/4 of activated CD8 T cells at onset were HCMV-specific, and influenza-specific CD8 T cells could not be detected until day 5. In addition, IL-15 preferentially activates memory CD8 T cells specific for chronic infections, augments anti-viral cytokine production with TCR stimulation, and is sufficient for spontaneous IFN-γ production	(50)

molecules, which likely act to minimize immunopathology (88, 102, 104–107). Alternatively, decreased numbers or diversity of lung CD8+ T_{RM} may lead to variant escape, impaired control of influenza virus growth, and immunopathology due to prolonged inflammatory immune responses.

CONCLUSION

Memory T cells specific for conserved influenza epitopes can be advantageous during an outbreak of an antigenically distinct viral strain. Indeed, several vaccine studies in mouse models show vaccines which boost memory CD8 T cells can confer protection during heterosubtypic challenge. However, while a robust memory CD8 T cell population can lead to a rapid immune response to secondary infection and protect against severe disease, detrimental effects are possible. Cross-reactive clones may dominate the response to heterologous challenge and lead to a narrowed TCR repertoire. As previously discussed, heterologous infection with LCMV and PV alters the immunodominance of the CD8 T cell response, such that a normally subdominant NP_{205} epitope becomes immunodominant (54). Additional work in this model shows the TCR repertoire is narrowed to the extent that it results in an escape variant (117). Furthermore, studies in various infectious disease models have demonstrated an excessively large CD8 T cell response may lead to enhanced immunopathology and more severe illness outcome (56, 61, 118, 119).

Collectively, the studies reviewed here demonstrate that various heterologous infection scenarios can alter the primary T cell response and establishment or maintenance of the memory CD8 T cell pool. Specifically, heterologous acute sequential and chronic co-infection may result in: early migration of CD8 T cells to the site of infection, altered immunodominance hierarchies, inclusion in the anti-viral response of cross-reactive and/or lower avidity CD8 T cells, changes in cytokine levels, altered transcriptional profiles of naive and memory T cells, and changes in the differentiation of APCs (**Table 2**). Augmented activation of APCs (i.e., higher expression of co-stimulatory molecules) and/or production of cytokines (by APCs or CD4s) important for CD8 T cell differentiation or bystander activation of memory CD8 T cells would lower the threshold of activation for inclusion into the anti-influenza T cell response, thereby increasing the diversity of the TCR repertoire by facilitating the inclusion of low avidity and/or cross-reactive clones that would otherwise not be present. Thus, additional studies which more accurately reflect pathogen encounters in humans are needed to optimize vaccination strategies by inducing diverse, local memory CD8 T cell responses, while minimizing loss due to attrition and preventing immunopathology due to excessive pro-inflammatory responses.

AUTHOR CONTRIBUTIONS

PT assisted in planning and editing the review. AS planned and wrote the review, and designed and created the figure.

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LFA-1 in T Cell Migration and Differentiation

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Maintenance of homeostatic immune surveillance and development of effective adaptive immune responses require precise regulation of spatial and temporal lymphocyte trafficking throughout the body to ensure pathogen clearance and memory generation. Dysregulation of lymphocyte activation and migration can lead to impaired adaptive immunity, recurrent infections, and an array of autoimmune diseases and chronic inflammation. Central to the recruitment of T cells, integrins are cell surface receptors that regulate adhesion, signal transduction, and migration. With 24 integrin pairs having been discovered to date, integrins are defined not only by the composition of the heterodimeric pair but by cell-type specific expression and their ligands. Furthermore, integrins not only facilitate adhesion but also induce intracellular signaling and have recently been uncovered as mechanosensors providing additional complexity to the signaling pathways. Among several leukocyte-specific integrins, lymphocyte function-associated antigen-1 (LFA-1 or $\alpha_L\beta_2$; CD11a/CD18) is a key T cell integrin, which plays a major role in regulating T cell activation and migration. Adhesion to LFA-1's ligand, intracellular adhesion receptor 1 (ICAM-1) facilitates firm endothelium adhesion, prolonged contact with antigen-presenting cells, and binding to target cells for killing. While the downstream signaling pathways utilized by LFA-1 are vastly conserved they allow for highly disparate responses. Here, we summarize the roles of LFA-1 and ongoing studies to better understand its functions and regulation.

Keywords: T cell migration, integrins, LFA-1, chemokines, T cell activation, T cell differentiation

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INTRODUCTION

Precise spatial and temporal regulation of adhesion and de-adhesion is critical for immune cell development, localization, and pathogen clearance. LFA-1 is a key T cell integrin that plays a critical role in the regulation of these functions. With this highly diverse set of roles, it is unsurprising that LFA-1 has been implicated in numerous autoimmune and inflammatory conditions including inflammatory bowel disease, psoriasis, diabetes, and arthritis (1, 2). Intriguingly, intracellular signals dictating LFA-1 activation are highly conserved between migration, T cell activation, and cytolytic activity suggesting that any alterations in the signaling may cause substantial biological consequences during the host immune responses. This review will discuss our current understanding of the role of LFA-1 during T cell activation, effector functions, and memory formation.

LFA-1 STRUCTURE

LFA-1 is composed of α- and β- subunits that together form a heterodimer expressed at the cell surface. These subunits include long extracellular domains, a single transmembrane domain, and short cytoplasmic tails (**Figure 1**). The extension of LFA-1, which resembles a switchblade-like motion,

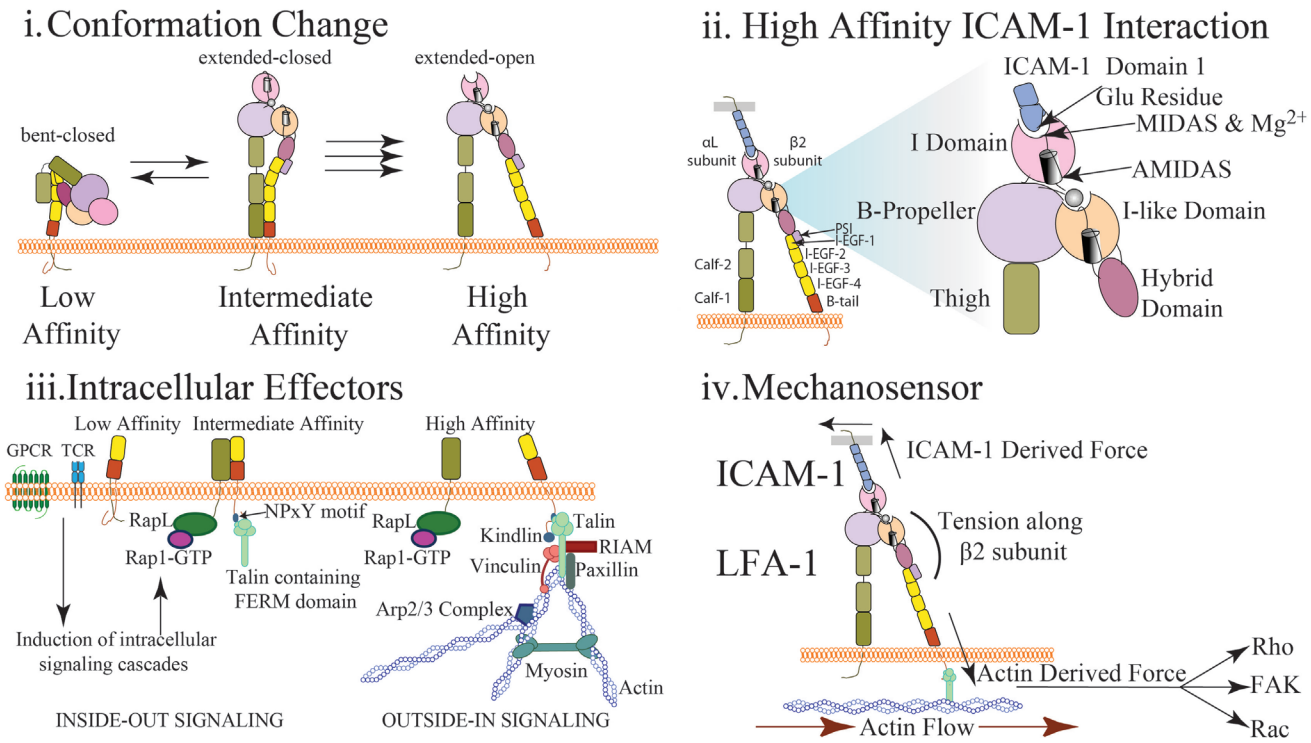

FIGURE 1 | Multidimensional regulation of LFA-1 affinity (i) LFA-1 affinity regulation is mediated *via* conformational changes to LFA-1 structure. In the low affinity state, the bent conformation causes the ligand binding αI domain to be inaccessible to interact with ICAM-1. In the intermediate affinity state, the extracellular leg domains are straightened allowing for low affinity interactions between LFA-1 and ICAM-1. Importantly, the intracellular domains of LFA-1 are not separated and the metal ion-dependent adhesion site (MIDAS) binding site closed. In the high affinity state, disruption of the salt bridge between the α and β cytosolic tails results in conformational shift along the β subunit and αI domain resulting in high affinity LFA-1 *via* the opening of the ligand-binding site. (ii) The αI domain contains the MIDAS within which resides Mg^{2+} coordinating the binding pocket. This site interacts with the glutamic acid-34 in Domain 1 of ICAM-1 to facilitate binding. This induces a shift in the α7 helix to cause the hybrid domain to swing out further stabilizing LFA-1 structure. Additional sites surrounding the MIDAS such as AMIDAS and ligand-induced metal-binding site assist with coordination of the binding pocket and stabilization of high affinity LFA-1. (iii) Upon T cell receptor or chemokine activation, RAP1-GTP recruits a number of factors including RAPL that interact with the α subunit of LFA-1 to induce integrin activation (inside-out signaling). Similarly, talin cleavage allows the FERM domain to interact with the NPxY motif of the cytosolic tail on the β subunit. This interaction causes a dissociation of the salt bridge inducing cytosolic tail separation. Kindlin also contains a FERM domain and interacts with the β subunit to further stabilize high affinity LFA-1. Molecules such as RIAM, talin, paxillin, and vinculin may interact with the cytosolic tails to recruit additional effector molecules and promote a scaffold to interact with actin and reinforce LFA-1 activity (outside-in signaling). Arp2/3 will promote continued actin filament growth while MyH9 functions to provide stress on actin fibers to induce LFA-1 dissociation from ligand. (iv) Interaction of LFA-1 with ICAM-1 and β-actin allows for force driven responses along the β subunit. Transmission of force (arrows) along the β-subunit has been measured in pN scale with actin flow functioning to direct the orientation and location of LFA-1 both at the immunological synapse and during cell migration. Stabilization of the integrin in the high affinity conformation *via* force generation requires adhesion to both the cytoskeleton and ICAM-1. The stiffness of the substrate may also alter the level of force generated thus altering the signaling response. Downstream signal is induced *via* outside in signaling generated through the stabilization of high affinity LFA-1. Phosphorylation of focal adhesion kinase through force generation may play a role in mediating cell adhesion and proliferation. Rho signaling, and thus actin polymerization, may also be altered through changes in force generation resulting in changes in actin dynamics and cell migration. Induction of Rac and CDC42 may also be altered through force generation resulting in changes to cell proliferation and survival.

requires substantial changes to the conformation of both subunits (3). LFA-1 has at least three separate conformational states that are conferred by movement of the extracellular and cytosolic domains: (1) closed/bent, where the integrin has low affinity for ligand and is conformationally unavailable to bind ligand; (2) closed/extended, where the integrin is extended allowing for interaction with ligand, but the cytosolic tails remain closed; and (3) open/extended, where the integrin has high affinity for its ligand and the cytosolic tails have separated (**Figure 1**i) (3–5).

Roughly half of all integrins, including LFA-1, express an αI domain, which is critical for ligand binding and contains a metal ion-dependent adhesion site (MIDAS) that binds Mg^{2+} to coordinate the binding pocket (**Figure 1**ii) (3). ICAM-1 will directly bind with the LFA-1 MIDAS and Mg^{2+} by interacting with a glutamic acid residue found in Domain 1 of ICAM-1 (**Figure 1**ii) (6). LFA-1 is also capable of binding ICAM-2 and ICAM-3 albeit with much lower affinity. Two additional sites, ligand-induced metal-binding site (LIMBS) and adjacent to MIDAS (ADMIDAS), have been shown to regulate cytosolic tail separation and reduce cell spreading, respectively (7–9). Two domains on the α subunit leg, calf-1 and calf-2, have a Ca^{2+} binding loop that is critical to the subunit bending. The β subunit consists of the I-like domain, which is homologous to the αI domain and plays a key role in determining specificity. The hybrid domain,

which connects the upper and lower portions of the β subunit, is critical for conformation change. The β subunit leg consists of a plexin/semaphorin/integrin domain that is connected to the βI domain and four integrin epidermal growth factor-like (I-EGF) domains, which facilitate β leg bending (**Figure 1**i–ii) (3, 5, 8–10).

To facilitate conformation changes, a number of structural modifications occur utilizing the abovementioned integrin components. As the α7-helix is displaced downward during integrin activation, the hybrid domain swings outward leading to separation of the extracellular legs (6). However, due to the flexibility of the extracellular β subunit leg, this separation facilitates only extension of the leg and does not separate the cytosolic tails. Complete cytosolic tail separation requires intracellular effectors, such as talin (**Figure 1**iii) and stabilization through interaction with actin and ICAM-1 (**Figure 1**iv). Fluorescent resonance energy transfer (FRET) studies have demonstrated cytosolic tails closely interact under resting conditions, and upon activation, the tails separate and induce an integrin conformational change to high affinity (11, 12). This process through which intracellular signals induce integrin activation is termed "inside-out" signaling. Similarly, integrin adhesion to ligand induces intracellular responses in a process termed "outside-in" signaling. High affinity integrin conformation can be achieved *via* either inside-out or outside-in signaling.

LFA-1 AND T CELL MIGRATION

Extravasation of immune cells from the vasculature is a highly organized process composed of five steps: (1) weak tethering/rolling, (2) firm adhesion, (3) crawling, (4) paracellular or transcellular migration through the endothelium, and (5) migration through the basement membrane (13, 14). Initial adhesion of cells to the endothelium is mediated by the expression of both selectins and addressins (15–19). Upon tethering, cells begin to decrease velocity and roll along the endothelium forming transient low-affinity interactions. Subsequently, immune cells may firmly adhere to the endothelium or be released back into circulation. Firm adhesion to the endothelium is induced by chemokine stimulations and high-affinity integrin activation *via* inside out signaling (20–23). The integrin conformation change can lead to as much as a 10,000-fold affinity increase of LFA-1 to its ligand ICAM-1 (3, 21). ICAM-1 is expressed at low levels and is highly upregulated upon damage or inflammation (24, 25). After secretion, chemokines bind glycosaminoglycan proteins, such as CD44 and syndecans, expressed on the endothelium (26, 27) to subsequently be presented to immune cells. Chemokine and LFA-1 engagement initiate a series of intracellular cascades that induce T cell polarization inducing subsequent migration (28, 29).

LFA-1-mediated adhesion plays essential roles in both naïve and activated T cells extravasation into the lymph node and tissue, respectively (30). Shulman et al. demonstrated that this process can be mediated *via* intra-endothelial chemokine stores at the immune/endothelial cell synapse and surprisingly, that T cell adhesion to the endothelium appeared independent of chemokine stimulation (31, 32). With single-dye tracking and conformation specific antibodies, Bakker et al. demonstrate that in resting monocytes that roughly 5% of LFA-1 is in nanoclusters that are in a fully active state and bound to the cytoskeleton, suggesting that low levels of LFA-1 activation may occur independently of chemokine stimuli (33). It is tempting to speculate from these data that integrin engagement may occur independent of chemokine stimulation suggesting that chemokine may simply act to reinforce integrin engagement and facilitate transmigration. While high affinity interactions are necessary for adhesion, constitutive expression of LFA-1 with the intermediate affinity I-domain led to impaired crawling and diapadesis through limiting detachment at the rear of the cell (34). This demonstrates a need for dynamic regulation of LFA-1 affinity. Indeed, studies have demonstrated that defects in LFA-1 adhesion and activation (changes in conformation, clustering, or cell signaling) through therapeutic treatments and genetic abnormalities can cause deficient immune response and autoimmunity (2, 35–37).

While LFA-1 plays a crucial role in adhesion to the vasculature, very late antigen-4 (VLA-4; α4β1) has also been implicated in T cell extravasation (14, 38–41). In tissues such as the CNS, LFA-1 inhibition is not sufficient to inhibit cell extravasation as cells also utilize VLA-4 (42, 43). However, in other tissues such as the retina, T cell infiltration was LFA-1 dependent and vastly VLA-4 independent suggesting that tissue-specificity plays a critical role in determining integrin-mediated extravasation (44). Indeed, in a bronchial epithelial model, inhibition of LFA-1 lead to a 75% decrease in infiltration whereas inhibition of ICAM-1 or ICAM-2 alone lead to a 50% reduction. However, when both ICAM-1 and ICAM-2 were blocked a 70% reduction in infiltration was observed (45). Additionally, immune cells may alter their integrin dependency. Glatigny et al. demonstrated in T regulatory (Treg) cells that when VLA-4 expression was blocked, cells were still capable of migration utilizing LFA-1 (46). These data demonstrates a highly diverse set of mechanisms, which dictate immune cell infiltration (43).

After firm adhesion, T cells crawl along the endothelium searching for a site to migrate across the endothelial monolayer into the tissue (47). Migration along the endothelium is primarily dictated by chemokine signals that direct cell chemotaxis *via* chemotactic gradients. However, LFA-1-ICAM-1 interaction also plays a critical role in regulating the direction of T cell migration in the blood vessel. T cells and hematopoietic stem/progenitor cells can migrate against shear flow on ICAM-1, while T cells mainly migrate with the flow on VCAM-1 (42, 48, 49). Diapadesis can occur through either paracellular (in between the junction of two cells) or transcellular (through a single endothelial cell) mechanisms. While most cells (~90%) are thought to utilize paracellular migration, the processes dictating para- vs. trans- cellular migration are still being investigated (50, 51). Evidence suggests that ICAM-1 density, monolayer organization (e.g., tricellular junctions), and previous cell diapadesis at the same location ("hot spots") are all implicated in dictating this phenomenon (50, 52–55). Additionally, cells have been found to survey the tissue with LFA-1/Wiskott–Aldrich Syndrome Protein-dependent protrusions, which have been observed to penetrate as deep as 600 nm into the endothelial cell to promote transcellular migration (56).

Additionally, endothelial cells may facilitate diapadesis *via* the lateral border recycling compartment (LBRC) (54, 57). Mediating changes in endothelial cell junctions to facilitate extravasation,

the LBRC has been shown to be essential for transcellular migration. Additionally, numerous diapadesis regulators, including cadherins, CD99, junction adhesion molecules, and platelet endothelial cell adhesion molecules are thought to determine and mediate cell extravasation and are thus a topic of continued research (58–60). We demonstrated that uropod elongation acts as the final step in leukocyte transendothelial migration. During this elongation, $CD18^+$ microparticles are left behind which may play a role in either prevention or promotion of leukocyte transmigration at the site (55).

Upon successful migration across the monolayer, T cells utilize a number of β4 and β1 integrins to migrate along the basement membrane composed primarily of collagen and laminin. Intriguingly, the basement membrane appears to be lost directly at the transmigration site (61, 62). While the exact reason for this loss remains under investigation, it is believed to help control cell migration, mediate cell death at the infiltration site, and maintain tissue structure. Following this last step of migration across the endothelial barrier, immune cells continue to migrate through the tissue interstitium to exert their effector function (63).

During T cell migration, LFA-1 engagement is primarily utilized in two-dimensional spaces. One study found that neither LFA-1 nor α_4 integrins support stable adhesions of naive T cells to neighboring T cells, DCs or stroma in the lymph node T cell zones (64). Indeed, studies have shown that in dense, 3D tissues dendritic cells are capable of migrating without integrin adhesion though actin-polymerization ("flowing") and myosin II-based contractions ("squeezing") (65). However, T cells appear to require integrin-mediated adhesion in the tissue microenvironments under inflammation (66). Therefore, it is likely that integrin-mediated T cell migration is determined by integrin/ligand expression and tissue density in which the cell is found. It is also important to note that, while LFA-1-independent migration occurs under depleting conditions within the lymph node, the outcome of the immune response may be altered. Additional studies demonstrated that LFA-1 blockade abolished directed, high velocity migration of naïve T cells (67), suggesting that LFA-1-mediated migration is important for the speed, and the pattern of T cell migration in the lymph node.

In addition to the conformational changes in LFA-1 (see Chapter 1), precise and dynamic regulation of LFA-1 recycling is a key to ensure efficient T cell migration and adapt to the constantly changing microenvironment (68, 69). While LFA-1 recycling occurs constitutively, as much as 75% of all integrins are internalized and redistributed within 15 min of cell migration onset (69). While integrins can utilize both clathrin-dependent and -independent pathways (69), LFA-1-dependent endocytosis is primarily mediated through clathrin-independent, cholesterol-sensitive mechanisms (68) and play an important role both in mediating cell migration and cell polarization through the partitioning of molecules near LFA-1 (68–70). Upon internalization, a series of steps determine the fate of intracellular LFA-1 (degradation vs. recycling). Integrins are generally thought to return to the cell surface through *via* either a direct exocytosis route (*via* Rab4 or Rab5) or the perinuclear recycling compartment route (*via* Rab11) (69, 71, 72). LFA-1 containing vesicles during T-antigen-presenting cell (APC) interactions have also been found to require Arf6 + Rab22 (72). LFA-1 can specifically utilize a Rab13-dependent pathway through which Rab13 associates with Mst1 to facilitate increased integrin activation, as evidenced by increased LFA-1 clustering and cell migration (69, 73). Additionally, LFA-1 recycling requires Rap2-expressing vesicles which work synergistically with Rab13 to mediate new adhesion, while Rap2 facilitates continuous adhesion (74). T cell activation may also be impeded through defects in LFA-1 recycling as demonstrated with Rab13 or Rab27 inhibition (73, 75, 76). However, determining the precise roles of each recycling mechanism during LFA-1-mediated cell migration and activation requires more investigation.

LFA-1 AND T CELL ACTIVATION

T cell activation is a highly organized process that can be divided into distinct events described by both T cell motility in the lymph node and T-APC interactions. The first phase is highly dynamic, as immune cells migrate along fibroblastic reticular cells (FRCs) while scanning for antigens. The second phase is defined by low motility and high interaction between the T cells and APCs. The final stage is characterized by regaining a high level of motility, effector differentiation, and proliferation (77).

T cells migrate along FRCs to sample antigens *via* random interactions with antigen-bearing dendritic cells. These short, transient interactions, termed kinapses, are characterized by reduced T cell migration (78). Interaction with APC is determined *via* affinity between the peptide-MHC (pMHC) complex and the T cell receptor (TCR). As these interactions are low affinity (1–100 nM Kd), they are highly specific for only 1×10^5–1×10^6 TCRs (79, 80). Upon recognition of cognate antigen, a T cell ceases migration and induces surface and cytosolic changes in both the APC and the T cell. This phenomenon is defined as the second phase of T cell activation and is characterized by a loss of motility, extended T cell/APC interaction and the formation of an immunological synapse (IS) (77, 78). These changes include a loss of polarity in the T cell and surface molecule reorganization referred to as supramolecular activation clusters (SMACs). The IS can be segregated into three distinct portions similar to a bullseye pattern. The center of the bullseye, aptly named center-SMAC, contains the TCR/CD3 complex and the co-stimulatory molecules CD28 and PKCθ. The outermost ring, or the distal ring (d-SMAC), is composed of the phosphatase CD45, and the center ring, or peripheral SMAC (p-SMAC) is composed of LFA-1 and talin (81). Surprisingly, it has recently been observed that under basal conditions, LFA-1 can be found in clusters prebound to the cytoskeleton suggesting this may help to induce initial cell adhesion and formation of nanoclusters upon TCR engagement (33, 82). Interactions at the IS can be defined by the duration of the interaction. Synapse formation (>5 min) and kinapse formation (<5 min) are determined by the affinity of the TCR/pMHC complex and the activation phase of the T cell. Evidence suggests that both types of interaction are required for complete T cell activation to balance activation signals imparted from the APC with differentiation and proliferation. Additionally, dysregulation of this process may lead to tolerance or autoimmunity through altering the balance of activation signals the T cell receives or

through altering the affinity threshold required to engage TCR/MHC complexes (78).

Interaction between TCR and pMHC induces a phosphorylation-mediated signaling cascade. This process activates LFA-1 at the IS leading to firm adhesion required for effective T cell activation. LFA-1 may be activated through a number of pathways that convert the small GTPase RAP1 to its active GTP bound form (83–85). Importantly, RAP1 activation is dependent on the context of activation (TCR vs. chemokine) with data demonstrating that RhoH functions as a rheostat with differential localization within the cell leading to alterations in LFA-1 activation (86). Many of these pathways require the LAT signalosome, including the PLCγ1 activation of DAG regulated-guanine exchange factor (GEF)1 (CalDAG-GEF), which directly acts on RAP1. Additionally the adaptor protein CRKII can interact with C3G, a GEF, to activate RAP1. CRKII-C3G can also be activated *via* the WASP family member 2 (WAVE2) actin related protein 2/3 complex (ARP2/3)-ABL complex. Upon conversion of RAP1 from the inactive GDP-bound form to the active GTP-bound form, it interacts with ADAP and the adaptor SKAP55 to recruit RAP1 to the plasma membrane (87). This allows for recruitment of the RAP1 effectors RAPL, Mst1, PDK, and RIAM to induce integrin activation. The RAP1/Mst1/Kindlin3 complex can be formed through inside-out integrin activation signals, but may also play a role in stabilizing outside in signaling (88–91). This process is essential for LFA-1 recycling, as RAP1 complexes play key roles in delivering LFA-1 vesicles to the cell surface (78, 81, 92, 93).

Antigen-presenting cell expression of ICAM-1 is also required for effective T cell activation. ICAM-1 expression on DC's plays a crucial role in mediating T cell migration and localization throughout the lymph node (94–96). Additionally, ICAM-1 clustering on APC is essential for effective LFA-1 engagement and T cell activation (97, 98). Interestingly, LFA-1 has also been directly implicated by $CD8^+$ DCs to facilitate T cell activation *via* acting as a scavenger receptor to collect antigen from antigen-bearing DCs (99).

Disregulation of LFA-1 expression can lead to changes in T cell activation and differentiation (28, 100–102). Stable engagement of LFA-1/ICAM-1 is required to re-enforce many pathways for complete T cell differentiation. LFA-1 crosstalk with Notch signaling has been shown to induce IFNγ production and re-enforce Th1 cell functions suggesting that LFA-1 engagement with tissue resident APCs will further strengthen T cell differentiation (103). Similarly, Tregs have been shown to require talin and LFA-1 activation to induce IL-2Rα upregulation, which is required for Treg function (104, 105). Additionally, we recently demonstrated that an intracellular pool of LFA-1 is relocalized to the cell surface upon initial T/APC interactions and plays a key role in T cell memory development (76).

LFA-1 AND T CELL CYTOTOXIC RESPONSE

Fully differentiated effector $CD8^+$ T cells kill infected/transformed target cells *via* caspase-dependent apoptosis (106, 107). Upon recognition of a target antigen *via* TCR/pMHC complex formation, $CD8^+$ T cell activates LFA-1 and binds to ICAM-1 expressed on the target cell. Important functions of LFA-1 during the cytotoxic response was demonstrated with LFA-1 blockers that inhibited target cell killing (107–109). Cytotoxic T cells form short, LFA-1 driven, kinapse-like interactions with infected cells to facilitate killing with interactions for as little as 10 min inducing apoptosis in target cells (110–112). Similar to the IS formed during T cell activation, TCR-derived (113–115), inside-out signals induce translocation of the microtubule organizing center (MTOC) toward the contact between IS and the target cell. The MTOC and microtubules interact with LFA-1 within the p-SMAC to define the ring shape structure observed during perforin/granzyme release (116–118). Organization of LFA-1 at the p-SMAC is thought to act as a "gasket" to prevent cytolytic granules from escaping (119). Furthermore, studies have indicated that the stability and strength of the LFA-1-mediated contact is critical for effective cytolytic activity (118). Additionally, CTLA-4 signaling has been shown to lead to RAP1-mediated increase in LFA-1 binding (120, 121). While the purpose is unclear, it is possible that this mediates low affinity TCR interactions or in cases of high stimulation, induces greater cell polarization and migration. Finally, galectin coating of the tumor infiltrating leukocytes (TILs) surfaces has led to decreased LFA-1 recruitment and activation at the IS and reduced cytokine secretion, further supporting a key role for LFA-1 in mediating TIL cytotoxic function (109).

LFA-1 AND T CELL MEMORY DEVELOPMENT

As described above, LFA-1 plays a critical role in facilitating naïve T cell activation and differentiation through T cell-APC contacts. Indeed, defects in LFA-1/ICAM-1 interactions have been shown to lead to impairment of memory formation (122, 123). Interestingly, ICAM-1 expression on T cells is important for T cell clustering during transient T–T interactions that provide additional cues for proliferation (124). ICAM-1 deficient cells resulted in higher levels of IFN-γ and granzyme B as well as, increased KLRG-1 expression suggesting increased differentiation toward short-lived effector cells (97). LFA-1 expression is required for the retention of tissue resident memory cells in the hepatic sinusoids and facilitate their migratory patterns unlike skin resident memory cells that are largely sessile (125). Additionally, numerous allograft rejection model studies have demonstrated both in mice and non-human primates that LFA-1 blockades reduce or delay memory cell mediated rejection (126–129). While the precise mechanism of this appears to be a combination of infiltration, proliferation, and cytokine secretion, this demonstrates LFA-1's multifaceted role in memory T cell function. Finally, it is important to note that this is not exclusive to LFA-1 as studies have suggested the integrin VLA-1 is required for memory T cell development in the airway against influenza infection (130, 131).

Intriguingly, several studies have shown that LFA-1, along with CD8, CD3, and CD43, are asymmetrically inherited into the two daughter (proximal vs. distal) cells upon initial T cell division (132, 133). This has been further studied with fate-associated factors such as IL-2Rα, IFNγR, and T-bet all being asymmetrically distributed (133). Importantly, we demonstrated that unequal inheritance of LFA-1 in daughter cells caused

differences in migration, T-APC contacts, tissue retention, and effector functions (76). This study further demonstrated that the unequal inheritance of LFA-1 plays an important role in memory generation and differentiation of T cells into both effector and memory subsets.

LFA-1 AS A MECHANOSENSOR

Recent evidence suggests a role for LFA-1 as a mechanosensor affecting cell signaling and integrin activation through the force generated by ligand binding. For example, it has been proposed that integrin adhesion occurs through a "catch bond" in which as the tension at the ligand binding site increases, the affinity also increases (134, 135). Recent work with a FRET-based LFA-1 tension sensor demonstrated significant tension across the β subunit of LFA-1 upon ICAM-1 binding resulting in the stabilization of active LFA-1 (136). Importantly, force generation requires adhesion to both ICAM-1 and actin to result in increased integrin constraint, cell tension, and cell signaling (33, 98, 136, 137). Actin remodeling *via* WASP-dependent mechanisms is essential for the assembly and distribution of high affinity LFA-1 clusters at the IS. The control of LFA-1 topology at the IS by WASP is related to both the control of the $CD4^+$ T cell stop signal (138) and the $CD8^+$ T cell cytotoxic activity (139). Further work has demonstrated that retrograde actin flow dictates LFA-1 orientation when bound to ICAM-1 on migrating Jurkat T (137). Similarly, TAGLN2 dependent inhibition of actin depolymerization is required for stable IS (140). Unsurprisingly, this force generation has been shown to be an important part of IS formation, cell cytotoxicity, and may modulate cell migration (42, 136, 141–143). As described in reviews by Sun et al. and Gauthier et al., the role of integrin tensile force requires continued study to fully elucidate its functions on in T cell activation, migration, and cytotoxicity (144, 145).

CONCLUSION

As this review has shown, LFA-1 functions are extremely varied but play a critical role in facilitating effective immune responses. Our understanding of the mechanisms through which LFA-1 mediates immune cell function have grown exponentially, yet many questions still remain. As our understanding grows, our capability to modulate this highly adaptive molecule to better treat autoimmunity, cancer, and allograft rejection will continue to improve.

AUTHOR CONTRIBUTIONS

BW and MK both researched the topic, wrote and edited the manuscript, and made the figure for this manuscript.

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Pathogen Recognition by CD4 Effectors Drives Key Effector and Most Memory Cell Generation Against Respiratory Virus

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Although much is known about the mechanisms by which pathogen recognition drives the initiation of T cell responses, including those to respiratory viruses, the role of pathogen recognition in fate decisions of T cells once they have become effectors remains poorly defined. Here, we review our recent studies that suggest that the generation of CD4 T cell memory is determined by recognition of virus at an effector "checkpoint." We propose this is also true of more highly differentiated tissue-restricted effector cells, including cytotoxic "ThCTL" in the site of infection and T_{FH} in secondary lymphoid organs. We point out that ThCTL are key contributors to direct viral clearance and T_{FH} to effective Ab response, suggesting that the most protective immunity to influenza, and by analogy to other respiratory viruses, requires prolonged exposure to antigen and to infection-associated signals. We point out that many vaccines used today do not provide such prolonged signals and suggest this contributes to their limited effectiveness. We also discuss how aging impacts effective CD4 T cell responses and how new insights about the response of aged naive CD4 T cells and B cells might hold implications for effective vaccine design for both the young and aged against respiratory viruses.

Keywords: CD4 T cells, ThCTL, T_{FH}, immune memory, influenza, virus, pathogen recognition

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THE GENERATION OF MEMORY CD4 T CELL RESPONSES TO INFLUENZA

An effective immune response to respiratory pathogens, such as Influenza A virus (IAV), requires coordination between the innate and adaptive immune system. Host defense to influenza infection begins when lung airway epithelial cells, dendritic cells (DC), and alveolar macrophages alert the host to the presence of virus through the activation of pattern recognition receptors (PRR) (1). This triggers the production of inflammatory cytokines, which activates antigen-presenting cells (APC). APC migrate to secondary lymphoid organs where they present antigen to T and B cells as soon as 2 days postinfection (dpi) (2). In a primary infection, CD4 and CD8 effectors are the most important for clearing virus, with Ab arising later. Effectors soon contract and a cohort become memory T cells that can persist in the host long-term and provide durable protection against the same virus.

Thereafter, re-encounter with the same virus goes largely unnoticed since neutralizing long-lived Ab, produced by long-lived plasma cells (LLPC), rapidly clears virus. However, when virus surface proteins hemagglutinin (HA) and neuraminidase (NA) can mutate sufficiently and escape

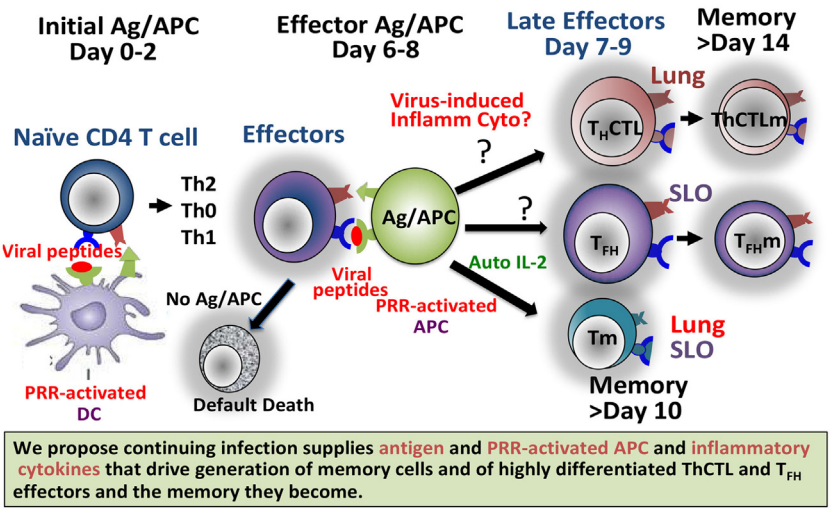

FIGURE 1 | Continuing infection, producing abundant antigen and pattern recognition receptor (PRR)-activated APC, and we propose inflammatory cytokines, drives generation of memory cells and of highly differentiated ThCTL and T_{FH} effectors and the memory they become. Providing such signals in a vaccine setting should result in longer-lasting more protective immunity. Known and likely roles for viral Ag and for PRR in the response to Influenza A virus are depicted.

recognition by the Ab. IAV also escape when new strain with a distinct HA and NA subtype (heterosubtypic) develops or by a high dose of exposure that overcomes Ab. When virus is incompletely cleared, memory T cells and B cells are induced to mount a secondary response, making secondary effectors that can protect against a broader range of influenza strains (3). Most studies have focused on T cell activation during priming, but it is unclear what continued signals are needed into the effector phase to generate memory. Although live virus is cleared between 10 and 13 dpi, viral antigen presentation is detected for up to 21 dpi (4), indicating Ag recognition could continue to drive T cell responses.

Indeed antigen presentation through 8 dpi drives effector CD8 T cells to expand and promotes memory formation (5, 6). We found that antigen presentation throughout the effector response was required for almost all CD4 T cell memory generated by IAV infection (7, 8). Antigen recognition induced autocrine CD4 effector IL-2 production that acted at days 6–8 postinfection at this "checkpoint" to downregulate Bim and to upregulate the IL-7Ra. These signals along with antigen recognition, promoted survival of CD4 effectors and drove their transition to memory (7, 8). Infection was not required at this stage, since antigen-pulsed APC were sufficient to support optimal memory CD4 T cell generation and maintenance that was able to protect a naïve host from an otherwise lethal influenza challenge (7). Antigen engagement at the memory checkpoint specifically upregulated the expression of CD25, Bcl-6, and phosphorylated STAT3 in the effector CD4 T cells (8). CD25 expression is needed for efficient IL-2R signaling, so that IL-2 can prevent effector cell death (9). Costimulation through CD27 is also important at this juncture (7) for the most efficient formation of CD4 memory, just as it is during priming (10). Correspondingly, a subset of DCs that express CD70 post-priming has also been identified which correlate with $CD27^{+}CD8$ effector expansion late into the IAV response (11).

The need for cognate interaction of effectors with Ag–APC during this phase potentially allows for the influence by Ag dose/avidity and by co-stimulatory ligands on the APC dependent on pathogen-associated molecular patterns (PAMPs). This ensures that strong memory develops only when there is no longer infection at the early effector phase, when necessary because of unresolved threats (**Figure 1**). We suggest that defining pathways that drive optimum CD4 and CD8 T cell memory will inform vaccine design to produce more protective T cell memory.

TISSUE-RESTRICTED EFFECTOR RESPONSES PLAY KEY ROLES IN ANTIVIRAL IMMUNITY

CD4 T cells responding to viral infections differentiate into a heterogeneous population of effector cells, with subsets that mediate viral clearance through distinct mechanisms including those that are cytokine-mediated (Th1, Th2, Th17) and that kill infected cells (ThCTL), as well as indirect mechanisms of help for B cell differentiation (T_{FH}) in germinal centers (GC) (3, 9, 12). Protection against lethal challenge with influenza can be mediated by synergy of CD4 T effectors acting *via* generation of Ab and by perforin-mediated lysis due to ThCTL (13).

It is well established that T_{FH} are required for GC formation and that they support GC B cell responses leading to isotype switching, somatic hypermutation, and selection of high affinity with the production of LLPC and memory B cells (14). As they recognize antigen on GC B cells, the T_{FH} in turn

become GC-T_{FH} and later can become memory T_{FH} (15, 16). The LLPC are responsible for producing the long-lived Ab that provides most rapid protection against viral infection. Thus, the tissue-restricted recognition of Ag by T_{FH} is critical to GC-T_{FH} development, for subsequent T_{FH} memory and for long-lived Ab-mediated protection. The critical T_{FH} functions and their transition to memory have been well reviewed (14–16). Understanding what signals from cognate interaction of T_{FH} and GCB are needed and how long they are needed is crucial to maximizing immunity.

Another tissue-restricted CD4 effector population is the cytotoxic CD4 T cells, ThCTL (17, 18). ThCTL lyse target cells by the same mechanisms utilized by cytotoxic CD8 T cells, including the perforin-mediated pathway. ThCTL are generated during influenza and many other viral infections (19). After IAV infection they are found in the lung and bronchoalveolar lavage (19, 20), suggesting they are restricted to the sites of infection. Two markers of ThCTL have been found: CRTAM and NKG2C/E. MHC Class I-restricted T cell associated molecule (CRTAM) can be expressed by IAV-specific CD4 T cells upon activation. $CRTAM^+$ CD4 cells, upregulate expression of granzymes and display peptide specific cytotoxicity, indicating these cells are ThCTL (21). While CRTAM marks cytotoxic CD4 T cells, its expression requires *ex vivo* activation, making tracking ThCTL more difficult.

NKG2A/C/E is a family of C type lectin receptors found on NK cells and CD8 T cells (22, 23). NKG2C/E, however, can be found on CD4 T cells directly *ex vivo* from infected mouse lungs (20). Isolating CD4 T cells based on their expression of NKG2C/E, indicates that cytotoxic activity *ex vivo*, as well as increased expression of perforin and granzyme is found only in the NKG2C/E-expressing effectors. NKG2C/E^{pos} ThCTL, readily degranulate and secrete high levels of IFNg when they recognize Ag, consistent with their potent antiviral activity (20). Although effector CD4 T cells infiltrate the tissues throughout the body (24), ThCTL are only found in the lung and we find that they upregulate a gene expression program consistent with tissue residency (20). Further, Ab to CD4 injected intravenously to assess relative accessibility at the effector stage, indicates most ThCTL are inaccessible, suggesting they are within the lung tissue (20). This location in sites of infection is important as local lung-resident memory CD4 T cells promote better protection than splenic memory CD4 cells against IAV infection (25).

Due to their protective capacity, in both mice and humans infected with influenza (13, 26, 27), it is important to identify the factors that support generation of ThCTL. ThCTL do not require standard polarizing cytokines during initial activation, but do depend on IL-2 (17). We find that CD4 T cells need Blimp-1 as a transcription factor to enable the cytotoxic phenotype. Loss of Blimp-1 in CD4 T cells leads to reduced ThCTL in the lungs (20), and, in others studies, reduced ability to prevent weight loss after influenza (26). Bcl-6, a transcriptional repressor of Blimp-1, is in contrast critical for T_{FH} generation (28–30), underlining the diverse transcriptional regulation of ThCTL versus T_{FH}. We note that the restriction of ThCTL to sites of infection and their late appearance (20) suggest that they, like T_{FH}, may require late cognate interactions to direct their final differentiation (**Figure 1**).

INNATE RESPONSES REGULATE THE GENERATION OF THE ANTIVIRAL CD4 T CELL RESPONSE

The innate immune system plays a critical role in initiating the cascade of the adaptive responses to viruses. Toll-, RIG-I- and Nod-like receptors (TLR, RLR, or NLR) on the initial infected cells are triggered by PAMP to signal the first wave of inflammatory cytokine production. These in turn activate various APC to effectively initiate T cell priming. IAV infection activates innate pathways primarily *via* the PRR, such as TLR3, TLR7, RIG-I, and Nlrp3. Previous reviews have discussed the role of PRR-signaling in IAV infection in detail (31, 32). However, little is known beyond the role of the PRR pathways acting early in priming and initiation of T cell responses (31–33). Here, we discuss recent advances in the fields of CD4 memory, T_{FH} and ThCTL that are making it clear that PRR pathways play a more global role in shaping CD4 effector and memory responses.

We find that the generation of CD4 memory does not require infection during the effector phase, as activated APC presenting peptides are sufficient to drive *in vivo* generated CD4 effectors to become memory in uninfected mice (8). However, the role of PRR pathways in generating specialized CD4 memory responses such as T_{FH} memory, ThCTL memory and CD4 T_{RM} is only now being studied. The gamma-chain cytokines, IL-2, IL-7, and IL-15, each play important roles in T cell memory (7, 34, 35). PRR pathways can induce high levels of IL-15 during infection (35). While we know that constitutive levels of IL-15 and IL-7 maintain homeostatic memory CD8 and CD4 T cell populations (35), the role of high levels of PRR-signaling such as that leading to type I IFN and other proinflammatory cytokines during active infection remains unclear. We find that IL-15 is required during the effector phase of the CD4 response for the generation of an IL-2 independent CD4 T_{RM} population (36). Another study indicates local inflammatory cues from IL-12 and IFNβ, made by intestinal macrophages, are involved in differentiation and persistence of the CD8 T_{RM} populations (37). While multiple PRR pathways promote T cell memory, the causal relationship between the memory subsets and the specific PRR has only been shown indirectly through the requirement for innate cytokines.

The TLR9 adjuvant CpG acts on TLR9/MyD88 signaling in both DC and B cells to promote optimum T_{FH} generation (37). Type I IFN produced by PRR-signaling, also promotes the T_{FH} genetic program. Type I IFNα/β induced Bcl-6, CXCR5, and PD-1 expression in CD4 T cells that were activated *in vitro* by Ab to CD3 and CD28, by a STAT-1-dependent pathway (38). During persistent LCMV infection, chronic Type I IFN supports T_{FH} formation (39). On the other hand, another study showed that the absence of STAT3 in CD4 T cells during an acute LCMV infection resulted in reduced T_{FH} differentiation caused by increased Type I IFN production, thus suggesting that Type I IFN indirectly inhibited T_{FH} differentiation (40). Type I IFNs have widely variable effects on T cell activation depending on whether they are present before activation, during activation or after activation and depending on whether they are present acutely or chronically (41). Thus, it is likely that the disparate

impacts of Type I IFN on T_{FH} generation in the studies above are due to differences in model systems used.

CD4 T cells stimulated *in vitro* in the presence of IFNα and IL-2 have increased cytotoxic potential (26). CD4 T cells in the lungs of IFNAR-deficient mice also expressed lower levels of Granzyme B and perforin, suggesting ThCTL generation also may depend on inflammatory cytokines. In support of roles for inflammation acting on T effectors, gene profiling following IAV infection indicate innate cytokines including Type I and Type III IFNs, are produced well into the effector phase of the response (42). Since innate inflammatory cytokines, such as Type I IFN, can be pathological (43) it makes sense that their production requires PRR activation, present only during continuing infection and that they could, then help further drive the differentiation of specialized effector CD4 T cells to ensure pathogen clearance.

Innate pathways also play an indirect role in promoting continued antigen presentation by inducing inflammation and enhancing APC activation. A recent study showed that type 1 IFN has differential effects on APC subsets by inducing different levels of co-stimulatory ligands, such as various TNFSF ligands, CD80, and CD86 on inflammatory APCs versus classical DCs, which control priming of CD4 T cells (44). Expression of many of these TNFR ligands such as OX-40, CD27, and 4-1BBL have been correlated with protective antiviral T cell responses (45, 46).

PATHOGEN RECOGNITION PROMOTES T CELL AND B CELL IMMUNITY

In addition to the roles of PRR-stimulation in driving the final differentiation of T_{FH}, discussed above, we recently showed that PRR-activated DC, acting as APC for CD4 T cells, could greatly augment both T_{FH} generation, generation of IL-21 secreting CD4 effectors. This indirectly enhances generation of GCB cells and most importantly of long-lived Ab in response to inactivated IAV vaccine (47). Vaccines, especially traditional inactivated or subunit flu vaccines often result in weak, barely protective Ab levels and low frequency T cell memory, especially when given to the elderly (48). In contrast, live infection is able to generate very long-lived and effective immunity of all types, suggesting that vaccines will need to be modified to provide the key signals inherent in live infection in order to improve efficacy. We found that when peptide-pulsed DC were activated by TLR-signaling and used to prime CD4 T cells specific for IAV, high levels of inflammatory cytokine were produced during the CD4:Ag/APC interaction. This enhanced otherwise weak helper T and B cell responses by an IL-6 dependent mechanism. This effect was apparent even in an unmanipulated mouse, where TLR-activated DC, presenting inactivated IAV, enhanced B cell IgG Ab response over several months (47). PRR activation in conjunction with BcR-triggering also can directly activate B cells and promote their differentiation to AbSC. This synergistic signaling drives T-independent B cell responses, but can also be involved in conventional B cell (B2) responses (48, 49). Thus, the presence of PRR signals acts at multiple levels in DC and in B cells to promote the initial B cell response, and we propose also later to promote T_{FH}, LLPC, and memory B cells.

AGED T AND B CELL RESPONSES BECOME MORE DEPENDENT ON PRR PATHWAYS

Animals undergo a dramatic shift in their immune responses as they age. The number of naive T cells (50) and naive follicular B cells (FOB) decreases and remaining naive cells are less responsive (51). So while previously established T and B memory cells often remain largely functional, responses to new viruses or strains of viruses not previously encountered is compromised. Our studies show that aged CD4 T cells have reduced responsiveness to IL-6 (47, 52) leading to weak generation of helper subsets. Providing high Ag doses on PRR-activated APC, markedly enhanced aged naive CD4 helper responses and indirectly improved B cell responses by a mechanism dependent on APC produced IL-6 (47). This study provided clear evidence that PRR signals acting on APC were responsible for improved CD4 helper responses and supported the concept that greater PAMP signaling is required to prime naive T cells as they age.

We wondered if the reduced responses by B cells in the aged could also be enhanced by high Ag dose and PAMP stimulation. We also noted that T-independent B cell responses, do not require the age-compromised T helper cells, might provide immune protection in the aged. A subset of B cells termed "age associated B cells" (ABC) that lack both CD23 and CD21 increase with age in mice (53). Studies showed that some of the ABC can develop from antigen-experienced FOB suggesting they are memory-like B cells (54). However, we find that most ABC in unimmunized mice express only surface IgD and IgM and lack the expression of key B cell co-stimulatory and activation markers, suggesting they are naive (51). These $sIgD^+$ ABC transferred to RAG-deficient mice, were driven by IAV infection into AbSC specific for IAV (51), indicating a T-independent pathway to Ab production. ABC identified in models of autoimmunity are dependent on BcR, TLR-7, and TLR-9 stimulation (53, 55), and memory ABC populations are implicated in mediating autoimmunity (56, 57). Thus like CD4 T cells, the ABC may require strong PRR-signaling to respond effectively. We propose that pathogens such as respiratory viruses can induce $sIgD^+$ ABC to generate protective Ab responses even in the aged (51). We are investigating whether the sIgD + ABC response to IAV infection can indeed contribute to a protective Ab response and if this is dependent on high levels of Ag and PAMP signals.

SPECULATIONS AND IMPLICATIONS FOR VACCINES

It has become increasingly clear that vaccines, especially inactivated, subunit, or recombinant protein vaccines, often result in weak, barely protective Ab levels and low frequency T cell memory, and they are even less effective in the elderly (48, 58). On the other hand, LAIV are able to elicit superior lung specific responses and T_{RM} (59). Various studies have indeed shown that that local antigen presentation and local inflammation are key factors that drive tissue-resident memory (60, 61). Live infection

is able to generate effective immunity of all types that often lasts several decades or more, suggesting that modifications to vaccines to provide key signals inherent in live infection should be able to improve vaccine efficacy (62). Here, we suggest that strong signals like those from replicating pathogens, including high doses of antigen persisting to the peak of the immune response, along with high levels of PAMPs acting on innate and B cells, are necessary to optimally trigger T cell effector and memory generation and B cell response. Replicating viruses that facilitate high and continued levels of antigen presentation and inflammation, in addition to tissue localization of the immune response induced by intranasal administration, may be key factors that determine superior vaccine induced protection. Though further definition of these pathways is needed to best inform vaccine strategies, we propose that they can ultimately be used to drive generation of increased immunity against respiratory viruses.

AUTHOR CONTRIBUTIONS

SS, PD, MJ, AV, OK-U, and JX wrote various sections in the manuscript. SS and PD conceptualized and organized the article and coordinated author contributions. MJ finalized the manuscript for submission. All other authors did experimental work that influenced the thinking behind the article. All authors contributed to manuscript revision and have read and approved the submitted version.

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REVIEW
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Tissue-Resident Memory CD8+ T Cells: From Phenotype to Function

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Tissue-resident memory CD8+ T cells are an important first line of defense from infection in peripheral non-lymphoid tissues, such as the mucosal tissues of the respiratory, digestive, and urogenital tracts. This memory T cell subset is established late during resolution of primary infection of those tissues, has a distinct genetic signature, and is often defined by the cell surface expression of CD69, CD103, CD49a, and CD44 in both mouse and human studies. The stimuli that program or imprint the unique gene expression and cell surface phenotypes on T_{RM} are beginning to be defined, but much work remains to be done. It is not clear, for example, when and where the T_{RM} precursors receive these signals, and there is evidence that supports imprinting in both the lymph node and the peripheral tissue sites. In most studies, expression of CD49a, CD103, and CD69 on T cells in the tissues appears relatively late in the response, suggesting there are precise environmental cues that are not present at the height of the acute response. CD49a and CD103 are not merely biomarkers of T_{RM}, they confer substrate specificities for cell adhesion to collagen and E-cadherin, respectively. Yet, little attention has been paid to how expression affects the positioning of T_{RM} in the peripheral tissues. CD103 and CD49a are not mutually exclusive, and not always co-expressed, although whether they can compensate for one another is unknown. In fact, they may define different subsets of T_{RM} in certain tissues. For instance, while CD49a+CD8+ memory T cells can be found in almost all peripheral tissues, CD103 appears to be more restricted. In this review, we discuss the evidence for how these hallmarks of T_{RM} affect positioning of T cells in peripheral sites, how CD49a and CD103 differ in expression and function, and why they are important for immune protection conferred by T_{RM} in mucosal tissues such as the respiratory tract.

Keywords: T cells, memory, tissue distribution, integrins, infection, immunity, cellular

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TISSUE-RESIDENT MEMORY CELLS

Tissue-resident memory CD8+ T cells (T_{RM}) are a distinct memory population that is generated and persists at the site of infection or vaccination (1–3). Upon exposure to the same or similar diseases, T_{RM} cells provide a first line of adaptive cellular defense and are indispensible in lethal challenge models (2, 4). Since CD8+ T cells mount responses aimed primarily at more conserved internal epitopes of pathogens, eliciting a T_{RM} response may provide increased protection compared with B-cell targeted vaccines, which some pathogens can escape by mutating antigenic sites (5). While research efforts have started to shed light on some of the requisite signals for maintenance of the T_{RM} population, less is understood in regard to the positioning within the tissue that is

required for the development of this subset and responses of T_{RM} cells after rechallenge. Here, we will provide an overview of the gross anatomical locations in which T_{RM} cells form, the known interactions that facilitate their development, and the consequences of reactivation, as well as the voids that remain in our understanding of this critical population. Closing these knowledge gaps will allow us to harness the full potential of T_{RM} and elicit improved protective responses to vaccination.

LOCATIONS

T_{RM} cells are most well known for their roles in barrier sites such as mucosal tissue and skin. While the populations in different tissues display heterogeneity in requirements for migration to the site of infection, the developmental program, maintenance within the tissue, and their surface phenotype, they ultimately perform similar critical functions. In the mucosa, T_{RM} cells have been identified in the intestines, female reproductive tract, salivary glands, tonsils, and lungs (1, 6–10). In addition, T_{RM} or T_{RM}-like cells have been described in a number of other non-mucosal sites, including lymphoid and peripheral tissues such as the thymus, spleen, lymph nodes, liver, kidneys, pancreas, heart, skin, and brain (6, 7, 11–14).

In most mucosal and barrier tissues, the T_{RM} population primarily homes to areas of epithelial surfaces, which represent the common site of infections in these tissues. Influenza, for example, infects the airway epithelium and Herpes simplex viruses, the epithelial cells of the skin or cervix (15–18). In other instances, however, such as with cytomegalovirus, the virus infects epithelial cells within an organ (salivary glands) rather than at the barrier surface (19, 20). As one might anticipate based on the name, salivary glands are home to extensive acinar epithelial-rich glandular structures that provide a location for the infection to persist, but also act as an ideal habitat for the formation and maintenance of T_{RM} cells (8). This opens up the possibility that internal glandular structures within other peripheral tissues could be similar targets for T_{RM} and warrants further investigation.

In the lung, $CD8^+$ T_{RM} cells preferentially localize to regions rich in collagen IV (ColIV), while $CD4^+$ T cells are biased to areas abundant in collagen I (ColI) (21). This coincides with the relative expression of the integrins specific for those collagen types, CD49a and CD49b, respectively (21). In non-mucosal organs, the positioning within the tissue is less well understood. As discussed later in this review, understanding the function of the surface receptors expressed on T_{RM} cells as well as their responses to different chemokine cues may better inform their roles and localization within non-mucosal tissues.

TRANSCRIPTIONAL REGULATION OF T_{RM}

Recent work has focused on identifying the transcriptional regulators of T_{RM}; however, these studies suggest that the specific requirements may vary between mice and humans. In mice, $CD8^+$ T_{RM} cells from a number of tissues express elevated levels of HOBIT (homolog of BLIMP-1 in T cells) compared with peripheral T cells (22). In conjunction with BLIMP-1, HOBIT promotes maintenance of the T_{RM} population through repression of genes associated with tissue egress. In addition, in mouse $CD8^+$ T_{RM}, it has been shown that T-bet and Eomes, two T-box transcription factors, needed to be down regulated for the cell to receive signals from TGF-β and upregulate CD103 (23, 24). However, a low level of T-bet expression is required to maintain expression of the IL-15 receptor β-chain (24, 25). Thus, these two T-box transcription factors control receptiveness to TGF-β and IL-15 signals, which are necessary for proper T_{RM} formation and function (9, 23–25).

In humans, a different set of transcription factors appear to be critical for T_{RM} development and maintenance. Similar to mice, Eomes and T-bet were not expressed in the T_{RM} subset (26). However, the link between HOBIT expression and T_{RM} cells is less evident. HOBIT is expressed in both circulating and resident $CD8^+$ T cell populations in humans, and when identified, associates more with cells lacking markers of residence. Reinforcing this notion, a more recent study evaluated gene profiles of $CD69^+CD8^+$ T cells derived from various human tissues and found low to absent levels of HOBIT (27). Instead, these cells expressed NOTCH-1 and HIF-1α, where NOTCH-1 regulated T_{RM} metabolism, which was suggested as its major function (26). In other cases, organ-specific transcriptional regulators were identified. In the lung, RUNX3, BATF, AHR, AP-1, RBPJ, and NF-κB were detected in the T_{RM} subset (26). Many of these regulate T cell effector functions and homing receptors. In the small intestine and vaginal mucosa, a requirement for mTOR was found through inhibition with rapamycin treatment (28). This defect was attributed to an inability to migrate to the site and respond to antigen, and less with a failure to be maintained within the tissue.

Despite some insight into the transcriptional control of T_{RM} cells, few of these transcription factors identified appear to be "master regulators" of T_{RM} differentiation as they are expressed in other $CD8^+$ effector or memory subsets. This suggests that T_{RM} differentiation and maintenance is likely controlled by complex combinations of several transcription factors, and the requirements may differ between mice and humans.

FUNCTIONS OF SURFACE PROTEINS THAT REGULATE T_{RM} LOCALIZATION

It has become clear that one of the only ways to concretely define a cell as "resident" is through the use of parabiotic mice, which allow for equilibration of all circulating cells, but not cells of residence (29, 30). However, the majority of studies in both mice and humans have used expression of a panel of T_{RM} identifiers: CD103 (integrins αE paired with β7), sphingosine-1-phosphate receptor 1 ($S1P_1$) antagonist CD69, collagen-binding CD49a (integrin α1 paired with β1), and hyaluronic acid (HUA) binding CD44 as surrogates. It is worth noting that not all populations of T_{RM} cells display all of these markers, suggesting there may be some nuance in T_{RM} subsets. The functional ramifications of expressing any of these molecules likely fit with the requirements for the appropriate positioning of the cells and long-term survival in the tissue.

CD69

The lectin CD69 is an antagonist of $S1P_1$ and limits egress by blocking responsiveness to sphingosine-1-phosphate gradients (31–33). It complexes with $S1P_1$ on the cell surface, which leads to its internalization and degradation (34). CD69 is initially upregulated on recently activated effector cells that have seen their cognate antigen, perhaps to limit egress from the lymph node, but constitutive expression is only observed on resident cells (35, 36). Downregulation of Krüppel-like factor 2 on these cells ultimately allows for the expression of CD69 within peripheral tissues (37). Interestingly, CD69 expression is not limited to $CD8^+$ T cells, and its presence on other immune subsets including natural killer cells and certain peripheral dendritic cells (DCs) plays a similar functional role of maintaining them within the organ (38, 39). While CD69 is expressed on the majority of T_{RM} subsets, absence of this lectin on $CD8^+$ T cells only limits the size of the population and does not result in complete ablation (12). This suggests that CD69 is not an absolute requirement, and while its expression may be advantageous, it is not mandatory. T cells, including T_{RM}, are dynamic in their movement in different tissue settings, and in peripheral tissues, multiple retention factors are important for maintaining the resident population. As previously insinuated, populations of T_{RM} cells exist within the salivary glands and female reproductive tract that are CD69 negative. This is interesting given the requirement for downregulation of the $S1P_1$ to establish the T_{RM} population in the salivary glands; however, it further suggests that other mechanisms of organ retention are at play (37, 40). So far, little is understood in regard to whether CD69 plays any role in how the cells are positioned within the tissue. In fact, it is quite possible that its primary and only role is limiting their exit from the organ to return to the blood and lymphatics.

CD103

Integrin αE (CD103) pairs with integrin β7 and is upregulated upon exposure to the active form of TGF-β (41, 42). The most well-known function of CD103 is as a receptor for E-cadherin, an adherans junctional protein interlocking epithelial cells (41). CD103:E-cadherin interactions can act as a tether, which may aid in positioning, retention, and the shape of cells within the epithelium (43, 44). Skin T_{RM} cells lacking CD103 are fewer in number and exhibit increased motility compared with their wild-type counterparts, corroborating this role *in vivo* (12, 45). Similarly, CD103 deficiency results in lower numbers of $CD8^+$ T_{RM} cells in the lung after influenza infection (46) and a decrease in intestinal $CD8^+$ T cells responding to oral *Listeria* infection due to a defect in initial accumulation (47). Since epithelial cells are the targets for a number of mucosal viral infections, adherence and localization of T_{RM} cells to the epithelium positions them to act as the first line of defense in subsequent exposures. In this regard, CD103 also facilitates the generation of a T_{RM} population at tumor sites such as in the case of melanoma (48). In fact, T_{RM} production by mucosal vaccination leads to inhibition of tumor growth in a preclinical model of head and neck cancer, which was substantiated through parabiotic experiments in mice (49).

While physical retention through ligand binding is the most obvious role for CD103, engagement of CD103 may have a number of other functional ramifications outside of adhesion. While the effects of CD103 binding have been primarily studied in tumor models, the identified features of this integrin are likely widespread throughout various disease states. $CD103^+$ tumor-infiltrating $CD8^+$ T cells are more capable of killing tumor cells (50). This is likely attributed to the fact that $CD103^+$ T cells form more stable synapses with target cells than their CD103-negative counterparts (51). Engagement of CD103 also positions cytolytic granules to organize in a polarized fashion, and the addition of signaling through the TCR results in lytic granule exocytosis (52, 53). Although these functions of CD103 are redundant in the presence of CD11a (LFA-1), T_{RM} cells, especially in the airways of the lungs, display low levels of LFA-1 (54). In fact, LFA-1 levels have been used to determine the age of the T_{RM} cells in the airway, functioning as a clock and decreasing over time (3). One hypothesis is that airway T_{RM} cells are not cytolytic because the synapse stability is affected by this defect. However, CD103 expression on T_{RM} may compensate for low LFA-1 levels and promote effective cytolytic responses to secondary infections.

Moreover, engagement of CD103 may also function to directly position the cells within a given tissue. As an example, it has been shown in the tumor microenvironment that binding of CD103 results in the upregulation of the chemokine receptor CCR5 (55). This suggests that the integrin/chemokine axis could greatly affect the downstream consequences of migratory cues received by a cell and looking at each pathway discretely may limit the overall understanding of the response. In the lung, CCR5 is critical for $CD8^+$ T cells to reach the airways (56). Therefore, it would not be unreasonable to hypothesize that CD103 deficiency may alter the localization of the $CD8^+$ T cells and delay clearance of the infection. On the flip side, binding of CCL25 through chemokine receptor CCR9 contributes to expression of CD103 on $CD8^+$ T cells in the intestine (57). While it is relevant that chemokine signals other than TGF-β may contribute to the upregulation of this integrin on the surface, due to limited expression of CCR9 on $CD8^+$ T cells in other organs, this may be a gut-specific mechanism.

In addition to E-cadherin expression on epithelial cells, flow cytometric analysis has demonstrated that the protein is expressed on the surface of specific immune populations such as DCs and, in some instances, T_{RM} cells (58–60). In the salivary glands and gut, E-cadherin is detected on virus-specific $CD8^+$ T cells, a phenomenon specific to mucosal compartments, as their lymphoid counterparts in the spleen do not display this phenotype (60). While it is unclear whether the $CD8^+$ T cells are producing E-cadherin naturally or acquiring the surface phenotype through a process such as trogocytosis, there are likely to be functional consequences of non-epithelial cells adorned with E-cadherin (61, 62). This may allow for the formation of more stable synapses, in this case between T cells and their APC, or potential cell:cell communication *via* engagement of

ligand on other T cells. Surface E-cadherin on the T_{RM} cell could alternatively lead to homotypic interactions (63) in the absence of CD103 expression; however, the downstream functional consequences of this interaction are unclear.

The brain and other epithelium-sparse tissues pose a conundrum in regard to understanding the role that CD103 plays on T_{RM} cells. In the brain, T_{RM} cells are localized to borders of different anatomical regions and in some cases in proximity to vasculature (64). Despite this, expression of CD103 does not appear to corroborate with positioning. However, as just posed, one possibility is that CD103 solely interacts with E-cadherin expressing immune cells in regions devoid of epithelial surfaces and potentially localized similarly to the profile of the T_{RM} cells. Another possibility is that CD103 has other unexplored ligands at play. Although only identified in an *in vitro* system, it has been suggested that CD103 can bind to microvascular endothelial cells derived from the intestine (65). While this did not hold true with endothelial cells derived from the dermis, it has yet to be investigated in the brain and other non-mucosal organs and could better identify a functional role for CD103 in these locations.

Overall, one of the caveats when studying the roles of CD103 *in vivo* is that deletion of the gene results in a lack of DCs expressing this marker. $CD103^+$ DCs are critical for the development of T_{RM} cells in the lung during influenza infection and are one of the APC population that drains to the mediastinal lymph node early during infection to present antigen (30, 53). One of the approaches to circumvent this issue is to transfer CD103 knockout transgenic cells into WT mice. This is the approach employed to establish its requirement in the skin, lungs, and gut as previously described (12, 46). However, none of these studies addressed whether CD103 signaling may be required during early stages of development and could ultimately alter the population prior to becoming established at a peripheral site. To fully examine the role of CD103 in the development and persistence of $CD8^+$ T cells, it would be necessary to develop an inducible knockout specific to $CD8^+$ T cells, so that the integrin could be eliminated at specific time points postinfection.

CD49a

The role of CD49a on memory T cells in peripheral tissues was discovered in 2004 (2). At that time, the term T_{RM} had not yet been coined, although it has since been associated with this subset of memory T cells (2, 9, 12, 66, 67). CD49a, or integrin α1, pairs with CD29 (integrin β1) to form the heterodimer VLA-1. VLA-1 is a collagen-binding integrin, with preference for the non-fibrillar form, ColIV (68, 69), although it can also bind to ColI, the fibrillar form present in the interstitium of almost all tissues (21). Early studies showed that VLA-1 was not only critical for adherence to ColIV but also migration of the cells along the collagen (68–70). The idea that two of the predominant integrins expressed on T_{RM} cells have opposing functions when interacting with the tissue is quite remarkable, and yet both CD49a and CD103 appear to contribute to the development and/or survival of this population.

While CD49a does not have a direct role in attaching to epithelial cells, its ligand ColIV is located in the lamina densa layer of the basement membrane of mucosal epithelium and is the surface to which the epithelial cells are attached (71). The motility features of this interaction could allow for migration along the basement membrane to access additional regions of the epithelium or may be essential for traversing the collagen to reach infected cells. In either scenario, this interaction is believed to be critical for persistence of the resident population as demonstrated in both the lungs and the intestines (2, 72). While some have assumed that CD49a acts to retain T cells at the epithelium, this has not been unequivocally demonstrated experimentally. As mentioned, it could be necessary to migrate within those sites. CD49a regulation of retention and motility is not mutually exclusive as the ability to stay in the tissue and perform surveillance are the functional hallmarks of T_{RM}.

In addition to fostering close proximity to the target cells, engagement of CD49a has a pro-survival role. In conjunction with signaling through the TNF receptor II, binding to CD49a works in a synergistic fashion to protect the cells from undergoing apoptosis (73). Blocking CD49a with antibodies results in a diminution of T_{RM} in mucosal sites (2). Similarly, genetic deletion of CD49a results in the resident population becoming limited (2, 72). Protection requires the presence of a sufficient number of T_{RM} cells, and mice deficient in CD49a become susceptible to secondary heterosubtyptic infections at an earlier time point after primary infection (2–4).

While the requirement for CD49a expression on T_{RM} cells became clear through mouse focused studies, T_{RM} from human subjects share many attributes. In the lung, T_{RM} expressing CXCR6, CD49a, CD103, and CD69 are known to be the major memory population (27, 74). In the skin of healthy individuals, $CD8^+$ T_{RM} cells expressing CD49a identify a population of cells that produce IFNγ (9). Unexpectedly, in addition to IFNγ, when stimulated with IL-15, they produce large amounts of perforin and granzyme B (9), perhaps offering a potential mechanism for their reactivation as effectors in secondary encounters. On the other hand, CD49a-negative $CD8^+$ T cells in the skin instead produced IL-17 and were associated with psoriatic lesions, indicating that CD49a expression defines different functional subsets. Whether CD49a was required to establish or maintain these cells was not investigated. In mice, $CD49a^+$ $CD4^+$ memory T cells in the lung provide rapid effector and innate like functions, probably related to more efficient recruitment of other effector cells. This effector function bias based on CD49a expression has not yet been reported for T_{RM} in other tissues.

CD44

A fourth marker of T_{RM} cells is CD44. While CD44 alone does not distinguish T_{RM} cells from other $CD8^+$ T cell populations, its continued expression suggests that similar to other surface receptors, CD44 may have a functional role in T_{RM} biology (75). CD44 is a C-lectin containing glycoprotein, which is expressed on various cell types, including leukocytes and epithelial cells (76). The most well-studied function of CD44 is as a receptor for HUA, a component of the extracellular matrix, and a

substance made by vascular endothelial cells and an array of immune cells (77, 78). HUA expression in peripheral tissues is upregulated during inflammation and increases hydration of the tissue (79). On $CD8^+$ T cells, CD44 is a classical marker of previous activation, expressed on newly generated effector cells as well as resting memory cells (80). Unlike other T cell markers, which are either on or off, CD44 is expressed in a gradient depending on whether a cell is naive, an effector, or a memory cell (81, 82). Of note, CD44 exists in alternatively spliced isoforms, with various posttranslational modifications, which result in differential affinity for ligand (83, 84). However, in mice, only the invariant form of CD44 has been identified on T cells, with only suggestions that alternative forms are transiently expressed during periods of immunological challenge (85).

In regard to accessing peripheral tissues during an immune challenge, CD44 can bind HUA expressed on vascular endothelial cells and facilitate transmigration (86). In addition, CD44 can interact with CD49d through their intracellular domains, which may enhance this process. However, complete deletion of CD44 does not limit the accumulation of $CD8^+$ T cells at the site of initial infection, suggesting that this role is redundant when the cells can utilize other selectins expressed on the cell surface. Once within the tissue, CD44 expressed on DCs improves TCR synapse stability, possibly through binding to $CD8^+$ T cell production of HUA (87, 88). Expression of HUA on other immune cell subsets could also aid in cell:cell communication.

Most relevant to migration of $CD8^+$ T cells is that CD44 has been shown to play a critical role in maintaining cell structure through adherence to ECM (89). In addition to binding HUA, CD44 can interact with other matrix proteins such as fibronectin, laminin, and collagen. Loss of CD44 during *in vitro* experiments demonstrated that cells no longer display stable polarization with extension of a uropod (89). This defect resulted in a decreased migratory capacity within peripheral tissue, which affects initial access of the tissue. CD44 ligand binding led to recruitment of a number of signaling molecules, which transmitted signals to the cytoskeleton to stabilize the cellular morphology (89).

In the T_{RM} population, CD44 is maintained at high levels, suggesting that it may be important for the T_{RM} population; however, the specific function has yet to be elucidated (75). One possibility is that CD44 could be acting as a general receptor for ECM, maintaining cell shape and polarity as the cells interact with collagen and epithelial cells (89). However, this has yet to be directly tested. An essential role for CD44 in survival of Th1 $CD4^+$ T cells has been demonstrated; however, this function was not identified on $CD8^+$ T cells (90). In fact, so far the majority of functional requirements for CD44 are enhanced on Th1 $CD4^+$ T cells and do not translate to the $CD8^+$ T cell compartment (90). However, its role within the T_{RM} subset has not been comprehensively interrogated. Understanding the biology behind the contribution of CD44 to the localization and/or maintenance of the $CD8^+$ T_{RM} population could shed light on a new pathway of regulation and warrants further investigation.

ROLES OF CYTOKINES AND CHEMOKINES IN POSITIONING THE CELLS WITHIN THE TISSUE

The roles of chemokines in proper positioning within lymphoid organs have been extensively studied (91). However, their contribution within peripheral tissues is less well understood. In the lung, absence of CCR5 on T cells prevents $CD8^+$ T cells from effectively reaching the airway and clearing influenza infection (56). Alternatively, CXCL12 packaged in neutrophil trails facilitates efficient migration of $CXCR4^+CD8^+$ T cells to the site of influenza infection (92). However, the chemokine cues that are required for the acute response may or may not translate to the T_{RM} population and long-term protection. Depletion of neutrophils delayed $CD8^+$ T cell infiltration into the infected lung but did not affect the development or protective capacity of T_{RM} (93). By contrast, it is clear in the intestine that expression of CCR9 and consequent binding to CCL25 is essential for migrating T cell subsets to localize to the epithelium and for the development of memory (57).

In the skin, treatment with pertussis toxin decreases the velocity of $CD8^+$ T_{RM} cells and alters their morphology, resulting in a rounded phenotype (45). This suggests that chemokines likely play a role in the formation of dendritic spines on the T cells and interactions with the tissue. Keeping with this, the development of the T_{RM} population in the skin greatly benefits from the presence of CXCR6, which is one of the "core" markers of bona fide T_{RM} from many tissues (27, 45). The chemokine that this receptor binds is CXCL16, which has been shown to position innate lymphoid cells and natural killer T cells in tissues and is released by DCs during viral infection (94, 95). Although a diminished population still develops in the absence of CXCR6, the great reduction suggests that it increases either entry into tissue or proper positioning for survival. Alternatively, in the tonsils constitutive expression of IL-15 in the T cell zones and the subepithelium retained virus-specific $CD8^+$ T cells within these two locations (96). Interestingly, in this organ, CD103 was only expressed on the T cells in close proximity to the epithelial cells and not in the extra-follicular region.

In many of these cases, signalizing through a cytokine or chemokine receptor may have other implications that indicate that the molecule itself is not directly necessary, but rather the downstream effect such as integrin activation or expression. For example, as previously addressed, interplay between TGFβ and CCL25 are associated with an upregulation of CD103 (57). Engagement of CD103 in turn leads to upregulation of CCR5 (55). CCR5 and CX3CR1 on $CD8^+$ T cells in the lung are important for positioning of the cells in proximity to highly inflamed sites and the presence of antigens (56). These consequences suggest that there are complicated signaling pathways and loops at play that have yet to be fully elucidated.

In addition, the presence of various cytokines may provide sufficient inflammatory signals to allow cells access to locations not previously available. Inflammation of the vascular endothelium and changes to the barrier epithelium can occur either through cytokines or early immune cytolytic responses to

infection (97, 98). Both of these events could dynamically alter the tissue landscape and allow T_{RM} cells to gain access to previously obscured regions and persist. To fully understand these changes, it is likely that *in vivo* imaging systems will need to be developed to examine alterations in the ECM over time.

FUNCTIONS OF T_{RM} CELLS

While this was touched on briefly earlier in the review, the ultimate role that T_{RM} cells play in protecting a host is not fully understood. One possibility is that the cells produce antiviral cytokines and control downstream immune responses to eliminate infected cells. Alternatively, T_{RM} cells could directly target the cells. In mice, T_{RM} cells in the lungs make large quantities of IFN-γ in response to antigen-specific rechallenge, and blocking of this antiviral cytokine is detrimental to survival (99). In this instance, cells do not produce granzymes or other markers of cytotoxicity. T_{RM} in the female reproductive tract display a decrease in motility after reexposure suggesting that the cells are interacting with antigen-bearing DCs; however, the direct antiviral outcome was not examined in this context (100). $CD4^+CD49a^+$ T cells in the lung activate within hours of secondary challenge with influenza and secrete an array of chemokines that attract other immune effectors (101). In line with this, $CD4^+$-resident memory cells in the lungs are also sufficient for promoting airway hyperresponsiveness in a house dust mite model of asthma (102); however, this response can be partially attributed to T_{RM} activation of local DC subsets (103).

Reactivation of human T_{RM} cells alternatively not only leads to IFNγ production but also degranulation of cytolytic granules, including granzyme B and perforins (9, 74). It is conceivable that the limited responses observed in the mouse models are due to the methods employed to evaluate the cellular responses. It is possible that T_{RM} cells are cytolytic *in vivo* with the proper stimulus. Other signals may be necessary for a similar response *in vitro*. For example, T_{RM} cells may require signals from CD103 as well as through the TCR for degranulation of lytic granules. To fully understand the responses, it is necessary to examine these cells *in vivo*. To achieve this, granzyme B reporter mice and other protein reporter mice are the ideal tools (104, 105). As an alternative approach, transcriptional reporters for cytokine production or calcium reporters to indicate productive interactions with APCs may suffice (106–108). Utilizing these tools in conjunction with reporter pathogens, all the cues and responses necessary for protection of the host can be elucidated (109).

OTHER OPEN QUESTIONS

With the current methods employed for examining T_{RM} cells, researchers are likely underrepresenting the populations as demonstrated by Steinert et al. (40). Using histology and/or other imaging methods will better reveal the totality of the T_{RM} cell presence and ultimately their response. Although T_{RM} cells are identified through expression of the discussed surface receptors, it is still not clear how the combination of markers alters the response of a given cell. Not all cells that express these markers at the resolution of an infection persist in the organ, suggesting

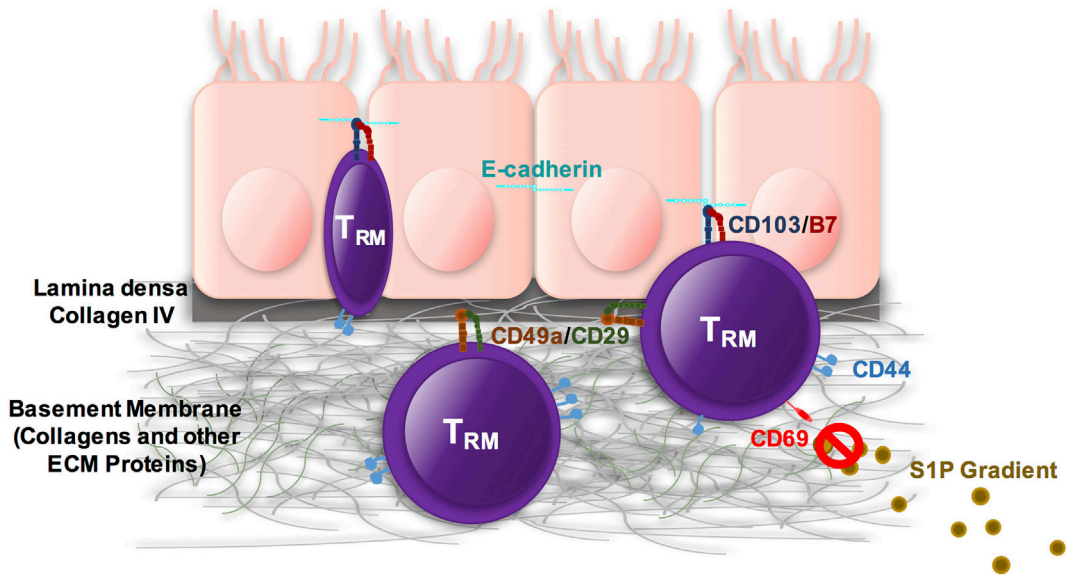

FIGURE 1 | TRM cells are heterogenous both within tissues and also between sites. They express some combination of CD49a, CD103, CD44, and CD69, which cooperate to position the cells and maintain them within the site of initial infection. CD103 can interact with E-cadherin within epithelial surfaces. CD49a interacts with collagens with a preference for collagen IV in the lamina densa underlying the epithelium. CD44 maintains cell shape and integrity and can interact with a number of different tissue components including hyaluronic acid as well as fibronectin and other ECM proteins. CD69 antagonizes $S1P_1$, essentially blocking any response to S1P gradients.

that it is a combination of expression profiles, localization within a tissue, and perhaps proximity to APCs, which may present persistent antigen.

While CD49a, CD103, CD69, and combinations of chemokine receptors are used to define T_{RM}, the precise positioning of the T cells in mucosal and glandular tissues remains to be directly examined, including demonstration that CD103 actually binds to E-cadherin at epithelial sites. Besides positioning, how these markers regulate cell motility versus retention in different tissues is not well defined. Intravital microscopy may be one approach to answer some of these questions.

It is also still not fully understood what population of cells lead to the T_{RM} subset. In the lung, evidence suggests that T_{EM} may be maintained within the parenchyma and refeed the transitory airway subset (30). Other T_{RM} populations may also be self-renewing, similar to other populations of resident immune cells which seed organs earlier during development (11, 110). In fact, recent evidence in the female reproductive tract suggests that T_{RM} cells divide during antigen-specific challenge and contribute to the secondary T_{RM} population more than circulating memory cells (100).

Given the common features of T_{RM} subsets in different tissues begs the question of whether there is a common developmental program, as no one specific pathway has been identified. How that program is triggered remains unclear, although TGF-β is required for CD49a and CD103 expression (23). Identifying key features, such as route of delivery, will be critical for optimally generating T_{RM}. Previous studies indicate that memory generated through direct infection or exposure of the tissue is distinct from cells that result from systemic priming (47). Ideally, production of T_{RM} could be an effective approach to problems like a universal flu vaccine; however, the field still struggles with eliciting an effective response in a vaccination setting.

CONCLUSION

T_{RM} cells are important for protection from secondary encounters with various pathogens. They can protect and preserve the integrity of barrier surfaces such as skin, gut, and respiratory tissues. Transcriptional regulation is different in mouse and human systems, although a set of "core" markers have been identified, each having a distinct role in establishing and maintaining T_{RM}. It is clear from these studies that T_{RM} cells are highly regulated, poised to respond, yet suppressed by molecules including PD-1 and CD101 to prevent aberrant activation (27). Although the specific cues that result in loss of that suppression have not been identified, it is likely a combination of signals that may include type 1 interferons, which are one of the earliest innate factors made during infections and TCR engagement. In terms of positioning, there is evidence that CD49a interacts with ColIV, and CD103 adheres to E-cadherin, both retaining the cells near the epithelial surface (**Figure 1**). CD69 and CD44 do not seem to have the same level of necessity, and they are not included in the list of core markers identified in humans; however, they both likely contribute to persistence through limiting egress and maintenance of cytoskeletal structure. To resolve many of these questions future work will need to be done to elicit a full understanding of how T_{RM} cells function to provide pathogen surveillance and protection.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Protective Role of Nuclear Factor Erythroid 2-Related Factor 2 Against Respiratory Syncytial Virus and Human Metapneumovirus Infections

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The pathogenesis of respiratory syncytial virus (RSV) infections is characterized by lower airway obstruction driven at great extent by the exuberant production of inflammatory cytokines. We have previously shown that RSV infection *in vitro* and *in vivo* results in production of reactive oxygen species along with reduction in the expression of antioxidant enzymes (AOEs), which are involved in maintaining the cellular oxidant–antioxidant balance. These events were associated with the concomitant reduction in nuclear factor erythroid 2-related factor 2 (Nrf2), a key transcription factor that controls AOE expression. The objective of the current study was to establish the role of Nrf2 in shaping innate immune responses, clinical disease, airway inflammation, and viral replication in established experimental models of intranasal RSV and human metapneumovirus (hMPV) infections, by employing mice genetically deficient for the *Nrf2* gene. Compared to control wild type (WT), mice genetically deficient in Nrf2 (Nrf2 KO) developed enhanced clinical disease, airway inflammation and pathology, and significantly greater lung viral titers following experimental infection with either RSV or hMPV. In particular, compared to control mice, RSV-infected Nrf2 KO mice lost more body weight and had increased airway obstruction at time points characterized by a remarkable increase in inflammatory cytokines and airway neutrophilia. Airway levels of AOEs and enzymes that regulate synthesis of the endogenous hydrogen sulfide (H_2S) pathway, which we showed to play an important antiviral function, were also decreased in RSV-infected Nrf2 KO compared to WT. In conclusion, these results suggest that Nrf2 is a critical regulator of innate, inflammatory, and disease-associated responses in the airways of mice infected with viruses that are members of the *Pneumoviridae* family. Importantly, the results of this study suggest that Nrf2-dependent genes, including those controlling the cellular antioxidant and H_2S-generating enzymes and cytokines can affect several aspects of the antiviral response, such as airway neutrophilia, clinical disease, airway obstruction, and viral replication.

Keywords: respiratory syncytial virus, human metapneumovirus, nuclear factor erythroid 2 related factor 2, antioxidant enzymes, hydrogen sulfide, lung injury, airway inflammation, airway obstruction

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INTRODUCTION

Acute bronchiolitis is a viral lower respiratory tract infection (LRTI) that represents the primary cause of hospitalization for children in the first year of life. The orthopneumovirus respiratory syncytial virus (RSV), a member of the *Pneumoviridae* family is the causative agent in more than 50% of the cases. In the United States, 75,000–125,000 hospitalizations related to RSV occur each year in children <1 year of age, and RSV infection results in approximately 1.5 million outpatient visits among children <5 years of age (1, 2). Given the fact that RSV infections do not provide permanent immunity, reinfections can reoccur throughout life and cause serious respiratory disease in certain adult populations, including frail elderly and subjects with chronic heart and lung diseases (3). There are no vaccines or specific antiviral therapies currently licensed to prevent or treat RSV infections. A significant obstacle to the development of therapeutic strategies is our still limited understanding of the pathogenic mechanisms that determine the severity of LRTI caused by RSV. Although infants with certain risk factors (prematurity, chronic lung disease, congenital heart disease, or immunodeficiencies) have an increased risk for more severe RSV disease, the large majority of infants with RSV infections that require hospitalization were previously healthy (4, 5). Therefore, the spectrum of RSV disease severity in otherwise previously healthy infants, points to both host determinants and virus-specific factors that determine the outcome of infection (4). Lung inflammation and airway obstruction, driven by an exuberant innate mucosal immune response and cytokine production is recognized to play a central pathogenic role (6, 7). Excess neutrophils are found in the airways of infants with acute RSV bronchiolitis as well as in the bronchoalveolar lavage (BAL) fluid of RSV-infected children (8, 9). At the same time, infants with greater viral quantities in the respiratory tract secretions are at greater risk for prolonged hospitalization, intensive care unit stay, and mechanical ventilation (10), possibly by causing direct lung injury.

Reactive oxygen species, such as the superoxide radical anion $\left(O_2^{\bullet-}\right)$ and hydrogen peroxide (H_2O_2), have been directly implicated in tissue damage, inflammatory disorders, pulmonary disease, and infections [reviewed in Ref. (4, 11)]. We have previously shown that RSV is a potent inducer of reactive oxygen species (ROS) in epithelial cells and in the airways but, contrary to what has been shown in certain viral infections, they do not contribute to the host anti-RSV response; rather they cause lung inflammation and clinical disease (12). In addition, we have shown that ROS function as intermediate signal in the transcriptional regulation of cytokine and chemokine genes and cause oxidative damage to the airways (12–14). Indeed, treatment of RSV-infected mice with antioxidant molecules reduces inflammation, clinical disease, and airway hyperresponsiveness (AHR) (12). Our most recent study shows that RSV infection in epithelial cells (15), in experimentally infected mice and in naturally infected children (16) causes a significant decrease in the expression of most antioxidant enzymes (AOEs) and as result it disrupts the pro-oxidant–antioxidant balance (4).

Transcription of many oxidative-stress-inducible genes is regulated in part through *cis*-acting antioxidant responsive element (ARE) sequences. This element has been identified in the regulatory regions of genes encoding detoxification enzymes, such as NQO1 (NADPH:quinone oxidoreductase), as well as many AOEs, including Cu/Zn-superoxide dismutase (SOD1), catalase, heme oxygenase 1, glutathione S-transferase, and glutathione-generating enzymes, such as glutamate cysteine ligase (GCLC) [reviewed in Ref. (17)]. NF-E2-related factor 2 (Nrf2) is an important redox-responsive protein that helps to protect cells from oxidative stress and injury. It is a basic leucine zipper transcription factor that is normally bound in the cytosol to a cytoskeleton-associated inhibitor called Kelch-like ECH-associated protein 1 (Keap1). Electrophile-induced release of Nrf2 is proposed to involve covalent modifications of Keap1 and/or Nrf2 in the cytoplasm. Such modifications include oxidation of key cysteine residues in Keap1, phosphorylation of Nrf2, and switching of Cullin-3-dependent ubiquitination from Nrf2 to Keap1, leading to the degradation of Keap1, and the stabilization and activation of Nrf2 (18). The released Nrf2 then translocates to the nucleus and binds to ARE sites to promote gene transcription (17). Cho et al. using Nrf2-deficient mice have shown that the Nrf2-ARE pathway plays a key role in antiviral host response to RSV (19) and we have shown in previous studies that decrease in AOEs expression in epithelial cells as well as in the lungs of mice infected with RSV was associated with a significant decrease in nuclear expression of Nrf2 (15, 16). Furthermore, children with naturally acquired RSV infections had significant increase in markers of oxidative injury and a significant decrease in AOE expression in their nasopharyngeal secretions (NPS), which correlated with the severity of clinical illness (16), suggesting that RSV-induced oxidative damage *in vivo* is the result of an imbalance between ROS production and airway antioxidant defenses. Therefore, the current study was designed to test the role of Nrf2 in shaping innate immune responses, clinical disease, airway inflammation, and viral replication in an established experimental model of RSV infection, by employing adult/aged mice that were genetically deficient in the *Nrf2* gene.

MATERIALS AND METHODS

RSV and Human Metapneumovirus (hMPV) Preparations

Respiratory syncytial virus long strain was grown in HEp-2 cells (American Type Culture Collection, Manassas, VA, USA) and purified by polyethylene glycol precipitation, followed by centrifugation on 35–65% discontinuous sucrose gradients as described elsewhere (20, 21). The virus titer of the purified RSV was determined by a methylcellulose plaque assay and ranged from 8–9 log10 plaque forming units (PFU)/ml. The hMPV strain CAN97-83 was obtained from the Centers for Disease Control, Atlanta, GA, USA, with permission from Guy Boivin at the Research Center in Infectious Diseases, Regional Virology Laboratory, Laval University, Quebec, Canada. Virus was propagated in LLC-MK2 cells (American Type Culture Collection) in minimal essential medium (without serum) containing 1 μg trypsin/ml, followed by purification on a 60% sucrose cushion (22). Virus titer was determined by a cell-based immunoassay.

For that, sucrose-purified virus was serially diluted (log10) on LLC-MK2 cell monolayers in 96-well flat-bottom plates. Forty-eight hours later, monolayers were washed and incubated with guinea pig anti-hMPV antibody (MedImmune, Inc., Gaithersburg, MD, USA) and stained with horseradish peroxidase-labeled anti-guinea pig antibody (Zymed, San Francisco, CA, USA). Infected cells were detected using 3-amino-9-ethyl-carbazole, infectious units being enumerated by light microscopy. The final viral titer was expressed as PFU/ml. Virus pools are aliquoted, quick-frozen in liquid nitrogen, and stored at −70°C until used. Viral preparations are routinely tested for LPS and cytokine contamination.

Neutrophil-Specific Probe

Neutrophil-Specific, near infrared (NIR) Fluorescent Imaging Agent (cFLFLF-PEG_{76}-Cyanine7) was purchased from Kerafast (Boston, MA, USA). The neutrophil probe was freshly prepared in PBS prior to use in mice according to the manufacturer instructions.

Ethics Statement

All procedures involving mice in this study were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch at Galveston.

Mice and Infection Protocol

The study has been approved by the UTMB IACUC (protocol number 9001002 and 0808049). Heterozygous $Nrf2^{+/-}$ mice (on C57BL6 background) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). In this $Nrf2^{-/-}$ strain, exons 4 and 5 of the mouse *Nfe2l2* gene, which encodes the basic leucin zipper domain that controls transcriptional activation, has been replaced by a LacZ reporter gene. Heterozygous mice were crossbred to generate homozygous Nrf2 $^{-/-}$ (Nrf2 KO) mice as well as wild type littermates controls [Nrf2 $^{+/+}$, wild type (WT)]. Genotyping for Nrf2 status was performed by PCR amplification of genomic DNA. Pathogen-free breeding colonies of $Nrf2^{+/-}$, Nrf2 $^{-/-}$, and Nrf2 $^{+/+}$ were maintained at the University of Texas Medical Branch at Galveston and experiments were performed using 12- to 15-month-old Nrf2 KO mice and WT age-matched littermates of both genders. Under light anesthesia, were infected intranasally (i.n.) with 50 µl of RSV diluted in PBS at a dose of 10^7 PFU or mock inoculated using the same volume of control buffer. Daily determinations of body weight and illness score, collection of BAL for differential cell counts and cytokines, chemokines, and type I interferons (IFNs) measurements, lung neutrophil counts by flow cytometry, lung viral titration, nuclear translocation of NF-κB/RelA in the lungs, pulmonary histopathology, and pulmonary function testing were performed as previously described (12, 23–25). Antioxidant (SOD1 and catalase) and hydrogen sulfide (H_2S)-generating enzyme (CSE, CBS, and 3MST) mRNA expression in lung tissue was analyzed by quantitative real-time PCR (qRT-PCR) (24, 26). In some experiments Nrf2 KO and WT mice, were infected i.n. with 5×10^6 PFU of human metapneumovirus (hMPV, CAN97-83 strain) for assessment of disease parameters, BAL differential cell count, cytokines and chemokines, and pulmonary function testing (27, 28).

Clinical Disease

Animals from all groups were evaluated on a daily basis for weight loss, illness score, and presence of any respiratory symptoms. The percentage of body weight change was plotted over time. A clinical illness score for mice was used to measure the severity of clinical disease (0—healthy; 1—barely ruffled fur; 2—ruffled fur but active; 3—ruffled fur and inactive; 4—ruffled fur, inactive, and hunched; and 5—dead). These parameters have been shown to closely correlate with lung pathology in experimental infection of mice (12, 24, 25, 27).

Broncholaveolar Lavage

Broncholaveolar lavage was collected *via* the trachea by flushing the lungs twice with 1 ml of ice-cold PBS. A total of 100 µl of BAL fluid was used for cytospin analysis, and the rest was immediately centrifuged and stored at −80°C. Total number of BAL cells was counted with a hemacytometer and viability was assessed by trypan blue. BAL differential cell counts were determined using morphogenic criteria under light microscopy of Protocol HEMA3 (Fisher Scientific) stained cytospins with a total count of 200 cells per slide.

Measurement of Cytokines, Chemokines, and IFNs

Levels of cytokines and chemokines in BAL fluid were determined with the Bio-Plex Pro Mouse Group I 23-plex panel (Bio-Rad Laboratories, Hercules, CA, USA). The lower limit of detection for all cytokines measured by this assay is 3 pg/ml. IFN-α and IFN-β were measured by commercial enzyme-linked immunosorbent assays (ELISA), following the manufacturer's protocol (PBL Biomedical Laboratories, Piscataway, NJ, USA). The range of sensitivity of the assay is 12.5–400 pg/ml for IFN-α, and 12.5–1,000 pg/ml for IFN-β.

RSV Titration of Lung Tissue

Lungs were removed from infected animals at day 5 after RSV infection. Tissue samples were homogenized in 1 ml of Dulbecco's modified Eagle's medium and centrifuged twice at 14,000 rpm for 1 min at 4°C. Serial twofold dilutions of the supernatant were determined by plaque assay on HEp-2 cells under methylcellulose overlay. Plaques were visualized 5 days later, and virus titers will be expressed as log10 PFU/gram tissue.

Pulmonary Histopathology

Mice were euthanized at days 5 and 7 post-infection, and the entire lung was perfused, removed, and fixed in 10% buffered formalin following by paraffin embedding. Multiple 4-µm longitudinal cross-sections were stained with hematoxylin and eosin (H&E). The slides were analyzed and scored for cellular inflammation under light microscopy by a board-certified pathologist with expertise in mouse lung, unaware of the infection status of the animals. Two separate grading systems were used to assess the lung inflammation. The first grading system, adapted from

Ref. (23), measured the percentage of abnormal perivascular spaces in the tissue sections. The second grading system assigned a 0–4 grade based on severity (0 = normal, 4 = severe pathologic changes) to four different parameters: perivasculitis, bronchiolitis, alveolitis, and necrosis. Ten high power fields were examined for each slide, and average grades were compared between groups and analyzed to determine whether observed differences were statistically significant.

Pulmonary Function

Airway hyperresponsiveness was assessed in unrestrained mice at different times after infection using whole-body barometric plethysmography (Buxco, Troy, NY, USA) to record enhanced pause (Penh), as previously described (12, 24). Penh is a dimensionless value that represents a function of the ratio of peak expiratory flow to peak inspiratory flow and a function of the timing of expiration. Respiratory activity was recorded for 5 min, to establish baseline Penh values. Mice were subsequently exposed to increasing doses of nebulized methacholine (3.25, 6.25, 12.5, 25, and 50 mg/ml) for 2 min, and data were recorded for another 3 min.

Flow Cytometry of Lung Cells

For flow cytometry analysis, lungs were collected at day 1 after RSV or mock infection and digested with collagenase, as previously described (24, 25, 27, 28). Cells were passed through nylon mesh to get a single cell suspension, and incubated with anti-FcγRIII/FcγRII mAb (anti-mouse CD16/CD32; BD Biosciences, San Diego, CA, USA) to reduce nonspecific binding, for 30 min at 4°C. After washing, cells were stained with the following anti-mouse antibodies: anti-F4/80 APC, anti-CD11b PerCP-Cy5.5, and anti-Ly6G FITC (for neutrophils). After incubation for 30 min at 4°C with the antibodies, cells were washed and then fixed in 200 µl of 1% paraformaldehyde in PBS. All the antibodies were purchased from BD Pharmingen, San Jose, CA, USA, except anti-F4/80 which was obtained from ebioscience, San Diego, CA, USA. Corresponding isotype Abs were used as controls. Cells were acquired with a FACS Canto flow cytometer equipped with Cell Quest software (both from Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Analysis was performed by using the FlowJo Software (Tree Star, NJ, USA).

Quantitative Real-Time PCR

Total RNA was extracted by using a ToTALLY RNA kit (catalog number AM1910; Ambion, Austin, TX, USA). RNA samples were quantified by using a NanoDrop spectrophotometer and quality was analyzed on an RNA Nano-drop by using the Agilent 2100 bioanalyzer (Agilent Technologies). Synthesis of cDNA was performed with 1 µg of total RNA in a 20 µl reaction mixture by using the TaqMan Reverse Transcription Reagents kit from ABI (catalog number N8080234; Applied Biosystems). Amplification was done using 1 µl of cDNA in a total volume of 25 µl using the Faststart Universal SYBR green Master Mix (Roche Applied Science #04913850001). Viral genome copy numbers and RSV N gene copy numbers (primer sequences and conditions) were measured as previously described (24, 26). The mRNA sequences for SOD1, catalase, CSE, CBS, and 3-mercaptopyruvate sulfurtransferase (3-MST) reported under GenBank accession numbers X06683.1, L25069, NM_145953, NM_144855.3, and NM_138670 were used to design amplification primers for qRT-PCR assay (24). 18S RNA was used as housekeeping gene for normalization. PCR assays were run in the ABI Prism 7500 Sequence Detection System. Triplicate cycle threshold (C_T) values were analyzed in Microsoft Excel by the comparative C_T ($\Delta\Delta C_T$) method according to the manufacturer's instructions (Applied Biosystems). The amount of target $\left(2^{-\Delta\Delta C_T}\right)$ was obtained by normalization to the endogenous reference (18S) sample. RNA isolation, primer design, and qRT-PCR assays were performed at Molecular Genomic Core, UTMB, Galveston.

Western Blotting

Nuclear extracts of uninfected and infected lungs were prepared as previously described (16, 23). Equal amount of proteins (20 µg per sample) were then boiled in 2× Laemmli buffer and resolved on SDS-PAGE gels. Proteins were transferred onto a polyvinylidene difluoride membrane (Amersham, Piscataway, NJ, USA), and non-specific binding sites were blocked by immersing the membrane in Tris-buffered saline–Tween (TBST) containing 5% skim milk powder, overnight at 4°C. After a short wash in TBST, membranes were incubated with the primary antibody NF-κB p65 diluted 1:1,000 overnight at 4°C, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz, CA, USA), diluted 1:5,000 in TBST, for 1 h at room temperature. Finally, after washing three times with TBST, proteins were detected by using an enhanced chemiluminescence system (RPN 2016; Amersham, GE Healthcare, United Kingdom). Membranes were stripped and reprobed with anti-β-actin antibody for loading control. Densitometric analysis of band intensities was performed using UVP VisionWorksLS Image Acquisition and Analysis Software 8.0 RC 1.2 (UVP, Upland, CA, USA). Antibodies used for Western blot assays were NF-κB p65 rabbit mAB (C2284) and anti-β-actin (4967) from Cell Signaling, Technology, MA.

In Vivo Imaging of Neutrophils

Real-time visualization of neutrophils in the lung of infected mice was performed using the NIR Fluorescent Imaging Agent (29). One hour prior to imaging, 2 nmol of the neutrophil-specific, NIR Fluorescent Imaging Agent was administered to Nrf2 KO and WT RSV-infected mice and mock controls by way of tail vein injection or intratracheal instillation. Lungs were excised and imaged *ex vivo* using an IVIS 200 Spectrum (Perkin-Elmer, Waltham, MA, USA) and analyzed using Living Image software (Perkin-Elmer, Waltham, MA, USA). Images were collected after 1 s of exposure utilizing a 745 nm excitation and 800 nm emission filters. To depict the differences in intensity of the signal, fluorescence are represented in the images with a pseudocolor scale ranging from yellow (most intense) to dark red (least intense).

Statistical Analysis

The data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test for samples with unequal variances (GraphPad Prism 5.02; GraphPad Software, Inc., San Diego, CA, USA). Results are expressed as mean ± SEM for each experimental group unless otherwise stated and $p < 0.05$ value was selected to indicate significance.

RESULTS

Nrf2 Deficiency Exacerbates Disease Severity and Airway Dysfunction in RSV Infection

To understand the role of Nrf2 in the context of RSV infection, Nrf2 KO and WT control mice were infected with RSV (10^7 PFU/mouse) and monitored daily for clinical disease (i.e., body weight loss). Mock-infected mice did not display any signs of sickness or weight loss over the 6-day monitoring period, indicating that Nrf2 deficiency alone does not lead to clinical illness in mice. On the other hand, in RSV-infected mice, Nrf2 deficiency resulted in an enhanced disease severity compared with WT mice (**Figure 1A**). Nrf2 KO RSV-infected mice exhibited significant more body weight loss (~17%) at peak of clinical disease (day 3) than WT mice (8%, with disease peaking at day 2 post-infection). In addition, recovery from weight loss at days 4 and 5 post-infection was delayed in Nrf2 KO mice when compared with WT controls. Similar body weight loss was observed in both groups at day 6 post-infection. RSV-infected mice showed ruffle fur, but no significant difference in total illness score was observed between WT and Nrf2 KO animals (data not shown).

Next, to assess the effect of Nrf2 deficiency on pulmonary function after RSV infection airway obstruction and AHR in response to methacholine challenges were assessed by whole-body plethysmography (Buxco Electronics, Inc., Sharon, CT, USA) and expressed as enhanced pause (Penh). Nrf2 deficiency did not alter baseline Penh values or AHR to methacholine in mock-infected animals when compared with WT control mice. However, as shown in **Figure 1B**, RSV-infected WT mice developed significant airway obstruction compared with mock controls animals at day 1 post-infection ($p < 0.05$), and returning to baseline by day 5 post-infection and later time points tested (days 7 and 14). In addition, Nrf2 KO mice had significantly more airway obstruction compared to WT RSV-infected mice starting at day 1 post-infection ($p < 0.05$). In the case of AHR, RSV-infected mice showed dose-dependent increase in airway AHR in response to aerosolized methacholine at day 5 post-infection compared to mock-inoculated mice, but there are no significant differences between Nrf2 KO and WT mice (data not shown). Similarly, no differences in AHR between Nrf2 KO and WT groups were observed at later time points of infection (days 7 and 14).

Increased Viral Replication in Nrf2-Deficient Mice

To determine whether Nrf2 deficiency would alter replication of RSV in the lung, Nrf2 KO and WT mice were sacrificed at days 1 and 5 after infection and lung tissue was collected to determine virus replication and titer by qRT-PCR and by plaque assay. Compared to WT mice, significantly higher RSV N gene and genome copies number were found in the lungs of Nrf2 KO RSV-infected mice at day 1 post-infection (**Figures 2A,B**). Moreover, RSV peak titers (day 5) were significantly greater in the lungs of Nrf2 KO mice compared to WT mice ($p < 0.01$) (**Figure 2C**).

Nrf2 Deficiency Exacerbates Pulmonary Inflammation in RSV-Infected Mice

Next, we investigated whether Nrf2 could be involved in RSV-mediated inflammatory response in the lung. Mock or

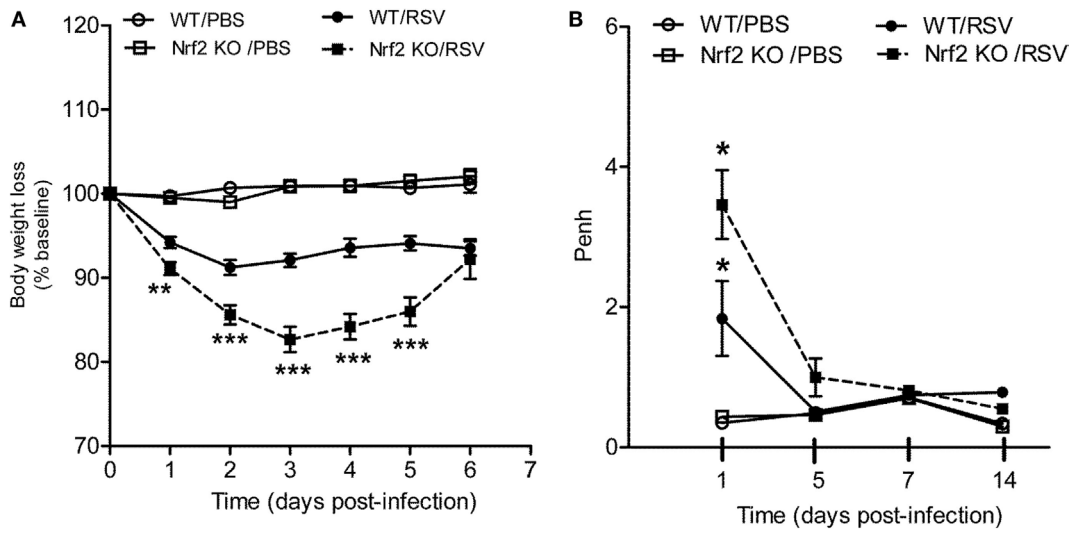

FIGURE 1 | NF-E2-related factor 2 (Nrf2) gene deficiency in mice exacerbates clinical disease and airway obstruction following respiratory syncytial virus (RSV) infection. Under light anesthesia, groups of Nrf2 $^{-/-}$ (KO) and Nrf2 $^{+/+}$ [wild-type (WT) control] mice were infected intranasally (i.n.) with 10^7 plaque-forming units (PFU) of RSV or PBS. **(A)** Mice were monitored daily for changes in body weight and disease manifestation. Data are expressed as mean ± SEM (n = 4–12 mice/group). $^{**}p < 0.01$, $^{***}p < 0.001$ when compared with WT/RSV at days 1, 2, 3, 4, and 5 post-infection. **(B)** Airway obstruction represented by baseline Penh was assessed by unrestrained plethysmography (Buxco Electronics, Inc., Sharon, CT). RSV-infected Nrf2 KO mice have increased airway obstruction compared to RSV-infected WT mice. Data are expressed as mean ± SEM (n = 3–8 mice/group). $^{*}p < 0.05$ WT/RSV compared with WT/PBS, Nrf2 KO/RSV compared with Nrf2 KO/PBS, and Nrf2 KO/RSV compared with WT/RSV at day 1 post-infection.

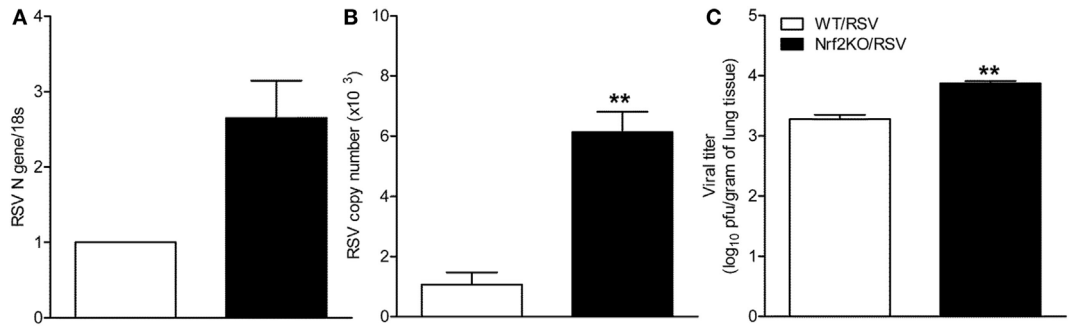

FIGURE 2 | NF-E2-related factor 2 (Nrf2) KO mice have increased viral replication in the lung. Nrf2 KO and wild type (WT) mice were infected intranasally with 10^7 plaque-forming units of respiratory syncytial virus (RSV) or PBS. Lungs were excised and viral replication was determined by quantitative real-time PCR (RT-PCR) and by plaque assay. At day 1 post-infection RSV N gene **(A)** and RSV genome copy number **(B)** in the lung were determined by RT-PCR. **(C)** Viral titration was performed at day 5 post-infection by a methylcellulose plaque assay. Data are expressed as mean ± SEM (n = 3–4 mice/group). $^{**}p < 0.01$ when compared with WT/RSV at days 1 and 5 post-infection.

RSV-infected Nrf2 KO and WT mice were sacrificed at different days post-infection to collect BAL samples for total and differential cell count. The total number of cells was significantly greater in BAL samples of Nrf2 KO compared to WT at 1, 5, and 7 days post-infection (**Figure 3A**). The increase in total BAL cells was primarily due to the increase in the number of neutrophils and lymphocytes in Nrf2 KO mice, which had concomitantly less macrophages compared to WT littermates (macrophages are mostly observed in uninfected BAL samples). No eosinophils were observed in BAL samples of either group. Furthermore, the effect of Nrf2 on pulmonary inflammation was confirmed by the pathology analysis. For histopathology studies, lungs from mock- and RSV-infected Nrf2 KO and WT mice were collected at days 5 and 7 after infection, formalin-fixed, H&E stained, and qualitatively and quantitatively analyzed using an established grading score (from 0, absent to 4, severe). Representative lung specimens for each group at day 7 after infection are shown in **Figure 3B**. Mock-infected animals from both groups had no inflammatory infiltrates in the lung (**Figure 3B**, upper panels). Lungs from RSV-infected WT mice showed mild perivascular infiltrates, mostly consisting of mononuclear cells (**Figure 3B**, left lower panel). On the other hand, lungs from RSV-infected Nrf2 KO mice showed diffuse inflammation with perivasculitis, peribronchiolitis, alveolitis, and vasculitis. Overall, quantification of the pulmonary inflammation by alveolitis and interstitial pneumonitis scores indicated significantly greater airway pathology at day 7 in RSV-infected Nrf2 KO mice compared to WT ($p < 0.05$, **Figure 3C**).

RSV Exacerbates Lung Neutrophilia in Nrf2-Deficient Mice

To further analyze the contribution of Nrf2 in the process of neutrophil recruitment to the lung observed at early time points after RSV infection, we conducted additional studies by flow cytometry. For that, lungs were harvested at day 1 post-infection. Lung single-cell suspensions were prepared from Nrf2 KO and WT mock- and RSV-infected mice and neutrophils were gated as $CD11b^+/Ly6G^+$ cells. Following RSV inoculation, Nrf2 KO mice had significantly higher total cell numbers (mostly neutrophils) compared with WT mice ($p < 0.01$) (**Figure 4A**). Neutrophil influx was significantly greater in Nrf2 KO mice ($9.55 \pm 0.428 \times 10^6$) compared to WT ($5.22 \pm 0.746 \times 10^6$). Next, to monitor neutrophil trafficking *in vivo* in real time we used the neutrophil-specific, fluorescent imaging agent, NIR. This reagent is a Cyanine7-conjugate, PEG-modified hexapeptide that specifically binds the formylpeptide receptor of neutrophils. One hour prior to imaging, NIR was administered to mock- and RSV-infected animals, either intratracheally or intravenously, and 24 h later animals were euthanized and their lungs were excised and imaged. As shown in **Figure 4B**, no fluorescence signal was detected in mock-infected Nrf2 KO or WT mice by either mode of administration of the NIR agent. On the other hand, in RSV-infected mice, the fluorescence signal was well detectable in the lungs and greater in Nrf2 KO animals compared to WT, as shown in **Figure 4C** (fluorescence intensity signal detected using the intratracheal administration of neutrophil-specific agent). Taken together, these studies provide strong evidence that RSV-induced neutrophil migration to the lung is greatly enhanced in the absence of Nrf2 gene.

Nrf2 Deficiency Increases the Release of Lung Cytokines and Chemokines After RSV Infection

Respiratory syncytial virus is a potent inducer of cytokines and chemokines, which have been shown to play an important immunoregulatory role and contribute to lung inflammation and disease severity. In our mouse model, the peak of chemokine production occurs during the first 2 days of infection (30). Thus, to investigate the role of Nrf2 in the regulation of RSV-induced inflammatory response, the concentration of cytokines and chemokines was measured in Nrf2 KO mice and compared with WT littermates. Mice were infected with PBS or RSV and at days 1, 5, and 7 after infection, BAL samples were collected from each group of mice and assessed for cytokines by a multi-plex cytokine array. Concentrations of inflammatory and immunomodulatory cytokines IL-1β, IL-6, TNF-α, IL-10, and IL-13 were significantly

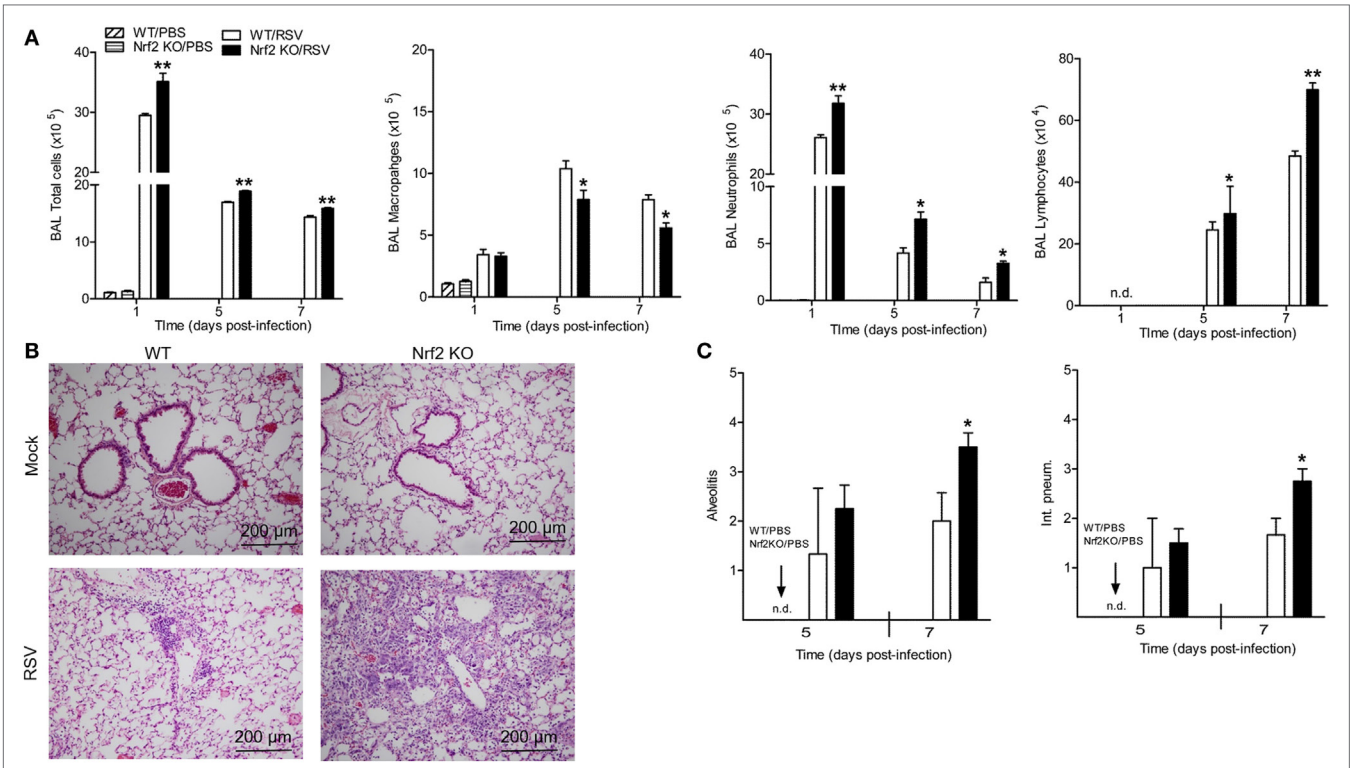

FIGURE 3 | NF-E2-related factor 2 (Nrf2) gene deficiency increases airway neutrophilia and lung inflammation in respiratory syncytial virus (RSV)-infected mice. Nrf2 KO and Nrf2 wild type (WT) mice were infected with RSV or PBS. **(A)** Bronchoalveolar lavage was collected at different time points after infection for differential cell counts by Protocol HEMA3-stained cytospins. **(B)** Lung samples were harvested at days 5 and 7 post-infection, formalin-fixed for slide preparation, and hematoxylin and eosin stained. Representative stained lung tissue sections from the indicated treatment at day 7 post-infection. **(C)** Alveolitis and interstitial pneumonitis scored on lung sections. The bar graph represents mean ± SEM (n = 2–4 mice/group). *$p < 0.05$, **$p < 0.01$ when compared with WT/RSV mice.

greater in BAL samples of Nrf2 KO mice compared to WT controls at day 1 after infection ($p < 0.05$) (**Figure 5A**). Also, we found strikingly increased levels of BAL IFN-γ at days 5 and 7 post-infection in Nrf2 KO mice compared to WT controls ($p < 0.05$). Similar results were observed with regards to chemokines, including CCL5 (RANTES), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL2 (MCP-1), and CXCL1 (KC) (**Figure 5B**). Concentrations of type I IFN-α and -β were also significantly greater in BAL samples of Nrf2 KO mice compared to WT controls (**Figure 5C**).

RSV Induced Greater NF-κB Activation in Lungs of Nrf2-Deficient Mice

The transcription factor nuclear factor (NF)-κB plays a major role in innate inflammation by controlling the expression of cytokines, inducible chemokines, as well as mucosal IFNs (23, 31, 32). We have previously shown, using a BALB/c mouse model, that RSV potently and specifically activates NF-κB *in vivo* and plays a central role in regulating RSV-induced disease and pathology (23, 33). Thus, we assessed NF-κB activation in RSV-infected mice lacking Nrf-2, as a possible mechanism underlying our findings of enhanced cytokines and inflammation in KO mice. Nuclear proteins were isolated from the lungs of Nrf2 KO and WT mice (23, 33, 34), that were either PBS inoculated or infected with RSV for 12 h were subjected to Western blot analysis with anti-NF-κB p65 antibody. The results of these experiments demonstrated a significantly greater abundance of lung nuclear p65 in RSV-infected Nrf2 KO compared to WT mice (**Figure 6**, $p < 0.05$).

Expression of AOEs and H_2S-Generating Enzymes Are Reduced in Nrf2 KO Mice in Response to RSV Infection

The Nrf2-ARE pathway regulates the expression of antioxidant and phase 2 metabolizing enzymes in response to oxidative stress (17) and RSV infection (16, 19). In addition, Nrf2 has been shown to regulate the expression of cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS) (35), two key enzymes that along with 3-MST are responsible for the synthesis of the endogenous gasotransmitter H_2S (36). We have recently shown that endogenous H_2S plays a critical antiviral function and mice that are genetically deficient in the CSE enzyme have increased RSV replication in the lung (24). Moreover, we have shown that RSV infection in human epithelial cells and in mouse lung is associated with decreased expression of AOE (16) and H_2S-generating enzymes (24). Thus, we determined whether mice lacking Nrf2 had a relative deficiency in AOE and H_2S-generating enzymes compared to WT controls following RSV infection. Lungs samples were harvested at day 1 post-infection to assess mRNA levels of catalase, Cu/Zn-superoxide dismutase

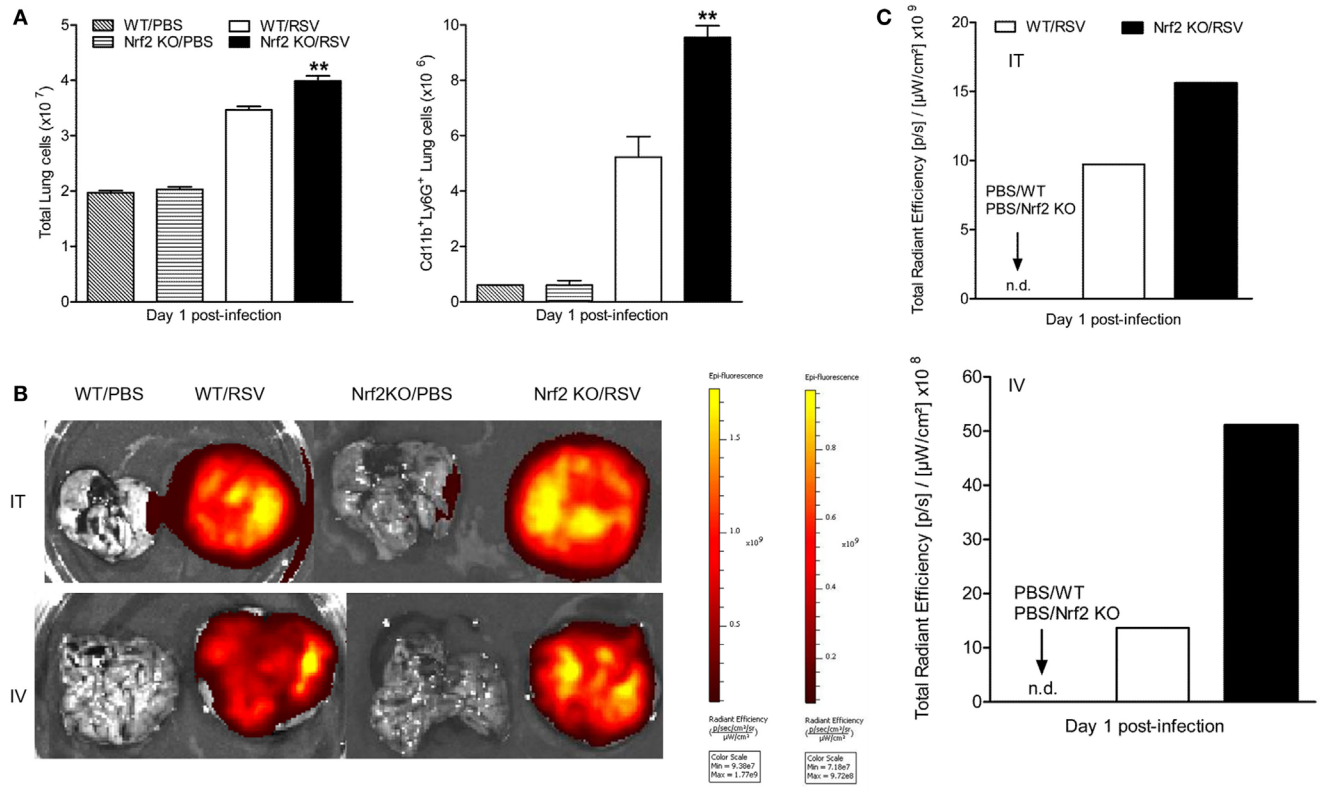

FIGURE 4 | NF-E2-related factor 2 (Nrf2) deficiency leads to increased neutrophil infiltration in the lungs in response to respiratory syncytial virus (RSV) infection. Nrf2 KO and wild typr (WT) mice were infected with RSV or PBS and lungs collected at day 1 post-infection. **(A)** Neutrophils ($CD11b^+Ly6G^+$) recruitment to the lung by flow cytometry analysis. Imaging **(B)** and quantification **(C)** of lung neutrophils using the near infrared fluorescent imaging agent by intratracheal (i.t., upper panels) or intravenous tail injection (i.v., lower panels). Fluorescence intensity is represented in the images with a pseudocolor scale ranging from yellow (most intense) to dark red (least intense) and quantified in specific total radiant efficiency per lung region of efficiency, using the Living Image software. The bar graph represents mean ± SEM (n = 2–4 mice/group). $^{**}p < 0.01$ when compared with WT/RSV mice.

(SOD1), CSE, CBS, and 3-MST. As shown in **Figure 7A**, lower levels of mRNA expression for catalase (left panel) and Cu, Zn-SOD1 (right panel) were observed in both RSV-infected Nrf2 KO and WT mice when compared with mock-inoculated mice. Noteworthy, mRNA levels for catalase, but not SOD1, were significantly lower in RSV-infected Nrf2 KO mice compared with WT ($p < 0.01$). In addition, mRNA levels of the H_2S-producing enzymes CSE, CBS, and 3-MST were significantly decreased in Nrf2 KO mice when compared with WT after RSV infection ($p < 0.05$).

Nrf2-Deficient Mice Have Greater Clinical Disease, Airway Obstruction, BAL Neutrophilia, and Cytokine Production Following Infection With hMPV

Like RSV, hMPV is a member of the *Pneumoviridae* family and we have previously shown that it induces a progressive decrease of AOE expression levels in airway epithelial cells (AECs) and in the lung of infected mice (16, 22). We, therefore, tested the contribution of Nrf2 to hMPV-mediated disease, innate responses, and pulmonary function. For that, Nrf2 KO and WT mice were infected i.n. with hMPV (5×10^6 PFU/mouse), monitored daily, pulmonary function measured, and BAL fluid analyzed at 12 h post-infection. As shown in **Figure 8A** and consistent with previous findings in experimental mouse models, hMPV-infected mice showed an initial phase of body weight loss, followed by a second phase, starting around day 5 post-infection. During both phases, hMPV-infected Nrf2 KO mice exhibited significantly more body weight loss and a delayed recovery, compared to hMPV-infected WT mice ($p < 0.05$ from days 2 to 5 p.i. and $p < 0.01$ from days 7 to 10 p.i.). Mock-inoculated mice from both groups did not play any significant weight loss over the 12 day monitoring period.

We have previously shown that hMPV infection in mice is also associated with airway obstruction and AHR, using both unrestrained whole-body plethysmography and invasive analysis of lung function (28). As shown in **Figure 8B**, hMPV-infected Nrf2 KO mice showed consistently increased baseline obstruction compared to WT-infected mice ($p < 0.05$), starting at day 1 post-infection, peaking around day 5, and returning to baseline by day 12 post-infection. They also exhibited a significant dose-dependent increase in AHR in response to aerosolized methacholine at day 1 post-infection, compared to WT mice,

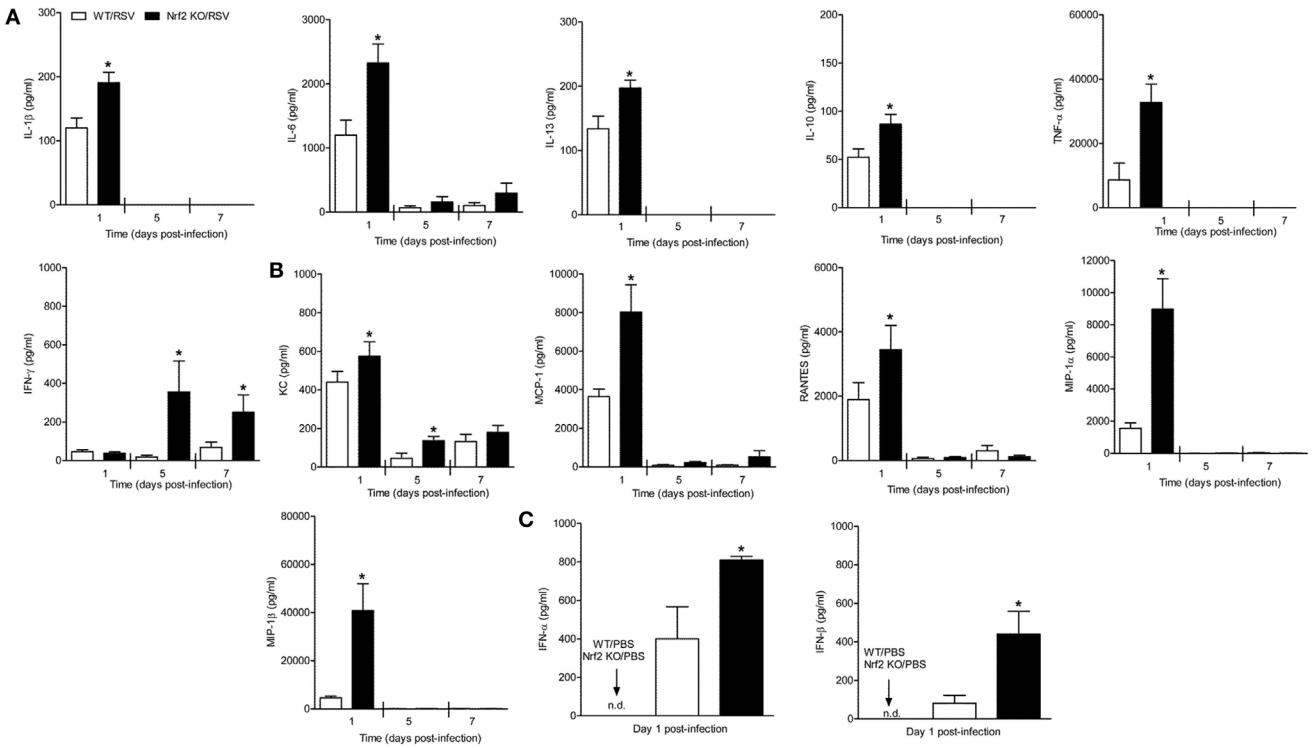

FIGURE 5 | NF-E2-related factor 2 (Nrf2) gene deficiency is associated with increased secretion of cytokines chemokines and type I interferon (IFN) in response to respiratory syncytial virus (RSV) infection. Nrf2 KO and Nrf2 wild type (WT) mice were infected with RSV or PBS. The bronchoalveolar lavage samples were collected at different time points after infection and analyzed for cytokines **(A)** and chemokines **(B)** by Bio-Plex, and for type I IFN by enzyme-linked immunosorbent assays **(C)**. The bar graph represents mean ± SEM (n = 3–6 mice/group). *$p < 0.05$ when compared with WT/RSV.

with no differences between hMPV-infected groups by day 12 post-infection (data not shown).

Airway neutrophilia is characteristic of experimental hMPV infection, including adult (28) and older mice (37). In this study, BAL samples were collected 12 h after inoculating mice with hMPV or PBS, and differential cell count was performed. We focused on the neutrophil recruitment at earlier time points post-infection based on previous observations that indicate that neutrophil recruitment peaks by day 1 after hMPV infection. As shown in **Figure 8C**, BAL neutrophil counts were greater in hMPV-infected Nrf2 KO compared WT mice ($p < 0.05$).

To further elucidate the role Nrf2 in hMPV infection, we determined the profile and concentration of cytokine and type I IFN levels and compare it to that of WT mice. Cell-free supernatants from BAL samples were analyzed by a Bio-Plex array, and tested for IFN-β production by ELISA. As shown in **Figure 8D**, levels of IL-6 ($p < 0.01$), IL-10 ($p < 0.01$), TNF-α ($p < 0.05$), CXCL1 ($p < 0.05$), CCL2 ($p < 0.05$), CCL5 ($p < 0.001$), and CCL4 ($p < 0.01$) were significantly increased in hMPV-infected Nrf2 KO compared with WT mice. No significant difference was observed in the production of other cytokines and chemokines in the array. Overall, similar to what we observed with RSV our results demonstrate that hMPV infection in Nrf2 KO mice causes increased body weight loss, airway obstruction, neutrophilic inflammation, and increased levels of proinflammatory cytokines.

DISCUSSION

The results of this study show that mice genetically deficient in Nrf2 developed enhanced clinical disease, airway inflammation and pathology, and significantly greater lung viral titers following experimental infection with RSV. In particular, we have shown that compared to control mice, Nrf2 KO mice lost more body weight and had increased airway obstruction at day 1 post-infection (**Figure 1**), a time point which was characterized by a remarkable increase in airway neutrophilia (**Figure 4**). As mentioned in the introduction, neutrophil migration to the airways is a feature of naturally acquired RSV infections in human, is considered a hallmark of bronchiolitis and is recapitulated by BAL neutrophilia in the murine model of experimental infection (38). These features of RSV infections were associated with an increased innate and inflammatory cytokines and chemokines, and overall reduced expression of the antioxidant machinery, compared to control WT mice (**Figures 5** and **7**). These findings are in agreement with our previous observations in Nrf2-competent BALB/c mice, in which RSV infection leads to a significant decrease in the expression of most AOE involved in maintaining cellular oxidant–antioxidant balance in the lung. This process is associated with a progressive decrease in nuclear protein levels of Nrf2 (16), *via* increased Nrf2 ubiquitination and its degradation through a proteasomal pathway, which

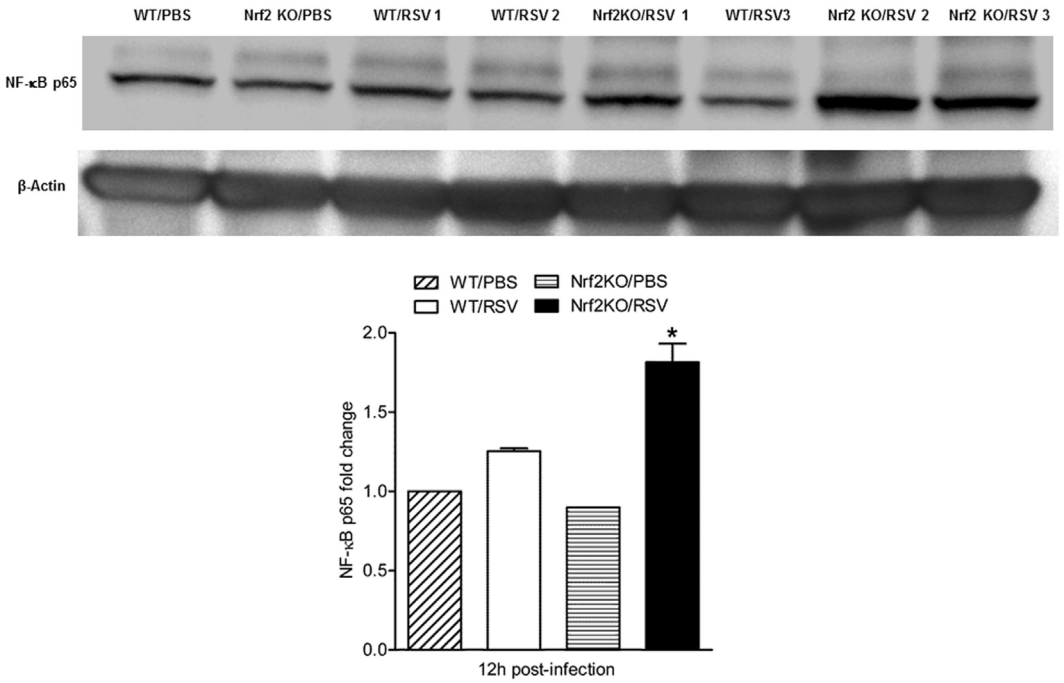

FIGURE 6 | NF-E2-related factor 2 (Nrf2)-deficient mice have enhanced NF-κB nuclear translocation in the lung. Nrf2 KO and wild type (WT) mice were infected with respiratory syncytial virus (RSV) or PBS inoculated for 12 h. Nuclear proteins were isolated from lungs and subjected to Western blot analysis with anti-NF-κB p65 antibody. For loading controls, membranes were stripped and reprobed with anti-β actin Ab. Densitometric analysis of NF-κB p65 band intensity is shown after normalization to β-actin. The groups were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. Data are shown as mean ± SEM. $^*p < 0.05$ relative to WT/RSV-infected mice.

we observed both *in vitro* epithelial cells and in RSV-infected mouse lung (39). Early innate/inflammatory events in the lung appeared to be critical in shaping the overall antiviral response and some of the disease features and as evident in our studies by the observation that peak lung viral titers at day 5 were higher in Nrf2 KO mice and lung histopathology at day 7 was characterized by significantly more alveolitis and interstitial pneumonitis compared to control Nrf2-competent mice. However, in agreement with another report (19), we did not observe sustained airway obstruction or AHR to methacholine challenge at the later time point tested.

The mechanisms underlying the increased RSV replication/gene copies in the lung of Nrf2 KO mice are unclear. Similar to our findings, adult Nrf2 KO mice on ICR background were shown to have increased peak viral replication and shedding (19). The relative defect in the antioxidant defense system and enhanced oxidative response in absence of Nrf2 (16, 19) could in part explain this finding. In previous work, treatment of AECs with the salen–manganese complexes EUK-8 or EUK-189, which possess superoxide dismutase, catalase, and glutathione peroxidase activity, strongly reduced RSV-induced ROS formation by increasing cellular AOE enzymatic activity and reduced viral replication when used at higher doses (40). In other models of experimental viral infections (influenza), exogenous treatment with the AOE catalase significantly reduced viral titers in the lung of mice (41). Nrf2 has also been shown to affect Th balance, i.e., favoring Th1 responses, while oxidative stress might be involved in the loss of naïve T cells and decrease in Th1 immunity (42). As such, changes in innate immunity, including macrophage and dendritic cell antigen presenting functions, which occur in mice deficient in Nrf2 and consequent increased oxidative stress has been shown to shift the Th1/Th2 balance (43). Although we have not conducted extensive work to address the robustness and nature of T cells responses in RSV-infected Nrf2 KO mice and potential involvement in the reduced antiviral response, we observed greater concentrations of IL-13 and IL-10 in BAL of RSV-infected Nrf2 KO at day 1 post-infection, while on the other hand levels of IFN-γ were greater in Nrf2 KO at days 5 and 7 compared to WT controls (**Figure 5**). Thus, cytokine levels in the BAL fluid suggest a skewed Th2 response only in the initial phase of infection. Choi et al. have reported a more prolonged increased of IL-13 and IL-10 in Nrf2 compared to WT mice (up to day 7 post-infection), which could explain the presence of BAL eosinophils in their study (rather than neutrophils as in our study). We suspect that the mouse background of our study (C57BL/6) and the previous study (ICR) (19), as well as the strain (long vs. A2) and dose of RSV infection (smaller dose in Choi's study) may be the reason for these contrasting findings.

Another potential explanation for the increased RSV replication is the relationship between Nrf2 and the H_2S-generating enzymes, as suggested by the significant reduction in expression of CSE, CBS, and 3-MST which we observed in RSV-infected Nrf2 KO mice (**Figure 7**). We have recently described antiviral properties of the cellular endogenous H_2S pathway. In particular,

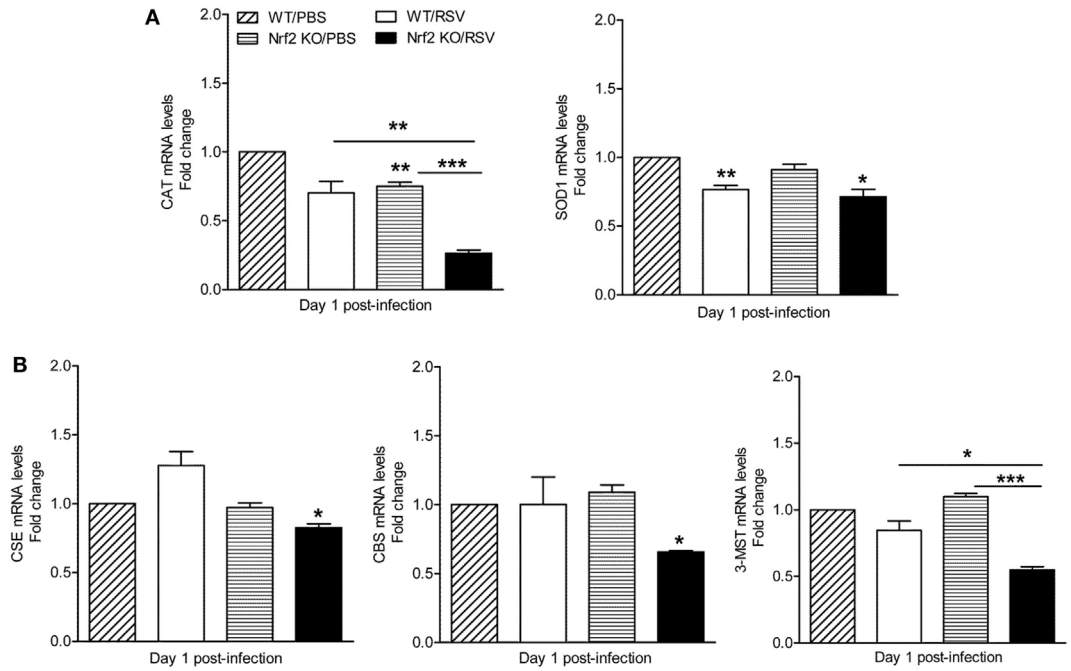

FIGURE 7 | Decreased levels of antioxidant enzymes and hydrogen sulfide enzymes in NF-E2-related factor 2 (Nrf2)-deficient mice. Nrf2 KO and wild type (WT) mice were infected with respiratory syncytial virus (RSV) or PBS and harvested at day 1 post-infection to isolate total RNA from lungs. **(A)** Catalase (left panel) and Cu/Zn-superoxide dismutase-1 (right panel) gene expression were quantified by quantitative real-time PCR (and expressed as fold change). The bar graph represents mean ± SEM ($n = 3$ mice/group). *$p < 0.05$ Nrf2 KO/RSV vs. Nrf2 KO/PBS, **$p < 0.01$ Nrf2 KO/PBS vs. WT/PBS, WT/RSV vs. WT/PBS, and Nrf2 KO/RSV vs. WT/RSV, ***$p < 0.001$ Nrf2 KO/RSV vs. Nrf2 KO/PBS. **(B)** CSE, CBS, and 3-mercaptopyruvate sulfurtransferase mRNA levels were measured by quantitative real-time PCR (expressed as fold change). The bar graph represents mean ± SEM ($n = 3$ mice/group). *$p < 0.05$ Nrf2 KO/RSV vs. Nrf2 KO/PBS, and Nrf2 KO/RSV vs. WT/RSV, ***$p < 0.001$ Nrf2 KO/RSV vs. Nrf2 KO/PBS.

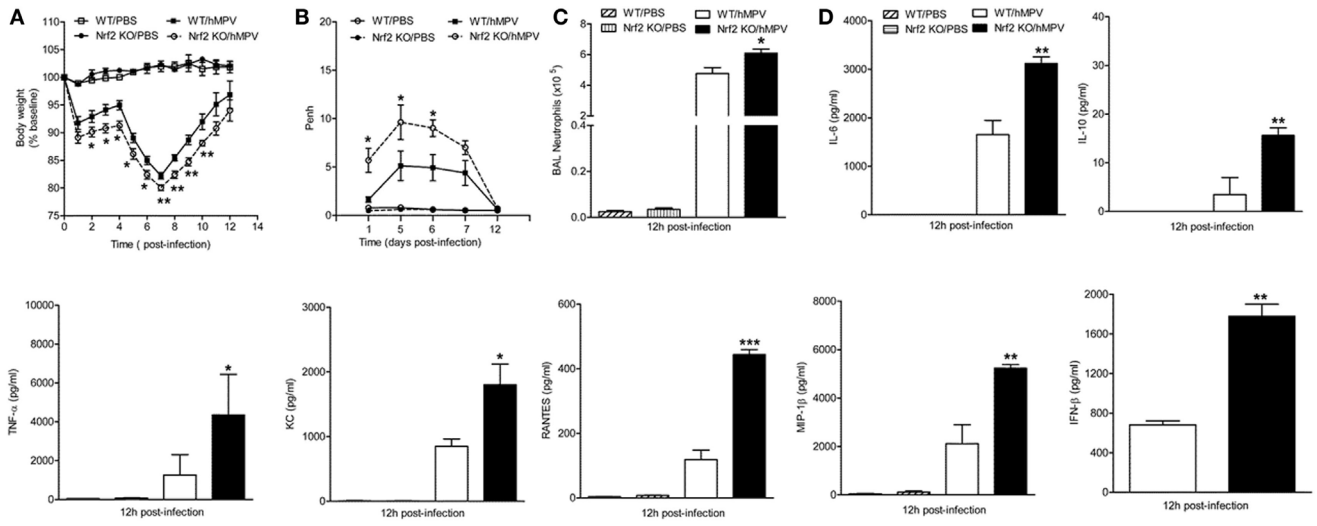

FIGURE 8 | NF-E2-related factor 2 (Nrf2)-deficient mice have greater clinical disease, airway obstruction, bronchoalveolar lavage (BAL) neutrophilia, and cytokine production following infection with human metapneumovirus (hMPV). Nrf2 KO and Nrf2 WT mice were infected with hMPV or PBS. **(A)** Mice were monitored daily for changes in body weight. **(B)** Airway obstruction was assessed by unrestrained plethysmography as baseline Penh. **(C)** Total number of neutrophils was determined by cytospin analysis of BAL samples collected at 12 h p.i. **(D)** hMPV alters cytokine/chemokine production in Nrf2-deficient mice. BAL samples were collected at 12 h p.i. from each group of mice and assessed for cytokine/chemokine production by Bio-Plex and type I interferon by enzyme-linked immunosorbent assays. Data are expressed as mean ± SEM ($n = 2$–4 mice/group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ when compared with WT/hMPV.

we have shown that inhibition of CSE, a key enzyme in the biosynthesis process of H_2S, is associated with enhanced RSV replication in human epithelial cells (26) and mice genetically deficient in CSE showed ~50% increase in RSV peak lung titer. Moreover, treatment of RSV-infected BALB/c mice with H_2S pharmacologic donors significantly reduced viral replication, indicating that endogenous H_2S plays an important role in controlling RSV replication (24). The involvement of the H_2S pathway in the observed increase in RSV replication in Nrf2 KO mice was further suggested by the significant reduction in lung titers when Nrf2 KO mice were treated with the H_2S donor GYY4137 (Figure S1 in Supplementary Material). Not surprisingly, based on the complex nature of interaction between RSV and type I IFNs, the increase in peak RSV replication in the lung of mice lacking Nrf2 occurred despite increased levels of secreted type I IFN compared to Nrf2-competent animals (**Figure 5C**).

Our results showed that levels of several innate cytokines and chemokines in BAL were significantly increased in absence of Nrf2 gene, suggesting that it plays a central role in modulating/inhibiting excessive viral-mediated production of pro-inflammatory mediators. There may be multiple mechanisms to explain the inhibitory activity of Nrf2, one being its inhibitory activity on the NF-κB transcription factor, which is the major arm of the innate immune response that controls the RSV-induced gene program in the lung. Indeed, our results in RSV-infected mice (**Figure 6**) and previous work in model of experimental sepsis (43) demonstrate greater nuclear translocation of RelA in the lung in absence of Nrf2. Although is not clear how Nrf2 interferes with RSV signaling to inhibit NF-κB, several lines of evidence, including our own work in epithelial cells and in mice indicate that the redox status of the cells modulates RSV-induced NF-κB activation (31, 44). Thus, overall reduction in the Nrf2-dependent cellular antioxidant system, which occurs in RSV infection (15, 16) and in Nrf2-deficient mice (19) and (**Figure 7**), would result in an unbalanced production of ROS in the airway mucosa (12) and contribute to the activation of NF-κB and other transcription factors, including STAT, and IRF proteins that control the innate/inflammatory gene network (13, 14). Other mechanisms underlying Nrf2-mediated anti-inflammatory properties in RSV infection could include its suppressive activity on macrophage cytokines. In this regard, alveolar macrophages (AMs) significantly contribute to the production of key innate and inflammatory cytokines in the lung in response to RSV, including type I IFN, IL-6, and TNF-α, as we showed in studies in which these cells were depleted prior to viral inoculation (25). Nrf2 has been shown to bind to the proximity of cytokine genes (including IL-6 and IL-1β) in macrophages and to inhibit RNA Pol II recruitment, a process independent of the Nrf2-binding to motif and ROS levels (45).

We have previously shown that similar to RSV, hMPV induces a progressive decrease in AOE expression levels in AECs (22) and in the lung of infected mice (16). As member of the *Pneumoviridae* family, hMPV was first isolated in 2001 and since been characterized as an important cause of acute respiratory tract infections in the young (46, 47). Recent prospective surveillance studies conducted in the US over 3–6 seasons have shown that the annual rate of hMPV-associated hospitalization in children is the same as the hospitalization rate associated with influenza virus (48). The pathophysiology of hMPV infection is largely unknown, and some of the scientific information has been extrapolated from the RSV research literature, rather than by direct or comparative side-by-side studies. Herein, we show for the first time that lack of Nrf2 in hMPV-infected adult mice is associated with increased secretion of cytokines in BAL, airway neutrophils, exacerbated body weight loss, and sustained airway obstruction (for more than a week), compared to control Nrf2-competent mice. Therefore, it appears that experimental infections with RSV and hMPV cause a spectrum of innate and inflammatory responses that are similarly affected by Nrf2-dependent pathways, although some of the evidence of oxidative-mediated injury that we have reported in human natural RSV infections has yet to be investigated in hMPV infections (16).

In conclusion, these results suggest that Nrf2 is a critical regulator of innate, inflammatory, and disease-associated responses in the airways of mice infected with viruses that are members of the *Paramyxoviridae* family. Importantly, the results of this study suggest that Nrf2-dependent genes, including those controlling the cellular antioxidant and H_2S-generating enzymes and cytokines can affect several aspects of the antiviral response, such as airway neutrophilia, clinical disease, airway obstruction, and viral replication. Translating mouse experimental models into human disease, this study and previous ones by us and others suggest a potential new mechanism of RSV pathogenesis driven by viral-mediated oxidative stress damage of the airways which is not balanced by an adequate AOE response due to direct degradation of Nrf2 by RSV and/or by functional deficits in Nrf2 and AOE gene expression, which are genetically determined or linked to age-dependent maturation (16, 19). In our previous study of nasopharyngeal secretions obtained from a relatively small population of RSV-infected children with different spectrum of airway disease severity, we found a significant increase in markers of oxidative injury and a significant decrease in AOE expression, which correlated with the severity of lung disease (16). The known developmental process of the AOE system and of the H_2S-generating enzymes that starts during fetal life and is characterized by a certain degree of immaturity in the neonatal period and early infancy may contribute to the severity of RSV infections that occur during this vulnerable period of life (49, 50). Therefore, these findings may have implications for the development of therapeutic interventions aimed to increase the antioxidant tone or control the redox state in the airways at the time of RSV infections, including measures to acutely increase Nrf2 activity. These approaches have shown significant benefits *in vitro* and in experimental animal models of RSV infections by modulating cytokine secretion, reducing lung inflammation, airway dysfunction, and viral replication (12, 19, 40).

ETHICS STATEMENT

All procedures involving mice in this study were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical

Branch at Galveston. The study has been approved by the UTMB IACUC (protocol number 9001002 and 0808049).

AUTHOR CONTRIBUTIONS

TI contributed to the design of the experiments, performed the experiments presented in this manuscript, and wrote the manuscript. RG provided the overall conceptual design of these studies, reviewed and edited the final version of the manuscript. AC contributed to the design of the study and supervised the experiments related to NF-κB assays. ES performed the pathology analysis of lungs. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00854/full#supplementary-material.

FIGURE S1 | GYY4137 treatment reduces respiratory syncytial virus (RSV) replication in NF-E2-related factor 2 (Nrf2) KO mice. Nrf2 KO and wild type (WT) mice were treated intranasally with GYY4137 (50 mg/kg body weight) or an appropriate volume of vehicle (PBS) 1 h before, 6 and 20 h after infection. Mice were inoculated with either RSV (1×10^7 plaque-forming units/mouse) or PBS. At day 5 after infection, lungs were excised and viral replication was determined by plaque assay. Data are expressed as mean ± SEM ($n = 3$ mice/group). $^*p < 0.05$ Nrf2 KO/PBS/RSV compared with WT/PBS/RSV, Nrf2 KO/GYY4137/RSV compared with WT/GYY4137/RSV, $^{***}p < 0.001$ WT/PBS/RSV compared with WT/GYY4137/RSV, and Nrf2 KO/PBS/RSV compared with Nrf2 KO/GYY4137/RSV.

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Adrenergic Signaling at the Interface of Allergic Asthma and Viral Infections

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Upper respiratory viral infections are a major etiologic instigator of allergic asthma, and they drive severe exacerbations of allergic inflammation in the lower airways of asthma sufferers. Rhinovirus (RV), in particular, is the main viral instigator of these pathologies. Asthma exacerbations due to RV infections are the most frequent reasons for hospitalization and account for the majority of morbidity and mortality in asthma patients. In both critical care and disease control, long- and short-acting β2-agonists are the first line of therapeutic intervention, which are used to restore airway function by promoting smooth muscle cell relaxation in bronchioles. While prophylactic use of β2-agonists reduces the frequency and pathology of exacerbations, their role in modulating the inflammatory response is only now being appreciated. Adrenergic signaling is a component of the sympathetic nervous system, and the natural ligands, epinephrine and norepinephrine (NE), regulate a multitude of autonomic functions including regulation of both the innate and adaptive immune response. NE is the primary neurotransmitter released by post-ganglionic sympathetic neurons that innervate most all peripheral tissues including lung and secondary lymphoid organs. Thus, the adrenergic signaling pathways are in direct contact with both the central and peripheral immune compartments. We present a perspective on how the adrenergic signaling pathway controls immune function and how β2-agonists may influence inflammation in the context of virus-induced asthma exacerbations.

Keywords: adrenergic receptor, asthma, rhinovirus, inflammation, cytokine

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RHINOVIRUS (RV)-INDUCED ASTHMA EXACERBATIONS

Asthma is a debilitating chronic disease that has a significant impact on society, including decreased quality of life, work productivity, and increased utilization of health-care resources. With total annual costs reported at $81.9 billion in the U.S. alone (1), asthma represents an enormous economic burden. Approximately 2 million annual emergency room visits and 500,000 hospitalizations have been attributed to acute asthma management in the U.S. (2), highlighting the substantial contribution of asthma exacerbations to the morbidity associated with this disease. Respiratory viral infections are commonly associated with asthma exacerbation episodes (3–6), and RVs have long been recognized as the most frequent viral contributors. The seasonality of RV-associated asthma exacerbations has also been well described, with predictable peaks of hospitalizations for asthma occurring during September epidemics of RV infection (7).

The mechanisms underlying this association between RV and exacerbations of asthma represent an area of intense investigation. The impact of the infection itself on the lung represents one potential mechanism. Although most commonly detected in upper airway samples, RVs have also

been demonstrated in lower airway fluids and cells following experimental infection of the upper airway (8–10). Paired with clinical evidence linking RV to lower respiratory tract infections in children (11–13), it is possible that RV infection directly injures airway tissues in the lower airway (14), potentially contributing to exacerbations of asthma. RV infection of airway epithelial cells (ECs) induces the expression of a range of chemokines and cytokines that promote ensuing inflammatory responses. These include such pro-inflammatory molecules as IL-8/CXCL8 (15–17), IL-6 (17, 18), CCL11/eotaxin-1, RANTES/CCL5 (19), IP-10/CXCL10 (20), and ICAM-1 (21). In turn, inflammatory cells recruited by these chemokines secrete IFN-γ and TNF-α, which in some cases can provide a direct antiviral activity in target cells mimicking type I interferon (22). Increased concentrations of inflammatory cytokines have also been demonstrated in airway samples (nasal samples and sputum) obtained from RV-infected individuals (21, 23, 24). RV-induced secretion of such chemokines may also promote asthma exacerbations by promoting an influx of immune cells such as eosinophils, neutrophils, lymphocytes, and macrophages (Mϕs) into the airway (25). Immune cells themselves have also been shown to contribute to the epithelial RV response; human monocytic cells amplify bronchial epithelial cell (BEC) chemokine production during RV infection (26) and could thus also influence asthma pathogenesis in the setting of RV infection.

In addition to the chemokines listed above, RV also induces type I IFN (IFN) expression in airway ECs. The demonstration of decreased IFN-β responses in RV-infected BECs from asthmatics led to the hypothesis that defective IFN antiviral responses could contribute to the pathogenesis of asthma exacerbations (27). While virtually all somatic cells have the capacity to produce IFN-α/β in response to infection, specialized plasmacytoid dendritic cells (pDCs) are the primary cell type to secrete IFN at high levels in response to viral infection. Furthermore, human pDCs also express the high affinity IgE receptor, enabling them to respond to both viral and allergic signals. Deficient viral-induced IFN responses have been demonstrated in virus-simulated whole-blood cultures (28, 29) and purified pDCs (30) from individuals with allergic asthma, providing further evidence for a potential role of IFN in asthma exacerbations. In addition, the link between IgE and pDC antiviral IFN responses could explain the increased risk of asthma exacerbations seen in the presence of atopy and respiratory viral infections. Allergic sensitization and elevated IgE levels are known risk factors for asthma exacerbations with RV infection (3). The magnitude of pDC IFN responses to *in vitro* viral challenge is inversely correlated with serum IgE levels. In addition, IgE cross-linking abrogates viral-induced pDC IFN production (30, 31). In a recent NIAID-sponsored trial of omalizumab in children with allergic asthma, RV-induced pDC IFN responses were significantly increased in the group who received this IgE-reducing treatment, and this improved antiviral response was associated with lower exacerbations (31, 32).

Since pDCs represent the major source of this antiviral cytokine (33), a defect in IFN production, this cell type could explain how viral infection promotes severe disease in patients with asthma. Another potentially significant effect of reduced pDC antiviral IFN production includes the effect on T helper type 2 responses. IFN has recently been shown to reverse the Th2 phenotype of CD4 lymphocytes *via* suppression of the Th2 transcription factor GATA-3 (34, 35) and to acutely inhibit IL-5 and IL-13 secretion from memory Th2 cells (36). Thus, a deficient IFN response during respiratory RV infection could contribute to the increased Th2 inflammation observed in individuals with allergic asthma.

CONTROL OF IMMUNE FUNCTION BY ADRENERGIC SIGNALING

While the use of corticosteroids and long-term β2-agonists are used for maintenance therapy for asthma sufferers, the front-line intervention for acute exacerbations driven by RV infections is the short-acting β2-agonist, ventolin (nebulized albuterol). The β2-adrenergic receptor (ADRB2) is expressed on smooth muscle cells surrounding the bronchioles, and activation of this receptor by both the natural ligand, epinephrine and norepinephrine (NE), as well as β2-agonists promotes smooth muscle cell relaxation and restored breathing capacity. Signaling through adrenergic receptors controls a myriad of physiological responses, including heart rate, respiratory capacity, and lung turgor. As such, both natural and synthetic ligands for adrenergic receptors have been chiefly used to control sepsis, heart disease, COPD, and asthma.

Innervating throughout most tissues and organs, post-ganglionic sympathetic neurons release the major neurotransmitter NE in response to various intrinsic and external stimuli. Diurnal fluctuations in the release of NE link the sympathetic nervous system to circadian rhythms. Sympathetic neurons also control the "fight or flight" response during periods of stress or fear. Upon ligand binding, adrenergic receptors can activate various G-proteins, depending upon the class of receptor and the specific cell types that express them. For example, the binding of adrenaline and noradrenaline to β2AR results in activation of Gαs (the stimulatory subunit of heterotrimeric G protein) and subsequently activation of adenylyl cyclase, increase in cyclic AMP (cAMP) concentration, and activation of cAMP-dependent protein kinase A (PKA). Depending on the cell that the receptor is engaged, PKA activation can lead to several physiological changes, including muscle contraction, cytokine secretion, and so on. Moreover, the same receptor can couple to the inhibitory Gαi and or signal through MAP kinase pathways (37–39). This complex behavior of the adrenergic receptors enables these receptors to induce cell- and context-specific physiological changes.

The ADRB2 is expressed widely on many types of immune cells, albeit at different levels of cell surface ligand binding sites (40). For example, Maisel et al. identified expression of beta-adrenergic receptor density on lymphocytes ranging from 1,000 to 2,000 receptors/cell (41). In general, ADRB2 signaling acts to suppress the level of inflammation and cytokine secretion in both innate and adaptive T cells (diagrammed in **Figure 1**). For example, recent studies demonstrated that $CD8^+$ T cell effector function was impaired in response to adrenergic receptor signaling

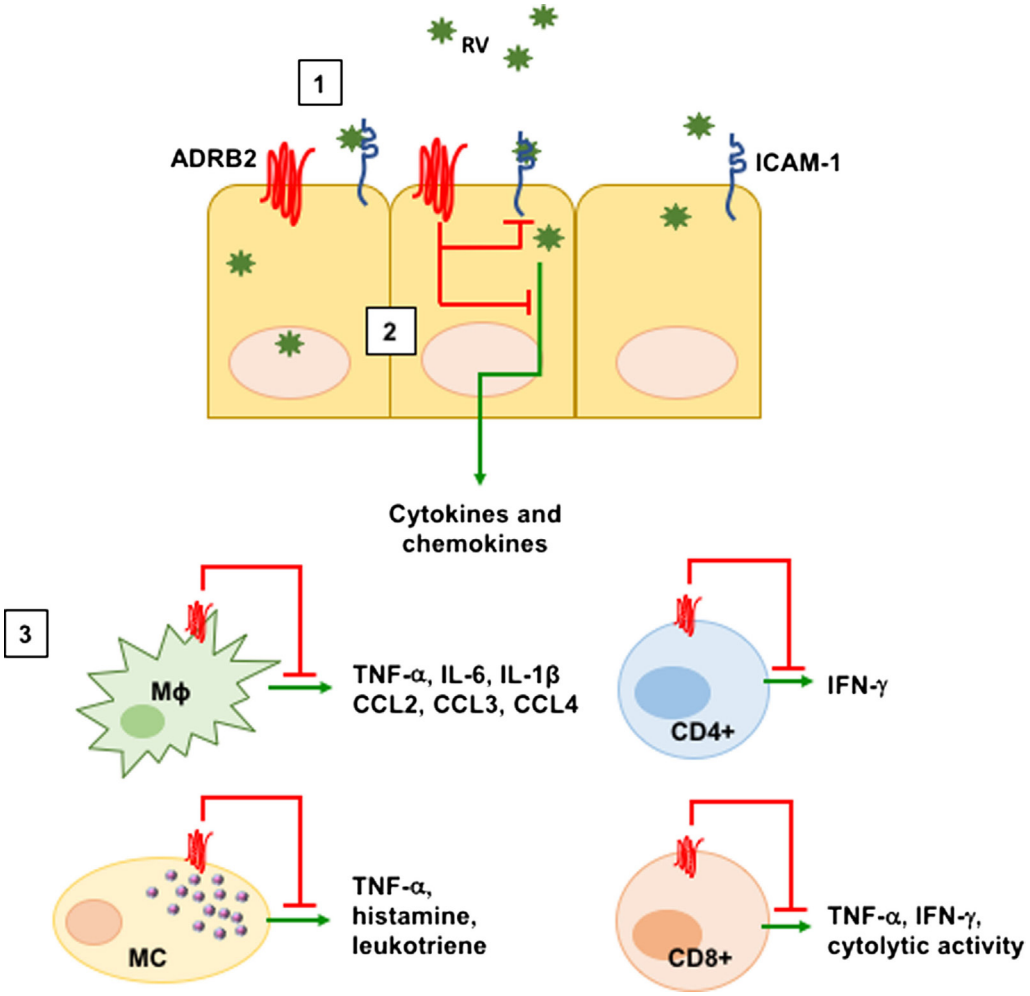

FIGURE 1 | ADRB2-mediated suppression of inflammatory processes. Adrenergic signaling through the ADRB2 inhibits various virus-induced immune mediators. [1] Rhinovirus (RV) infects the upper airways by binding to ICAM-1 on the surface of lung ECs. RV infection of ECs upregulates ICAM-1 as well as IL-8, IL-6, CCL5, CCL11, and CXCL10 to recruit inflammatory cells. [2] Activation of the ADRB2 by either the natural ligands epinephrine and norepinephrine or by β2-agonists downregulates ICAM-1 as well as IL-8, CCL5, and GM-CSF from ECs. [3] ADRB2 signaling additionally inhibits pro-inflammatory mediators in innate and adaptive immune cells. Abbreviations: EC, epithelial cell; MC, mast cell; Mϕ, macrophage.

(42–44). Presence of β2-agonists such as albuterol reduced TCR-induced IFNγ and TNFα production, as well as cytolytic activity of both human and murine T cells (43). Similarly, use of beta-blockers increased the frequency of intratumoral $CD8^+$ T cells and increased the efficacy of anti-PD-1 treatment (45). In $CD4^+$ T cells, the presence of NE increases IFN-γ production from Th1 cells (46). Although Th1 cells have been reported to be affected by NE, Th2 cells are less responsive to NE due to the reduction of ADRB2 expression during differentiation and lack of the receptor expression on mature Th2 cells (47, 48). In addition to suppressing T cell effector function, previous studies have demonstrated that ADRB2 signaling can also inhibit TNF-α and IL-12 secretion from innate cells including dendritic cells and Mϕs (49–52) perhaps through direct inhibition of TLR-mediated NF-κB activation (53, 54). Finally, ADRB2 signaling has been shown to enhance the suppressive function of Treg cells (55), which may have significance for clinical effectiveness in asthma.

ROLE OF β2-AGONISTS IN THE CONTEXT OF RV-MEDIATED INFLAMMATION

Rhinovirus infects human airway ECs by binding to ICAM-1 (**Figure 1**). As discussed earlier, the natural course of inflammation and cytokine expression increases ICAM-1 expression, allowing additional migration of inflammatory cells into sites of infection. This process likely contributes to RV-induced exacerbations in allergic subjects. Interestingly, both natural ligands of adrenergic receptors (epinephrine and NE), as well as synthetic agonists of the ADRB2 (salbutamol and terbutaline) downregulate ICAM-1 expression on monocytes (56, 57). Furthermore, human BECs reduced the expression of ICAM-1 in response to fenoterol, a β2-agonist (58), suggesting the use of β2-agonists might help patients with RV-induced exacerbations by downregulating the entry receptor on various cell types. Moreover, human airway parasympathetic neurons also downregulate expression of

ICAM-1 (59), raising the possibility that the use of β2-agonists has a broader effect than previously appreciated.

Smooth muscle cell responsiveness to β2-agonists is critical for emergency intervention during exacerbations. However, RV infection has been shown to reduce expression of the ADRB2 on airway smooth muscle cells *via* indirect actions on infected ECs. RV drives secretion of prostaglandins from ECs, which act in a paracrine fashion on smooth muscle cells to suppress ADRB2 expression (60). In this context, COX2 inhibitors tended to restore adrenergic responsiveness. Airway ECs express functional adrenergic receptors (61, 62). Stimulation of the ADRB2 increased the beat frequency of cilia (63), and fenoterol, a β2-agonist downregulates ICAM-1 (58). Sabatini and colleagues reported that salmeterol downregulated VCAM-1 in addition to ICAM-1. In addition, RANTES, IL-8, and GM-CSF were inhibited in response to adrenergic stimulation (64). In murine models, airway EC-specific expression of ADRB2 can recapitulate IL-13-induced airway hyperresponsiveness, mucus production, and cellular infiltration (65), which contrasts to the suppressive effects of β2-agonists seen in human cells.

Victoni et al. demonstrated that β2-agonists can downregulate TNFα, IL-6, and IL-1β from human monocyte-derived Mϕs; however, lung Mϕs are resistant to suppressive effects of β2-agonists (66). Similarly, chemokines CCL2, CCL3, and CCL4 were downregulated in human monocyte-derived Mϕs, yet lung Mϕs were not affected. One possible mechanism of cytokine suppression may involve targeting cytokine mRNA transcripts. For example, β2-agonist salbutamol increases the expression of tristetraproline (TTP) in murine and human Mϕ cell lines. TTP can bind to AU-rich elements in 3′UTR of several pro-inflammatory cytokine transcripts, including TNF and GM-CSF. This interaction might account for the reduction in pro-inflammatory cytokines in response to adrenergic signaling (67). Although lung Mϕs had similar levels of ADRB2 transcript, the ADRB2 protein was not expressed, which can explain why lung Mϕs may not respond to β2-agonists as efficiently as their splenic and circulating counterparts (66). β2-Agonists inhibit release of histamine and leukotriene from mast cells (MCs) *in vitro* and *in vivo* (68–70). Similarly, β2-agonists reduce histamine release from human lung MCs when cocultured with airway smooth muscle cells (71). IgE-mediated release of TNFα is also reduced in response to β2-agonists (72). These findings suggest that MC mediators that are involved in acute inflammatory responses can be controlled by adrenergic receptor agonists.

In IFN-γ-primed human dendritic cells, salbutamol inhibited IL-12, IL-1α, IL-1β, IL-6, and TNFα; however, IL-10 was unaffected. When naive T cells were primed with dendritic cells exposed to salbutamol, commitment to Th1 lineage significantly reduced (possibly due to the reduction in IL-12) (49). This is accompanied by an increase in IL-4$^+$ Th2 cells in the coculture. This suggests that use of β2-agonists may skew lung T cells to the pathogenic Th2 lineage. Similarly, in murine bone marrow-derived dendritic cells, epinephrine enhanced differentiation of IL-4- and IL-17A-producing T cells (73). In addition to T cell priming, β2-agonists also alter phagosomal degradation of antigens and cross-presentation of dendritic cells (74). Finally, Yewdell and colleagues recently demonstrated that chemical sympathectomy increased CD8$^+$ T cell responses to influenza infection in mice (42). Furthermore, ADRB2 antagonists enhanced CD8$^+$ responses, and while a direct role for the ADRB2 on CD8$^+$ T cells was not examined, this study suggests that adrenergic signaling acts to limit the response to viral infections.

FINAL COMMENTS AND FUTURE AREAS OF INTEREST

Although β2-agonists are widely used in the management of asthma and COPD, many questions remain regarding their ability to suppress inflammation in the context of exacerbations. As mentioned earlier, stimulation of ECs *in vitro* with β2-agonists downregulates ICAM-1 expression. This indicates that the use of β2-agonists can potentially reduce RV entry and spread within the lungs. Yamaya and colleagues reported that pretreatment of human tracheal ECs with tulabuterol, a long-acting β2-agonist, for 3 days before RV-14 exposure reduced the expression of ICAM-1 and viral replication in ECs (75). By contrast, Bochkov and colleagues reported that pretreatment of BECs with budesonide (a corticosteroid), formoterol (a β2-agonist), or in combination for 24 h did not alter replication of RV-16 in asthmatics and healthy subjects (76). However, the authors did not present data on the level of ICAM-1 protein. It would be beneficial to assess the role of β2-agonists *ex vivo* during RV infections to eliminate the variation from *in vitro* settings. Also, no studies to date have investigated the role of adrenergic receptor signaling on expression of RV viral proteins. Moreover, β2-agonists promote an anti-inflammatory phenotype in innate and adaptive immune cells by suppressing production of antiviral cytokines (43) and downregulate a plethora of chemokines (64) that can contribute to recruitment of inflammatory cells to the lungs. This raises the issue of the benefits versus costs of the use of long-term β2-agonists to control asthma symptoms. If β2-agonists generally suppress innate immune function, does their use allow for a more receptive environment for infection? By contrast, in the contest of overt RV-driven inflammation, β2-agonists can certainly dampen the magnitude of inflammation, which is also thought to be the main benefit of corticosteroids. Additional studies are warranted to determine the long-range effects of β2-agonists in the context of both RV susceptibility and the acute effects these drugs have on suppressing inflammation during exacerbations.

AUTHOR CONTRIBUTIONS

DA wrote drafts of Sections "Control of Immune Function by Adrenergic Signaling" and "Role of β2-Agonists in the Context of RV-Mediated Inflammation." MG wrote Section "Rhinovirus (RV)-Induced Asthma Exacerbations." JF conceived of the subject, wrote the abstract, and edited the manuscript.

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REVIEW
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Memory of Natural Killer Cells: A New Chance against *Mycobacterium tuberculosis*?

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Natural killer (NK) cells are lymphocytes of the innate immune system, which play an important role in the initial defense against a wide variety of pathogens, including viruses and intracellular bacteria. NK cells produce cytokines that enhance immune responses directed toward pathogens and also exert cytotoxic activity against infected cells, thereby eliminating the reservoir of infection. Their role in defense against *Mycobacterium tuberculosis* (*Mtb*) has been recently studied, and there is increasing evidence that highlight the importance of NK cell function during pulmonary tuberculosis (PTB), especially in the absence of optimal T-cell responses. Additionally, in the last years, it has been observed that NK cells mediate secondary responses against antigens to which they were previously exposed, an ability classically attributed to lymphocytes of the adaptive branch of immunity. This phenomenon, called "innate memory," could have important implications in the efforts to develop therapies and vaccines to improve the initial phases of immune reactions against different microorganisms, especially those to which there is not yet available vaccines to prevent infection, as is the case for tuberculosis. Therefore, the possibility of inducing memory-like NK cells ready to act prior to contact with *Mtb* or during the earliest stages of infection becomes quite interesting. However, our understanding of the mechanisms of innate memory remains incomplete. Here, we review recent literature about the mechanisms involved in the formation and maintenance of NK cell memory and the role of these cells in the immune response during tuberculosis. Finally, we discuss if the current evidence is sufficient to substantiate that NK cells exert more rapid and robust secondary responses after consecutive encounters with *Mtb*.

Keywords: natural killer cells, innate memory, memory-like natural killer cells, *Mycobacterium tuberculosis*, innate immunity

INTRODUCTION

Natural killer (NK) cells are innate lymphocytes with cytotoxic activity that cannot be classified within T- and B-cell lineages, as they differ from these lymphocytes in expression of CD56 (NCAM) and a lack of CD3 and CD19 (1). NK cells are part of group 1 of innate lymphoid cells (ILC1) (2)

and were originally referred to as "natural killers" due to their intrinsic ability to induce lysis of target cells without previous antigen exposure (3, 4). Although NK cells use non-antigen specific mechanisms to exert effector functions, a growing body of evidence supports the idea that NK cells can exert recall responses against previously recognized antigens (5). This phenomenon, referred to as "innate memory," remained unnoticed for a long time as NK cells are constitutively "ready to act" and were not thought to improve their effector capacity after repeated antigenic exposure. In contrast to this belief, multiple studies have demonstrated that NK cells become more efficient during secondary responses to viruses, haptens, and intracellular bacteria (6–12). Nonetheless, the evidence of secondary responses mediated by NK cells against mycobacteria remains controversial.

Mycobacteria are pathogens that have co-evolved with and caused infection in mammals over thousands of years, resulting in the development of many strategies to evade protective innate and adaptive immune responses (13, 14). As NK cells play a role in host defense against *Mycobacterium tuberculosis* (*Mtb*) and there is increasing evidence of the importance of NK cell function during pulmonary tuberculosis (PTB), the possibility that NK cells may develop more efficient responses upon secondary exposure to *Mtb* becomes an interesting question that needs to be experimentally explored. If NK cells were found to be more effective upon secondary exposure to antigen, this aspect of NK cell biology could be manipulated for clinical application. For example, exposure of NK cells to antigens from different strains of *Mtb* would be helpful in improving vaccine efficacy and perhaps enhancing innate immune responses against *Mtb* in the clinical landscape of tuberculosis pathogenesis. However, the mechanisms responsible for induction, maintenance, and regulation of memory-like NK cells specific for *Mtb* must be extensively investigated using *in vitro* assays, experimental animal models, and eventually in human clinical studies. In this review, we focus on the evidence generated during the last decade about NK cell memory and critically discuss the experimental data which support the hypothesis that NK cells exert more rapid and robust secondary responses after consecutive encounters with *Mtb*.

BIOLOGY OF NK CELLS

Natural killer cells represent 5–15% of circulating lymphocytes in peripheral blood and their half-life, both in humans and mice, is about 2 weeks (15). They can be found in the spleen, liver, lung, thymus, uterus, lymph nodes, and they are also recruited to locally-inflamed peripheral tissues (16). Their "homing" under normal conditions depends on the expression of chemokine receptors which guide them to various sites (17, 18).

Natural killer cell function is determined by a balance between signals from both activating and inhibitory membrane receptors. These receptors are germline encoded and do not undergo somatic rearrangement like T-cell receptors. Hence, these cells have a limited repertoire, which allow them to recognize a reduced number of ligands, many of which are related to molecules of the major histocompatibility complex class I (MHC-I) (19, 20). Induction and maintenance of NK cell function is determined by NK cell receptors binding to their respective ligand, leading to subsequent activation of intracellular tyrosine-based activating motifs or intracellular tyrosine-based inhibitory motifs (ITIM), resulting in an activating or inhibitory effect, respectively (21, 22). Under homeostatic conditions, upon NK cell receptor binding of MHC-I expressed by normal (i.e., non-tumorigenic or uninfected) cells, SHP-1 and SHP-2 protein tyrosine phosphatases are recruited to ITIM present in the intracellular domain of inhibitory NK cell receptors. This results in the arrest of tyrosine kinase-based activation signals, and no further action is taken (20). These inhibitory NK cell receptors belong to four different families of membrane-bound receptors, which recognize MHC-I molecules: the killer-cell immunoglobulin-like receptors (KIR), the immunoglobulin-like transcripts (also known as leukocyte immunoglobulin-like receptors, or CD85), the C-type lectin-like receptors (CLR) of the Natural Killer Group 2 [NKG2 heterodimers (i.e., CD94/NKG2A)], and the killer-cell lectin-like receptor subfamily A (also known as Ly49) (21, 23).

Alternatively, when mature NK cells interact with target cells expressing an abnormal or diminished level of MHC-I (such as in the case of viral infection), SHP-1 and SHP-2 are not recruited and activation signals mediated through the NK cell activating receptors are not suppressed. This leads to a predominance of activating stimuli, triggering the cytotoxic effector function of NK cells and subsequent target cell death, which promotes clearance of the infected cells. NK cells express a large range of activating receptors, which can be classified into four groups based on function and ligand interaction. First, they express the low affinity activating Fc receptor, CD16a (also known as FcγRIIIa), which recognizes the Fc region of immunoglobulin 1 (IgG1) and 3 (IgG3) and mediates antibody-dependent cellular cytotoxicity (ADCC). Second is the expression of natural cytotoxicity receptors (i.e., NKp30, NKp44, and NKp46), which belong to the immunoglobulin superfamily. The last groups of activating receptors are of the same families as some of the inhibitory receptors: the CLR-NKG2 family (i.e., NKG2D) and the KIR family. These two families of receptors induce cytokine production and cytotoxic activity after binding by MHC-I-associated molecules expressed on the surface of infected or damaged cells (i.e., ULBP, MIC-A, MIC-B, RAE1, H60, and MULT1) (23) and viral proteins that are MHC-I-like [i.e., m157 encoded by mouse cytomegalovirus (MCMV)] (24). Moreover, NK cells can directly recognize microorganisms through the interaction of Toll-like receptors (TLR) with pathogen-associated molecular patterns expressed by the pathogen (25, 26).

In addition to the wide variability of receptors that regulate NK cell activity, there are several subpopulations of NK cells with different degrees of maturation, and hence, with diverse functional profiles. In mice, the extent of expression of CD11b and CD27 discriminates between subsets of different maturity. $CD11b^{low}$ $CD27^{+}$ cells are present in bone marrow and lymph nodes and have a more immature phenotype, while $CD11b^{+}$ $CD27^{low}$ NK cells localized in blood, spleen, liver, and lung have cytotoxic capacity, the ability to produce cytokines in response to stimulation, and decreased replicative potential (27). In humans, NK cells are distinguished by the expression of CD16 and varying levels of expression of CD56, which can be used to discriminate different subpopulations of NK cells. Those cells expressing low

levels of CD56, or $CD56^{dim}CD16^{+}$, are mature, differentiated cells in the periphery with high cytotoxic activity. The $CD56^{bright}CD16^{-}$ NK cells are associated with a lower level of development and poor lytic function and produce cytokines when stimulated with IL-12, IL-15, and IL-18 (28). These subpopulations are frequently located within lymph nodes, sites which have been considered centers of production and maturation of NK cells (such as the thymus is for T cells), and required for the development of NK cell "self-tolerance" (29–33).

Although much of the role of NK cells in tumor surveillance and in the defense to infectious agents is related with their cytotoxic properties, cytokine production is also a prominent function that places these cells as key orchestrators and regulators of innate and adaptive immunities. Along with IFN-γ and TNF-α, the main NK cells-derived cytokines that exert proinflammatory effects on other hematopoietic and non-hematopoietic cells in response to infection (34, 35), there is a growing list of soluble mediators that can also be released after activation of NK cells in different settings, including regulatory IL-5, IL-10, IL-13 (36–38), pro-inflammatory IL-17 (39), epithelial regenerative and protective IL-22 (40, 41), the growth factor GM-CSF (42), as well as chemokines MIP-1α, MIP-1β, IL-8, and RANTES (43–46). Such ability is triggered principally in response to other exogenous cytokines that supports the proliferation and function of NK cells (47), but as occurs with the cytotoxic activity, recognition of membrane-bound ligands on target cells also regulates the production of different mediators by certain NK cell subsets in humans (48). Moreover, NK cells can produce IL-2 to promote their proper activation after clustering in multicellular groups and come into cell-to-cell homotypic interactions (49).

ROLE OF NK CELLS IN THE IMMUNE RESPONSE AGAINST *M. tuberculosis*

Natural killer cells play an important role in the immune response against viruses and intracellular bacteria due to their ability to kill infected cells and to eliminate intracellular pathogen reservoirs (50). There are several examples in which NK cell activity is crucial for disease control; possibly, one of the most well-documented examples is the role of NK cells during cytomegalovirus (CMV) infection (34, 50–54). However, the participation of NK cells in the immune response during PTB has been underappreciated, despite the fact that NK cells are an important source of IFN-γ, a cytokine vital to the immune response against *Mtb* through activation of macrophages, and subsequent enhancement of bactericidal activity (55). Furthermore, NK cells produce IL-22, a cytokine that has been shown to have a protective role during chronic stages of infection by emergent hyper-virulent strains of *Mtb* (56, 57).

In spite of the historic lack of attention received by NK cells during *Mtb*-associated immunity, mounting evidence suggests that NK cell function is important during PTB. First, several studies in humans have revealed that the risk of developing the active form of disease is related with the phenotype and functional state of NK cells isolated from peripheral blood. In fact, there is a higher prevalence of the KIR group A haplotype (expressing more inhibitory KIR) among patients with active tuberculosis when compared with resistant individuals. Indeed, certain KIR genes have increased correlation with the likelihood to become ill (i.e., KIR3DL1, KIR2DL3, KIR2DS1, and KIR2DS5), whereas others associate with a protective phenotype (i.e., KIR3DS1, KIR2DS2) (58–61). Portevin et al. confirmed these observations by demonstrating NK cells from healthy individuals' respond to direct contact with *Mtb* and *M. bovis* BCG and that the degree of such responses was dependent on the KIR haplotype. Also, they observed that NK cells are recruited into the lung lesions of patients with chronic *Mtb* infection (62).

When cultured in the presence of live *Mtb* bacilli, both subpopulations of human NK cells ($CD56^{bright}$ and $CD56^{dim}$) respond and exert effector functions (63). However, in patients with active PTB, there is reduced frequency of $CD56^{bright}$ cells in the peripheral leukocyte population, accompanied by decreased expression of NK cell activating receptors (NKp30, NKp46), resulting in declined functional capacity (64, 65). This functional impairment of NK cells is also associated with an increase in $CD4^{+}$ $CD25^{+}$ regulatory T cells (T_{reg}), which may regulate the activity of NK cells (66). It is unknown if such alterations in NK cell functionality are related to the risk of bacterial dissemination to extrapulmonary sites.

Although the evidence mentioned earlier supports a role for NK cells in the defense against *Mtb* in patients with chronic infection, it is important to remember that these cells are innate in nature and act early during microbial defense (50). Functional assessment of human NK cells during initial stages of PTB is difficult, as most patients with pulmonary disease are diagnosed long after initial contact with the bacillus. Therefore, studies in different animal models have been conducted to evaluate NK cell activity in the early phases of the immune response against tuberculosis, with contradictory results. Specifically, Feng et al. showed that during *Mtb* infection of *Rag1*$^{-}$ deficient mice (which lack T and B cells), NK cells drive the immune response and resistance against PTB by secreting IFN-γ (67). In contrast, when *Mtb* infection occurs in T- and B-cell sufficient animals, NK cell depletion does not influence *Mtb* bacterial burden in the lungs or disease severity (68). This is in agreement with the evidence, both in mice and humans, of the redundancy of ILC activity in the context of a complete adaptive immune system (69, 70). Despite the perhaps redundant role NK cells play during tuberculosis in immunocompetent individuals, their role in protection against *Mtb* could be of particular relevance in cases of T-cell dysfunction, i.e., in individuals infected with human immunodeficiency virus (HIV). This concept is of great importance, as HIV/AIDS is the leading comorbidity in *Mtb*-infected individuals, and according to the 2015 WHO Tuberculosis report, 35% of HIV-related deaths were due to PTB (71).

Recent data suggest that NK cells may modulate the development of mycobacteria-specific adaptive immune responses. In this regard, Vankayalapati et al. demonstrated that NK cells isolated from human peripheral blood can regulate effector functions of $CD8^{+}$ T cells in response to *Mtb* infection. NK cell depletion reduced the frequency of $CD8^{+}$ IFN-γ^{+} T cells and decreased their capacity to lyse infected macrophages after *in vitro* exposure to *Mtb* bacilli (72). In addition, NK cells induced lysis of expanded

$CD4^+$ $CD25^+$ T_{reg} after incubation with *Mtb*-infected monocytes; this lysis was shown to be dependent on NK cell NKG2D recognition of ULBP-1 on the T_{reg} surface (73).

In vitro assays have also revealed specific interactions between NK cells with infected phagocytes as well as with bacilli in their extracellular form. Specifically, it was shown that NK cells efficiently recognize certain *Mtb* and *M. bovis* BCG cell wall components through TLR-2 and NKp44 receptors (26, 74, 75). Of note, NKG2D and NKp46 activating receptors are ligated by ULBP-1 and vimentin, respectively, whose expression increases on the surface of macrophages that have ingested bacteria (76–78). After recognition of these ligands, NK cells release IFN-γ and IL-22, increasing the bactericidal capacity of infected phagocytes and may perform cytotoxic measures against these infected cells to eliminate intracellular pathogen niches (79, 80). Moreover, activated NK cells kill extracellular mycobacterial bacilli by releasing perforin and granulysin, *via* a mechanism dependent on intracellular signaling pathways mediated by kinases such as ERK, JNK, and p38MAPK (81). Production of proinflammatory cytokines, specifically IFN-γ, also may be triggered by direct contact with *Mtb* antigen; this activity is enhanced by cell-to-cell interaction with dendritic cells and associated IL-12 signaling (82).

Finally, NK cells play a role during the development of protective immunity conferred by vaccination. Murine exposure to BCG was shown to increase the number of IL-22-producing NK cells, and NK cell depletion induced an expansion of $CD4^+$ $CD25^+$ T_{reg} during re-challenge with *Mtb* H37Rv as well as increased bacterial burden and diminished T-cell responses (83).

IMMUNOLOGICAL MEMORY MEDIATED BY NK CELLS

Natural killer cells are classically categorized as members of the innate immune system, as it was thought that repeated encounter with antigen did not augment or enhance their effector functions. Notably, in 2006, Leary et al. demonstrated that NK cells had adaptive memory qualities by using a mouse model of hapten-induced contact hypersensitivity (CHS) (5). In the absence of both T and B cells, NK cells were sufficient to mediate CHS responses in a hapten-specific manner, as CHS only occurred in those instances where the hapten used during challenge was the same hapten used during sensitization. This study also supplied a more structured understanding of NK cell function and delineated the mechanisms NK cells use to circulate within lymphatic vessels and access lymph nodes, where antigen priming occurs. Once NK cells have been activated in secondary lymphoid organs, they migrate to the liver and reside there until antigen re-exposure, then home to sites of antigenic challenge. Moreover, transfer of liver-resident and not spleen-resident memory-like NK cells into healthy mice resulted in transfer of the hypersensitivity phenotype (5). Further analyses revealed later that liver NK cells with the ability to exert secondary responses to haptens belong to a specific subset (denoted by $CD49a^+$ $DX5^-$) and that recall responses to subsequent contacts with haptens are dependent on the activity of NLRP3 inflammasome (84, 85).

Sun et al. subsequently found that MCMV infection elicited an immune response mediated by NK cells, which emulated all the hallmarks of a traditional memory response (7), characterized by an initial proliferation phase with clonal expansion of NK cells expressing the Ly49H receptor (which recognizes the m157 protein of the MCMV). This clonal expansion was dependent on Ly49H/DAMP12 signaling (receptor ligation, or "Signal 1") and IL-12 priming through STAT-4 activity (cytokine help or "Signal 2"), as well as co-stimulation by DNAX accessory molecule 1 (co-stimulation or "Signal 3") (7, 86). After this proliferation phase, contraction of effector NK cells occurred by mitochondrial apoptosis and was followed by the development of a pool of memory cells, which survived cell death through mechanisms of mitophagy (87). Similar to T-cells (88), NK cells required IL-15 signaling as well as the regulatory activity of miR155 on Noxa and SOCS1 for optimal differentiation into memory-like NK cells (89, 90). This pool of memory-like NK cells resided in the spleen and other lymphoid and non-lymphoid organs. Interestingly, transfer of MCMV-memory-like NK cells into neonate mice resulted in protection from a lethal challenge of MCMV (7).

Similar to the findings in MCMV-infected mice, clonal expansion of CD94/$NKG2C^+$ NK cells was observed in humans seropositive for human cytomegalovirus (91–93) as well as in transplant recipients who either underwent CMV reactivation or were seronegative and had received organs from seropositive donors (94, 95). These data show that transfer of NK cells with long-lasting survival and enhanced antigen-specific activity is a viable immune mechanism in humans as well as mice. Clonal NK cell expansion in humans was also observed in response to other viral infections, such as hantavirus, chikungunya, hepatitis B virus, hepatitis C virus, and HIV (96–100). Further evidence of memory and clonal expansion of NK cells in response to viral infection was found using mouse models of immunization with antigens of genital HSV-2 virus (12), influenza virus, vesicular stomatitis virus, and even HIV, a pathogen that does not infect mice naturally. Moreover, this virus-specific immune response by NK cells and the associated protection are mediated only by those NK cells isolated from the liver expressing CXCR6 (6). Interestingly, influenza vaccination of healthy humans enhanced the amount of IFN-γ produced by NK cells up to 6 months post-immunization (101). These data support that human NK cells, apart from expanding during antigen encounter, also display a better response in subsequent exposures to viral antigens, similar to that which occurs in mice. Although most of these recall responses occurred in the context of viral infection, it was recently documented that memory-like NK cells were expanded in response to infection with the intracellular bacterium *Ehrlichia muris* (9). This pathogen belongs to a genus of which it was recently reported that NK cells play an important role in the associated inflammatory responses (102). In addition, human and mouse NK cells display enhanced activity when they are primed with cytokines and later re-exposed to the same stimuli in conjunction with engagement of activating receptors by antibodies or cognate ligands (103–105).

Collectively, these studies support that immunological memory is an intrinsic property of NK cells conserved in vertebrates. Of note, NK cells have secondary responses against both previously

unencountered antigens and antigens that have not represented an evolutionary pressure, as in the case of haptens and HIV infection, respectively, in murine models (5, 6). In addition, it has been observed that HIV elicits memory of long duration in macaques, a mammalian species that, as the mouse, is not the natural host of this virus (8). Nevertheless, there are several aspects of innate memory of NK cell subsets that remain poorly understood. One question is whether NK cells with the ability to mediate memory responses belong to a specific subgroup of conventional NK cells or if they are in fact lymphocytes of a different subset within the group 1 ILC? Second, what markers and patterns of expression can be used to identify these memory-like NK cells? To date, it is well established in mice that memory-like NK cells are located preferentially in the liver, but their specific phenotype has only been described in mouse models using haptens and MCMV (5, 7, 84). It will be important to address if there is a specific NK cell subset induced by certain paired NK cell receptor-ligand interactions, or associated with different classes/types of pathogens and subsequent responses.

EVIDENCE OF MEMORY AGAINST *M. tuberculosis* WITHIN NK CELL SUBSETS

Different groups have recently sought to identify memory responses mediated by NK cells against mycobacteria, and the results have been contradictory, proving it difficult to define the importance of human NK cells in the immune pathogenesis of tuberculosis. NK cells isolated from pleural fluid of subjects with pleural effusion associated with active PTB express the memory marker CD45RO and produce higher amounts of IFN-γ and IL-22 in response to stimulation with interleukins and *M. bovis* BCG when compared with CD45RO- cells (106, 107). As these NK cells were isolated from a specific inflamed anatomical site, it would be of great interest to assess whether peripheral and tissue resident NK cells also express CD45RO and if this expression confers enhanced effector functions against different species of mycobacteria.

Additional evidence in favor of memory against *Mtb* within NK cell subsets has been provided by Suliman et al., who found that BCG re-vaccination of subjects latently infected with *Mtb* induced long-term responses in NK cells, which persisted for up to 1 year after re-challenge (108). In this study, they also found that BCG vaccination of humans at birth induced augmented activity of NK cells in 5-week-old neonates. Another study also showed that BCG vaccination in healthy subjects promoted the production of proinflammatory cytokines, particularly IL-1β, by NK cells in response to *Mtb* and other fungal pathogens 3 months after the initial exposure (109).

Meanwhile, Kawahara et al. were unable to demonstrate enhanced secondary responses by NK cells isolated from mice vaccinated with BCG (110). Using a mouse model of immunization with BCG, this group did not find functional differences between NK cells isolated from the spleens of vaccinated and unvaccinated animals, particularly in their capacity to produce cytokines after *in vitro* exposure to *Mtb* H37Rv. Also, NK cells stimulated *in vitro* with BCG antigens did not have increased cytotoxicity against *Mtb*-infected macrophages (110). As Leary et al. had shown that the liver-resident NK cells were the subset responsible for enhanced responses (5, 111), it would have been beneficial to assess memory responses in NK cells isolated from tissues other than spleen, i.e., the liver, in these BCG vaccinated mice.

However, a recent study using an experimental model of *Mtb* infection in mice showed that BCG vaccination promoted an IL-21-dependent expansion of a $CD3^{-}NKp46^{+}$ $CD27^{+}$ $KLRG1^{+}$ memory-like NK cell subset residing in lymph nodes and spleen. These cells produced higher amounts of IFN-γ compared to other subsets of NK cells and conferred protective responses against *Mtb* upon transfer to unvaccinated animals. This finding was also confirmed *in vitro* using latently infected human cells: $CD3^{-}CD56^{+}$ $CD27^{+}$ NK cells were able to reduce *Mtb* CFU when co-cultured with autologous infected macrophages more effectively than $CD3^{-}CD56^{+}$ $CD27^{-}$ NK cells. In addition, the responses of these $CD3^{-}NKp46^{+}$ $CD27^{+}$ $KLRG1^{+}$ cells were specific as *in vitro* exposure to *Candida albicans* did not induce NK cell expansion, as seen with *Mtb*. This study is possibly the first evidence of the existence of a subpopulation of NK cells with the ability to mount recall responses against repeated exposures to *Mtb*, highlighting the potential usefulness of memory-like NK cells to improve the efficacy of vaccination against *Mtb* (112).

CHALLENGES

It is important to mention that studies in humans that observed memory-like responses in NK cells isolated from pleural fluid in the context of tuberculous pneumonia are only descriptive and did not evaluate the parameters which characterize an effective adaptive immune response. This is the same situation for the work done by Kawahara et al. (110) whose data, although debatable, cannot be ignored. The fact that certain subsets of human NK cells respond more robustly after re-exposure to several strains of mycobacteria and to activation with non-antigenic stimuli does not necessarily mean that these are memory cells, even when such cells express markers classically attributed to adaptive lymphocytes with the ability to improve their function after subsequent contacts with pathogen-derived antigens. Although they might belong to the memory-like NK cell subset supposedly induced by cytokines, which mediate secondary responses to specific and non-specific re-challenges, the exact mechanisms that trigger their development and maintenance have not been vigorously evaluated. Additionally, we cannot rule out the possibility that these $CD45RO^{+}$ NK cells were expanded in response to other pathogens and became involved in the immune response to *Mtb* through a bystander mechanism.

At the same time, the lack of differences observed by Kawahara et al. in the splenic NK cell response to *in vitro Mtb* exposure between BCG vaccinated and unvaccinated animals does not mean that these cells do not "remember" previous antigen encounter and cannot exert secondary responses. Perhaps it is that these cells are simply not the subset capable of mounting a secondary response and it is only a matter of finding cells from an anatomical location, which harbor the effective subset, such as the liver.

Therefore, an animal model is needed, which allows detailed evaluation of clonal NK cell proliferation in response to PTB, as that which occurs in case of MCMV infection (7). However, this will be difficult to achieve as there are no sufficient mouse models of tuberculosis that strictly emulate the natural infection, which occurs in humans, and there is a lack of an NK cell receptor that can easily identify or define clonal NK cells with antigen specificity. In addition, we still have no markers to delineate exactly which subpopulations of NK cells are able to develop memory-like qualities in mice. In this regard, it will be important to identify mouse NK cell subsets currently known to expand in response to *Mtb* infection, as well as those populations which possess surface molecules implicated in previous studies with other pathogens. Also, pathogen species-difference specificity will need to be assessed to understand the breadth of memory NK cells induced upon antigenic stimulation. Moreover, this model must address whether memory-like NK cells migrate to a specific anatomic site and evaluate whether these presensitized NK cells confer protection when transferred to animals that have not previously come into contact with the pathogen. This last point is likely the most important aspect that future research should evaluate. Although the pleural effusion human studies support the existence of NK cell memory, they also call into question whether memory responses mediated by NK cells play a protective or pathogenic role, as the patients in this study developed severe disease complications. Moreover, although Suliman et al. observed that vaccination of neonates with BCG at birth induced enhanced responses in NK cells 5 weeks later (108), long-term follow-up is needed to find out if these responses persist indefinitely and whether they influence the risk of active PTB in the future, independently of the induction and activity of memory T cells.

In this regard, the study performed by Vankayalapati and colleagues utilizes almost all of the features that we propose an experimental model must possess to characterize immune memory mediated by NK cells in the context of *Mtb* infection.

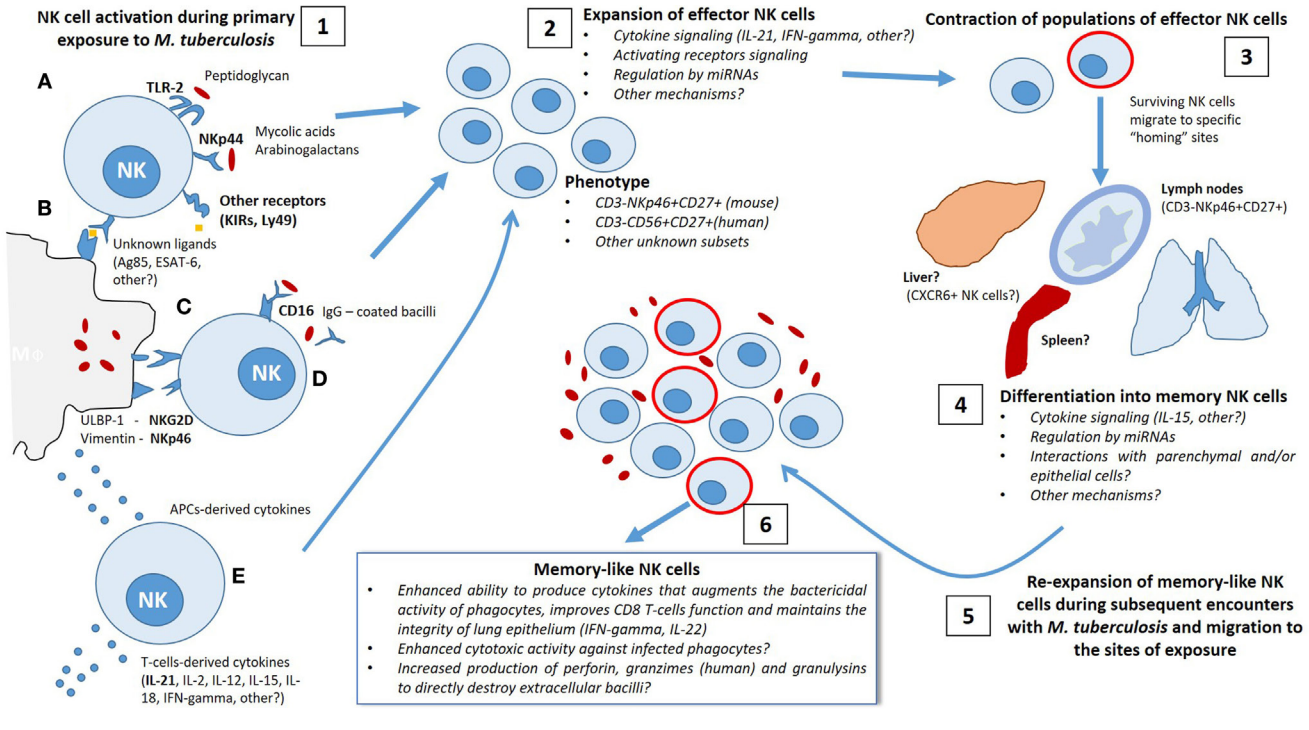

FIGURE 1 | Possible mechanisms implicated in the differentiation of memory-like natural killer (NK) cells during the immune response elicited by *Mycobacterium tuberculosis* (*Mtb*) infection. 1. After primary exposure to *Mtb*, either by natural infection or vaccination, NK cells become activated by distinct antigenic and non-antigenic stimuli, allowing involvement in the initial defense against the bacillus. Direct antigenic stimulation may be provided by **(A)** direct interaction of *Mtb*-derived antigens (i.e., peptidoglycan, mycolic acids, arabinogalactan and other unknown ligands) with activating NK receptors or by Toll-like receptors engagement or **(B)** NK receptor binding to antigen presented in the context of major histocompatibility complex class I expressed on the surface of infected macrophages and/or dendritic cells. Non-specific activation of NK cells could be induced by **(C)** interaction with danger signals expressed at the membrane of infected phagocytes, such as Vimentin/NKp46 interactions, or **(D)** CD16-mediated antibody-dependent cellular cytotoxicity triggered by binding to the Fc regions of opsonized IgG-coated bacilli, as well as **(E)** priming with macrophage-derived and T-cell-derived cytokines. 2. Activation of NK cells elicits a rapid expansion of effector clones with an specific phenotype ($CD3^{-}NKp46^{+}$ $CD27^{+}$ $KLRG1^{+}$, others) in a cytokine-dependent manner (IL-21, IFN-γ, others), which may require additional mechanisms of regulation, potentially by miRNA. 3. Following the initial phase of proliferation, there is a contraction of effector NK cell population and the surviving cells become long-lived and migrate to specific "homing" sites to differentiate into memory-like NK cells. 4. It is possible that certain tissues contain the specific requirements needed by different subpopulations of NK cells to improve their function and to exert secondary responses. 5–6. Subsequent encounters with *Mtb* trigger a re-expansion of several subsets of memory-like NK cells with enhanced ability to respond to antigenic and non-antigenic stimuli depending on the signals received during the first mycobacterial exposure.

However, it would be of great interest to address if there is expansion of additional subsets of memory-like NK cells in tissues other than spleen and lymph nodes (i.e., the liver) and if such cells confer protection against *Mtb* infection. Also, other mechanisms regulating the proliferation and differentiation of memory-like NK cells must be evaluated, including a broader spectrum of cytokines that might be involved in the expansion of $CD3^{-}NKp46^{+}$ $CD27^{+}$ $KLRG1^{+}$ NK cells as well as other subpopulations with distinct phenotypes. Moreover, further analysis is needed to reveal the receptors and *Mtb*-derived antigens implicated in the activation and expansion of memory-like NK cells. Use of blocking antibodies and/or knockout animals lacking different NK cell membrane receptors would allow further study of these mechanisms. Finally, to determine whether expanded NK cell subsets protect from disease in humans, a comparative study is required that includes, in addition to subjects latently infected with *Mtb*, both individuals with active PTB and those with disseminated disease.

In **Figure 1**, we show the possible signals involved in induction of NK cell secondary responses by several specific and non-specific stimuli. We also highlight the mechanisms implicated in the differentiation of a subset of memory NK cells capable of responding more efficiently during repeated contact with *Mtb*.

ARGUMENTS IN FAVOR OF THE EXISTENCE OF NK CELL MEMORY AGAINST *M. tuberculosis*

The fact that NK cell responses against haptens, viruses, and intracellular bacteria are characterized by hallmarks of adaptive immunological memory supports the hypothesis that NK cells can become sensitized and memory like in response to repeated exposure to *Mtb*. Despite current controversial evidence supporting the existence of memory-like NK cells and their role in the immune pathogenesis of tuberculosis and that these findings must be confirmed in experimental animal models and in humans, we consider there are at least two reasonable arguments to support this assertion. First, some of the studies that defined memory-like qualities of NK cells were performed using animal models with infection by pathogens to which it is still unclear whether NK cells play an important role in the defense against them (6, 8). Second, this memory-like quality of NK cells was identified using a model of delayed hypersensitivity induced by haptens (5) and there is little evidence to support why NK cells would have need to "remember" a hapten encounter. Thus, it would appear that memory is an intrinsic characteristic of NK cells, independent of the target antigen.

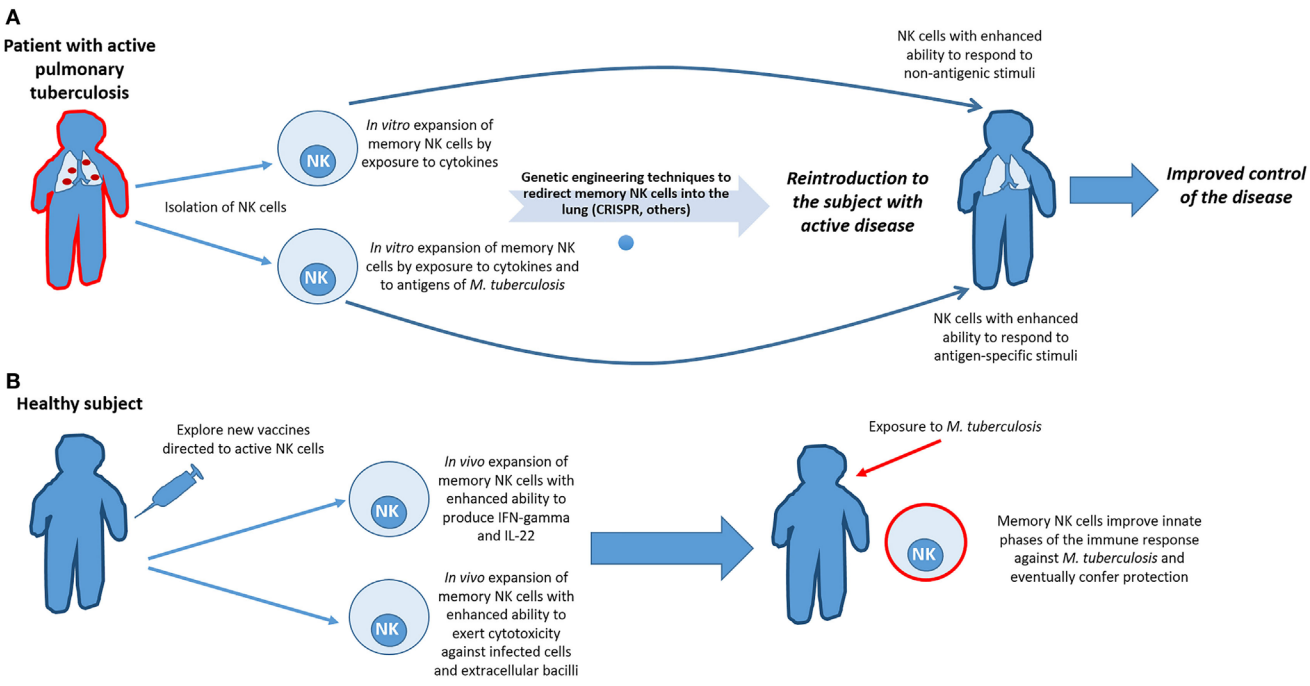

FIGURE 2 | Harnessing the adaptive features of natural killer (NK) cells to improve the clinical evolution of tuberculosis and to augment vaccine efficacy. **(A)** In patients with active pulmonary tuberculosis, and even in those latently infected, NK cells could be isolated and primed through *in vitro* exposure to cytokines and/or *Mycobacterium tuberculosis* (*Mtb*) antigens, in order to expand subsets of memory-like NK cells with the enhanced ability to respond to antigenic and non-antigenic stimuli after recurrent bacterial encounters. Redirecting them to the sites of exposure would be benefit from the use of techniques of genetic engineering currently available. However, due of the cost of such proceedings, clinical applicability would be reserved for patients with T-cell deficiencies or for those patients infected with multidrug-resistant strains of *Mtb*. **(B)** Another approach to improve the initial phases of the immune response against *Mtb* is to explore novel ways of inducing vaccination to specifically activate NK cells. If vaccination could indeed induce proliferation and differentiation of memory NK cells that would create a subset of cells ready to act prior to encounter with *Mtb*, thereby enhancing innate immunity against the bacillus and perhaps, eventually prevent infection.

Although they do not possess antigen receptors generated by genetic rearrangement, NK cells do have receptors which allow direct antigenic contact, resulting in subsequent cellular activation (74, 75). Of note, while direct contact with target antigen is an important step in the generation of memory T cells, this step is not necessary for the development of memory-like NK cells. NK cells also undergo secondary responses following activation by cytokines (103, 104). In the context of proinflammatory environments found at the sites of infection, this cytokine stimulation may augment NK cell function, as is seen when $CD8^+$ T cells differentiate into antigen-non-specific memory cells after being activated with cytokines through a "bystander" mechanism (113).

Finally, species of the mycobacterium complex have infected hominids for the past 3 million years and have supplied a constant evolutionary pressure for innate and adaptive cells of the mammalian immune system (13, 14). There is evidence of immunological memory existing prior to the emergence of lymphocytes (114, 115), and NK cells, as well as their progenitors, are more ancient with respect to the cells of the adaptive immune system (116). Therefore, during mammalian evolution, there has been ample time for NK cells to learn from contact with these ancient pathogens and modify responses against them, but, at the same time, *Mtb* may have acquired evolutionary advantages to evade such responses (117).

CONCLUSION AND PERSPECTIVES

The adaptive-like qualities of NK cells make them new targets for the development of cellular-mediated therapies to enhance innate phases of the immune response against different pathogens and improve the protection conferred by currently available vaccines. Specifically, in the case of *Mtb* infection, one approach to achieve this purpose would be to prime NK cells with cytokines that augment function and allow improved responses against new antigen-dependent or -independent signals within the lung lesions of tuberculosis patients. An alternative approach would be to sensitize and expand NK cells using *in vitro* bacterial exposure, followed by autologous transfer of the sensitized cells back into to the infected individual. Although these cells would not act in the innate phase of infection, as patients are already infected, the NK cells may have enhanced function that would be of special importance in individuals with impaired T-cell responses. Finally, the fact that NK cells are a source of IL-22 during PTB provides rational support for the use of NK cells as a target of new vaccines to improve IL-22 production. This approach could provide patients with a cytokine involved in maintaining lung epithelium integrity from the early stages of the disease and help to limit bacterial dissemination (see **Figure 2**).

For years, efforts to understand why the human immune system cannot eradicate *Mtb* have focused on T-cell biology. However, the emerging role of NK cells during immunopathology of tuberculosis, and the antigen-specific recall responses of these cells, appeals for a redirection of attention to this lineage of lymphocytes which may represent "a new chance" against *Mtb*. Nevertheless, it is clear that much work remains to elucidate the molecular mechanisms, which regulate memory-like NK cell development and maintenance in response to *Mtb* infection. Also, immunologist has the task of ruling out possible collateral damage before implementing NK cells in protective strategies against *Mtb* and other pathogens since there are certain instances in which it has been possible to observe an association between the activity of NK cells with worsened outcomes and increased immunopathology (118–120). Nonetheless, current evidence does not support this scenario in the context of tuberculosis, as the data suggest that dysfunction of these cells is related with increased likelihood to become ill; in addition, there are discrepancies in the results of animal models and human studies of other infectious and non-infectious disorders that remain unclarified (121–123). Once such gaps in the knowledge have been overcome, the inherent memory-like qualities of this innate subset of cells may in fact be an old and well-known weapon, which could be used to further enhance innate phases of immunity against tuberculosis, improve vaccine design and efficacy, and perhaps, even to help to prevent infection.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the manuscript and approved it for publication.

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Trying to See the Forest through the Trees: Deciphering the Nature of Memory Immunity to *Mycobacterium tuberculosis*

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The purpose of vaccination against tuberculosis and other diseases is to establish a heightened state of acquired specific resistance in which the memory immune response is capable of mediating an accelerated and magnified expression of protection to the pathogen when this is encountered at a later time. In the earliest studies in mice infected with *Mycobacterium tuberculosis*, memory immunity and the cells that express this were definable both in terms of kinetics of emergence, and soon thereafter by the levels of expression of markers including CD44, CD62L, and the chemokine receptor CCR7, allowing the identification of effector memory and central memory T cell subsets. Despite these initial advances in knowledge, more recent information has not revealed more clarity, but instead, has created a morass of complications—complications that, if not resolved, could harm correct vaccine design. Here, we discuss two central issues. The first is that we have always assumed that memory is induced in the same way, and consists of the same T cells, regardless of whether that immunity is generated by BCG vaccination, or by exposure to *M. tuberculosis* followed by effective chemotherapy. This assumption is almost certainly incorrect. Second, a myriad of additional memory subsets have now been described, such as resident, stem cell-like, tissue specific, among others, but as yet we know nothing about the relative importance of each, or whether if a new vaccine needs to induce all of these, or just some, to be fully effective.

Keywords: vaccination, memory immunity, memory T cell subsets, BCG, *Mycobacterium tuberculosis*

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INTRODUCTION

The purpose of vaccination is to establish a long-lived state of immunological memory to a given pathogen which can mediate an accelerated response to that pathogen if it returns (1). That immunological memory exists in some sort of form is long known; Thucydides, in describing the Peloponnesian war in 430 BC wrote that a plague affecting the citizens of Athens never attacked the same man twice. Far more recently, Panum, a Danish doctor, observed that elderly residents of the Faroe Islands exposed to measles in 1781 were immune to a second outbreak 65 years later. However, it was only 50 years or so ago that the work of Gowans began to focus down on a particular white blood cell, the lymphocyte, as the actual mediator of immunity, and only 30 years since the first T cell transfer studies (2) suggested that one component of the host response in mice infected with *Mycobacterium tuberculosis* exhibited a longer lived phenotype, with subsequent studies (3) showing that it remained present even if the infection was cleared by chemotherapy, indicating a long-lived phenotype not dependent on continued exposure to specific antigen.

These early studies with *M. tuberculosis* made the reasonable assumption that generation of long-lived immunity was mediated by a discrete T cell, the memory T cell. This assumption, we now

know, was wrong, and the field since then began to recognize that there are at least two major subsets of memory cells, distinguishable both in terms of phenotype and tissue distribution. Then, more recently, this has further evolved into evidence for further subsets, as will be discussed below.

A further issue regards the system/model in which one can study these cells in the context of tuberculosis. Chronic disease, which can be studied in mice, generates cells in the lungs that have phenotypic characteristics of memory immunity (4, 5). In turn, BCG vaccination induces memory T cells in relatively similar numbers to chronic disease in terms of memory T cell subsets. Infection with *M. tuberculosis* followed by clearance with drugs induces a strong memory T cell response, but if these animals are re-challenged, then the two major memory T cell subsets are both triggered to essentially equivalent levels (6). In the latter case, we would expect this immunity to be stable and result in further expansion of memory immunity, but in fact the reverse is true. This further illustrates our minimal understanding of these events.

WHAT DO WE KNOW ABOUT MEMORY T CELLS IN GENERAL

As our knowledge of memory immunity developed, the concept quickly emerged that memory cells "marginate"—move from their initial sites of sensitization and spread out across the body to provide an early warning system should their specific pathogen reappear (7, 8). Memory B cells become distributed throughout the lymph node system, and T cells have an even wider distribution within lymph nodes and peripheral lymphatic tissues. This redistribution includes a particular emphasis on the two main mucosal tissues in the gut and the respiratory tract. The important findings of Sallusto and her colleagues (9–11) that there were two separate main subsets of memory T cells provide an additional element of overall design to this complex system, based upon a division of labor in which effector memory T cells (TEM) protected the periphery while central memory T cells (TCM) represented a "rapid response team" based in more central tissues such as the spleen and bone marrow.

As this concept of margination developed into the newer TEM/TCM model, it was still unclear to what degree each population remained essentially cessile, or whether despite a favored niche (gut, lung, for example), they continued to have some degree of recirculation properties. This is still very much under investigation today and has led to the potential identification of further subsets of T cells, discussed below.

As noted above, there is good consensus that there are at least two major subsets of memory T cells (9, 11, 12). TEM are found in peripheral sites such as the lungs, gut, and skin, where they represent a "first line of defense," whereas TCM are found in lymphoid organs such as the spleen and the bone marrow, and are thought to represent the second line should pathogens reach that far. This paradigm has proved to be workable and useful and is further helped by a clear phenotypic difference between the two—TEM are $CD44^{hi}$ $CD62L^{lo}$ $CCR7^{lo}$ while TCM are $CD44^{hi}$ $CD62L^{hi}$ $CCR7^{hi}$. Memory T cells in general can express an array of co-stimulatory molecules, including CD27, CD28 [which appears critical (13)], ICOS, 4-1BB, OX40, and CD40L, and various regulatory markers such as PD-1, BTLA, and CTLA-4.TEM are $CD44^{hi}$ $CD62L^{lo}$, T-bet^{int}, $CD27^{+}$, and KLRG-1^{neg}. They are more responsive to IL-2R signaling, express higher T-bet levels, but lowered Bcl-6 and CXCR5, whereas TCM are the reverse. TEM lack CCR7, and can rapidly produce key cytokines including IFNγ and IL-2.

ORIGINS OF MEMORY T CELLS

Our general concept is that infection with a given pathogen generates the clonal expansion of antigen-specific lymphocytes, which differentiate into effector cells. If/when the pathogen is cleared, the response contracts as most of these cells die, but some cells remain viable and become long-lived memory cells (14). If the pathogen then reappears, there is a subsequent transition in which memory cells become secondary effectors, exhibiting kinetics far faster than the emergence of primary response effectors (15). As yet, however, there still is no clear consensus on whether memory cells arise from a small percentage of effector cells, or arise independently, nor is there much known about the fate of re-stimulated memory cells and the secondary effectors some of them then become.

Memory T cells arise after stimulation by common gamma-chain cytokines, which triggers homeostatic expansion of this population. Signals from MHC molecules are required, as is CD70 engagement, as well as production of autocrine IL-2 to prevent clonal contraction; this also depresses potential apoptosis while upregulating expression of the IL-7 receptor (16). In the case of $CD8^{+}$ TCM, these cells require exposure to IL-7 and IL-15 to survive in a state of interphase and undergo occasional cell division without requiring signals from MHC molecules. This is different to other memory subsets, indicating that homeostatic control of the memory response is heterogeneous (17).

WHERE DO MEMORY T CELLS COME FROM?

There are currently two main models of memory T cell generation, and which one applies to our models of tuberculosis infection is still currently unknown. In the first model, TM differentiation occurs concomitantly with effector T cell generation right from the offset, when antigen presentation determines early programming of cells that will become memory cells, and which then become dominant after primary effector immunity has contracted. In the second model, TM arise later, possibly during the contraction phase, and either arises independently of effector T cells, or from a longer lived subset of them. There is no doubt that, at this time, the signals that control memory immunity development, maintenance, longevity, and function, are as yet still poorly defined (18), plus it is also probably true to say that we know much more about CD8 memory as opposed to CD4 (19, 20). A current concept is that competition for limited amounts of antigen presented by MHC-II is thought to be a limiting factor in CD4 memory T cell generation, with the model predicting

that the more activated the cell becomes, the less likely it will become a TM (suggesting control by receptor signaling strength). If the antigen availability is high enough, T cell priming occurs rapidly in the presence of activated dendritic cell (DC), and this thought to drive effector memory T cell emergence, whereas the generation of central memory cells may be driven by more mature DC. This seems consistent with our knowledge that BCG is only slowly cleared from vaccinated mice, so sufficient antigen potentially remains to generate TEM after initial immunity has contracted. This contrasts with various virus infection models (21), as well as malaria models (22), in which rapid clearance of the infection and rapid contraction of effector immunity favors TCM generation (15, 23–25). At the cellular level, this further correlates with evidence for increased sensitivity of TCR/MHC engagement driving high-affinity TM cells, more efficient TCR triggering, altered CD3 clustering, and increased Zap70 signaling.

In the context of TH1 responses, Harrington and others (26) have provided evidence using cytokine reporter mice, which indicates that memory T cells arise from IFNγ-positive primary effector cells—a more satisfactory explanation than the scenario in which memory cells arise despite minimal reaction to the pathogen.

RECENTLY DEFINED NEW MEMORY T CELL SUBSETS

As discussed above, there is general consensus that TEM populate the periphery as a "first line of defense," whereas TCM occupy a more centralized distribution in the spleen, bone marrow, and lymph nodes (8, 9, 11, 12, 27–29). Despite this, the overall nomenclature is becoming more complicated, with cells in the lungs described (30–32) as resident T cells (TRM), which may also include memory precursor effector cells—as well as in addition, spleen-associated stem cell-like memory cells (TSCM), which we may have accidentally tripped over in 2005 (33), and since (5), although these tend primarily to primarily located in the spleen and lymph nodes.

The idea of memory T cell margination inevitably evolved into our current concept of resident memory T cells, cells expressing specific receptors/ligands directing them into specific tissues, in which they then become retained (34). The dynamics of these events are still unclear, given that we still do not know the distinction between truly migratory TEM and TCM—coming in and out of lymphoid tissues, or feeding peripheral sites from central reservoirs, versus cells that are truly resident—a confusion that exists in reviews even now (35). However, as parabiosis studies indicate (in virus infection models at least), a long-lived resident/cessile population exists and may be regarded as a separate subset to TEM and TCM, leading to the recent suggestion (30) that CD27 and CD43 staining could be used to better define these. In the context of CD8 cells, expression of CD103 and CD69 seem reasonable markers, although this is yet to be firmly established for CD4 T cells (7) In addition, CD69 seems key to maintaining TRM, in the context of preventing tissue egress (36). Exactly "where" in the lungs such cells reside is still unclear, but associated with the base of the bronchial epithelium seems one possibility (**Figure 1**).

In fact, the TRM subset may be far more widely distributed in the body than previously thought, given that recent estimates have calculated that a similar subset found in skin dermal tissues may be in excess of 10^{10}! As such, therefore, this represents a truly huge peripheral defense system. Although the majority of data to date has focused on CD8-positive skin-resident memory T cells, as above, these subsets differ phenotypically from TEM and TCM (37, 38). Again, they express CD103, which is consistent with this molecule being able to bind to E-cadherin expressed by skin keratinocytes, as is CD49a, which binds to various types of collagens, and they express other "stay put" ligands as well such as CCR4 and E-selectin.

In our own studies in BCG vaccinated mice, we made the unusual observation that a $CD4^+$ $CD62L^{lo}$ $CCR7^+$ subset slowly increased in number over time (5). Interesting, a more recent study looking at human skin CD4 subsets, a population with the same phenotype was observed in the dermis (39), with about a third of these also expressing CD69. The authors of this study named these "migratory memory T cells" and argued that they may represent a subset that recirculates bidirectionally between the skin and the blood or the lymphatics (40). While obviously not proven the cells, we observed in the lungs may be a subset of TRM with increased migratory properties. While confusing, this illustrates that the whole memory T cell population is probably a very dynamic event, rather than all cells finding a tissue niche and just staying there.

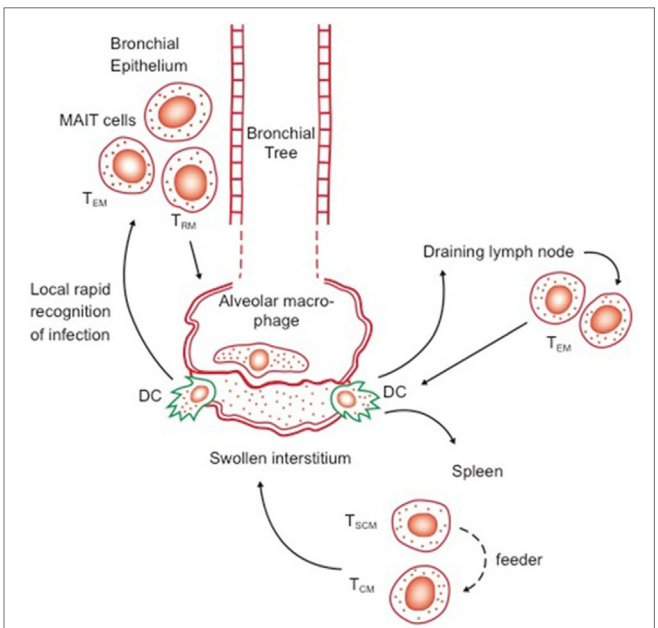

FIGURE 1 | Possible distribution of key reactive lymphocytes in the bronchial epithelium (first line of defense) and lymph nodes and spleen, in memory immune animals. Transport of bacilli and/or antigen out of the alveolus or swollen interstitium by dendritic cells (DC) will trigger responses by local responses by effector memory T cells (TEM) and resident T cells (TRM), as well as potentially by local mucosal-associated invariant T cells, as well as rapid responses by TEM and central memory T cells (TCM) from the draining lymph nodes and spleen. Whether TSCM act directly or feed the expansion of TCM is as yet unknown.

Cells now identified as stem cell-like memory T cells [TSCM] followed the realization that they had similarities to conventional hematopoetic stem cells; they share similar core transcriptional signatures, can readily proliferate, and gave rise to heterogenous cellular progeny (41)—in fact, they can reconstitute the full diversity of memory T cells. TSCM have a naïve-like phenotype and express high levels of the SCA-1 marker, CD122 (IL-2Rβ), and CXCR3 [14].

The concept of TSCM first arose in studies in 2005 (42) looking at CD8 responses in a mouse model of human GVHD. These cells had a "naïve/unactivated" $CD44^{lo}$ $CD62L^{hi}$ phenotype, but they were long lived, required IL-15 (suggesting they were memory cells), had the ability to efficiently self-renew, and were multipotential, in that they could apparently give rise to both effector and central memory populations—even while maintaining their own pool by self-renewal. Whereas initially, it was thought that these were just a component of the central memory response, they were shown subsequently (43) to be a distinct subset with a much higher proliferative capacity. More recently, a similar CD8 subset has been observed in macaques (44).

Available information to that point was limited to CD8 responses, but in 2011, a CD4 T cell subset with stem cell-like properties was identified in a tumor rejection model in mice (45). This subset was further identified as a component of the TH17 response based on secretion of this cytokine. This was of interest, not the least because TH17 are highly "plastic" in that they possess substantial flexibility in their developmental programming, and as a result, can acquire properties similar to TH1 T cells. In the tumor models, TH17 can also directly mediate rejection by themselves (with the caveat that they first have to be polarized *in vitro*), and can express both IFNγ and IL-17. Finally, these studies found parallels between CD4 and CD8 memory cells with stem cell-like properties, in terms of shared signaling *via* the Wnt/β-catenin pathway (the expression of which can be used to help identify them). Similar observations have been made using human cells, in which, inhibition of mTOR signaling or the Wntβ-catenin system induces the expansion of TSCM (46).

It is unclear if a "niche" exists for TSCM. The bone marrow and spleen may serve this purpose, and it has been suggested that they can be found associated with fibroblastic reticular cells within lymph nodes (which provide them with signals) (41). Their patterns of systemic recirculation and tissue distributions seem similar to naïve T cells, and recent studies (47) in humans seem to indicate a state of continuous flux and renewal of TSCM, even in elderly individuals.

TSCM are minimally differentiated phenotypically and functionally, and appear to fall midway between naïve and conventional memory cells. Right now, the debate continues as to where TSCM fit in the overall cell family tree. A working model is that TSCM arise from naïve T cells and act as feeder cells for other more differentiated cells. Because TSCM and TCM share similar abilities for rapid cell expansion, then, it is proposed that TSCM turn into TCM as needed. TCM, then, in turn, seed peripheral sites with TEM—now expressing ligands needed to identify target sites (lungs, gut, skin, etc.) where many probably become cessile TRM. If the pathogen reappears, these subsets mediate a rapid response, with many becoming secondary effectors. However, this is far from clear, since it is possible that TSCM can give rise to TEM as well. For instance, in our chronic tuberculosis murine model (5), one would predict that TSCM would increase the numbers of TCM present in lungs as the infection progressed, but this does not happen.

A further question yet to be determined is whether there is any relationship between memory $CD44^{lo}$ $CD62L^{hi}$ TSCM cells and CD4 cells secreting IL-17 (TH17). Recent studies have shown (48) that TH17 can be directly protective in a mouse model of tuberculosis. In that study, $Rag^{-/-}$ mice were infused with TH17 or TH1 CD4 T cells and both showed cell activation and subsequent protection against a challenge infection. Interestingly, while immunity transferred by TH1 cells was transient and contracted, TH17 cell transfer was stable and gave much longer survival (very consistent with the idea that TSCM are an "early" memory response). The only drawback was an increasingly florid inflammatory infiltrate characterized by an excess of neutrophils [as our laboratory has also noted (6)]. These data, the authors concluded, directly indicated that TH17 cells had the capacity to transfer protective immunity. The question yet unanswered is whether this was due to TH17 cells directly (perhaps acquiring an IL-17/IFNγ double phenotype?), or mediated by a TSCM subset arising from the overall TH17 population (45)? More recently, an important study from Denmark showed (49) that two vaccine candidates could induce TH17 responses in mice, that these were long lived (18 months), stable, and upon challenge started to show traits associated with TH1 responses. Thus, if TH17 cells have a memory component, is this part of or independent of the TSCM/TCM/TEM interrelationship discussed above?

Finally, follicular helper T cells (Tfh cells) control germinal center host responses at both the cellular and humoral levels. There is increasing information that this includes a memory T cell component (memory Tfh). These cells are driven by Bcl-6, and by exposure to IL-6 and IL-21 (50). Recent evidence supports the view that antigen-specific memory Tfh cells express CXCR5, but lack Bcl-6, ICOS, and expression of several other markers seen in primary responses.

MEMORY IMMUNITY TO TUBERCULOSIS

As recently extensively reviewed (1, 51–54), any successful vaccine against tuberculosis will need to generate memory T cells. We now know that the T cell response is phenotypically heterogeneous, and in the context of memory in tuberculosis, it is almost certain that more than one subset is both involved and important. The primary focus has been on the CD4-based immune response—since CD8 responses seem to play a much more minor role—and in particular on CD4 cells that secrete IFNγ.

It goes without saying that understanding memory immunity in the context of *M. tuberculosis* infection is imperative if we are going to design better vaccines, compounded by the fact that results from some of the current candidates are rather underwhelming. However, the blunt fact is that our knowledge of this parameter is woeful, and mainstream Immunology is gradually discovering new subsets of memory T cells on a steady basis. The TB research field is responding to this partially, certain vaccines

induce TCM, BCG given by a different route induces TRM, and so forth, but what is utterly lacking is an understanding of which ones are the most important. Added to this complexity is the vaccine "type" itself (54)—live vaccine A might work best if it induces TM subset X, while protein in adjuvant candidate B works best if it induces TM subset Y. If there are, say, five TM subsets, do we need to trigger all of them, just 3 or 4, or is just one? Does there need to be a balance between them? Are we deluding ourselves by continually comparing experimental outcomes directly to BCG (as our positive control in animal models)? For instance, candidate X might take much longer to induce TM compared to BCG, but in long-term studies, the longevity of the immunity it generates may be far superior. However, because of the standard assays we employ (mostly short, usually for economic reasons), we would miss the latter and discard this candidate (55–57).

If there was (as we believed for a while) a single "memory T cell" subset, then matters would be uncomplicated—one could perform parallel studies of vaccination with candidates followed by challenge, then determine which vaccine gave the strongest and most long-lasting memory immunity. This is unfortunately now not the case, however, because there appear to be multiple subsets involved, and we do not know which ones are critical for protection in vaccinated individuals, nor is there much consensus. This has the potential for being a further serious impediment for TB vaccine development.

In the first attempt to define host memory immunity involved in resistance to tuberculosis after vaccination, Lefford et al. gave rats the BCG vaccine, then used treatment with isoniazid to remove any surviving vaccine bacilli (58). He then challenged these animals and showed that they had increased resistance, thus showing that a form of immunity persisted even when the antigenic stimulus had been removed. A similar approach was then tried a decade later, but here, mice were directly infected with *M. tuberculosis* before the application of isoniazid chemotherapy. As before, these animals showed evidence of substantially increased resistance to a homologous challenge, and parallel passive cell transfer experiments showed that this resistance was mediated by CD4 T cells (3). This was followed by studies showing that the length of time needed to establish a state of memory immunity after BCG vaccination was inversely proportional to the vaccine dose, but that once established the level of immunity was equivalent (59). Soon thereafter, it was shown (60) that memory immunity involved T cells that were antigen-specific (in this case, to ESAT) and secreted IFNγ. In addition, in one of the first applications of flow cytometry to these questions, it was found that T cells changed their phenotype over the course of infection, with the gradual emergence of cells expressing the $CD44^{hi}$ $CD45RB^{lo}$ phenotype (61).

METHODS FOR STUDYING MEMORY IMMUNITY IN TUBERCULOSIS

Memory Cells Established by Vaccination

Mice are usually vaccinated with BCG by the subcutaneous route, at a dose of ~10^6. Some initial protection can be detected 10–15 days later, but an initial peak is not reached until ~25–30 days, with the latter being a conventional time point used in most vaccine testing studies (62–64) despite the fact that assays at this time are measuring effector T cell activity, not memory. BCG given *via* this route can occasionally reach the spleen and sometimes the lungs in very small numbers, and so, protective T cells present in this organ 2–3 months later can reasonably be regarded as memory T cells.

If after this time, the animal is infected with *M. tuberculosis*, there is an accelerated expansion of activated T cells, many of which are secreting IFNγ, and more rapid control of the infection as illustrated lower bacterial loads in the lungs, and by smaller and more lymphocytic granulomas, indicating accelerated expression of memory immunity. The source of this immunity was investigated more recently in mice that were vaccinated with BCG but not subsequently challenged (5). This showed that in such mice there was a slow increase over the first 100 days in the numbers of T cells recoverable from the lungs that had a $CD44^{hi}$ $CD62L^{lo}$ phenotype (and, therefore, TEM under the Sallusto definition); moreover, 90% of these cells were CD4-positive. Only 10% or so expressed a $CD44^{hi}$ $CD62L^{hi}$ TCM phenotype. These observations led to the hypothesis that BCG predominantly established a TEM population in the lungs, and this subsequently led to the proposal of a hypothesis that the apparently lower induction of any TCM may reflect an inherent weakness of the vaccine (65).

Recent studies have drawn attention to the route of BCG administration. While an earlier study (66) saw no difference between the efficacy of BCG in mice given the vaccine by aerosol or subcutaneously, more recent studies have suggested otherwise. In a potentially important breakthrough, Perdomo and her colleagues (67) demonstrated the induction of TRM in the lungs following instillation of BCG *via* the trachea, with these cells expressing CXCR3, CD103, and CD69; in addition, these cells were IFNγ-positive. However, these data should be viewed in comparison with results from the same laboratory in which a new rBCG vaccine candidate preferentially elicited $CCR7^+$ TCM T cells (68). In contrast, in a rhesus macaque model, Sharpe and her colleagues (69) found that intravenous BCG vaccination was the most effective, with strong induction of IFNγ- and TNFα-positive TEM. If anything, this further emphasizes our point above that different candidates can make different memory responses, and that even the same type of vaccine can give different responses when given by different routes.

Memory Cells in Chronically Infected Mice

After 40–50 days following low dose aerosol infection with *M. tuberculosis* the bacterial load in the lungs stabilizes at around one million bacteria. This establishes a chronic disease state in which there is a progressive but very slow increase in bacterial numbers over the next 100–200 days, concomitant with a slow degeneration of the lung granulomatous structures (70). Over this time, there are dynamic changes, both in terms of T cell subsets and macrophage/dendritic cell populations (71).

As with BCG vaccination, most cells recoverable from the lungs over this time are $CD44^{hi}$ $CD62L^{lo}$ CD4 T cells. Most of these are certainly TEM/TRM subsets, but there is probably

a further subset of secondary effector cells as well due to the continued presence of antigen. This is mediated by IFNγ, as illustrated by the continued presence of macrophages staining positive for NOS2 (72).

The distinction between TEM and effector cells is further suggested by the observation (73) that activated T cells in the chronically infected lung are PD-1^{+}, and transition into KLRG-1^{+} "terminally differentiated" cells (but not the reverse). It is increasing clear that these PD-1^{+} cells, once thought to be exhausted cells, in fact represent a major lung T cell subset, with further analysis (74) showing that these cells depend on ICOS and Bcl-6 expression. However, one note that these results are different to a further study in which KLRG-1-negative CD4 cells predominated; these expressed CD62L^{hi} and presumably, therefore, are part of the TCM response (75).

Given these discrepant findings, it is clear that the role of KLRG-1 and PD-1 subsets needs to be further clarified—an obvious starting point being their comparison in chronic infection models versus those of vaccination. A further complication is the newly proposed distinction between "parenchymal" and "intravascular" T cells in the lungs of *M. tuberculosis*-infected mice (76). In that model, cells expressing KLRG-1 and IFNγ are retained in the vasculature, whereas cells that mediate protection—most of them IFNγ-negative—are found in the lung parenchyma (76, 77). However, not only is this a misuse of the term "parenchymal,"[1] but the actual flow cytometry staining technique, which is based on the injection a few minutes earlier with anti-CD45, does not take into account lung blood capillary transit time (78). This takes a finite time (neutrophils, which are similar size take several hours) because the lymphocyte has to deform so it can pass into the capillary. Lymphocytes are 6–8 μm in diameter and can be twice as large if activated blast cells, and have to pass through capillaries that are only 5-μm in diameter (much narrower than capillaries in other body organs). Our own interpretation of these observations (see **Figure 2**) is that the cells that stain positive for IFNγ are actually at the proximal end of the capillary bed—and have not reached the lesions yet—whereas the "parenchymal" cells in the lesions are IFNγ low or negative because they have already released this cytokine. In addition, we would propose that KLRG-1 expression is to enable cells to bind cadherins in the extracellular matrix to contribute to the developing granuloma.

[1] Parenchymal tissue refers to functional tissue within a given organ. In the lungs this is the bronchial tree, the airspaces, *and* the blood supply.

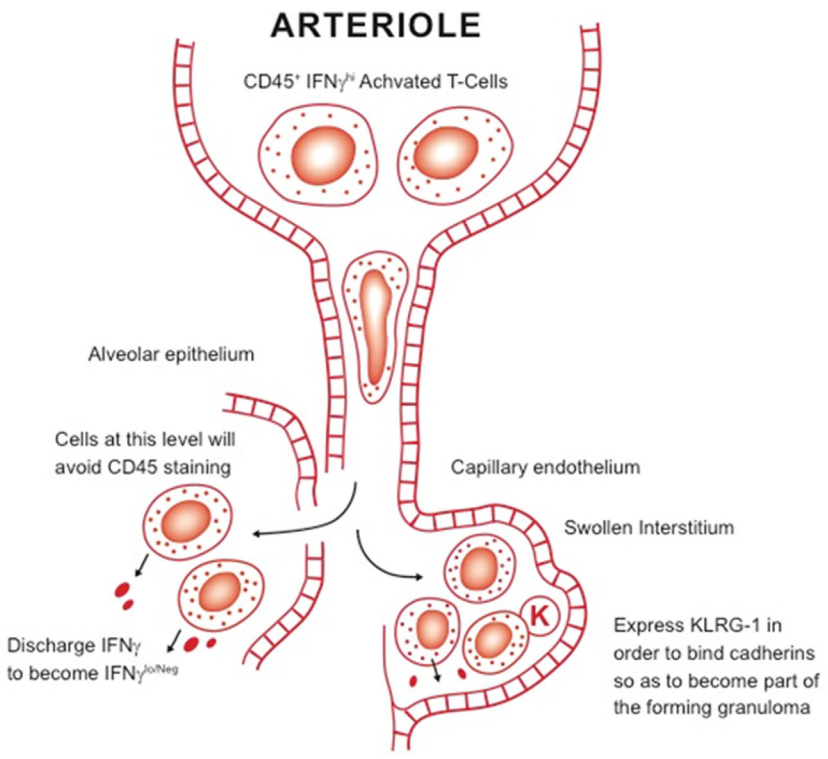

FIGURE 2 | Our working hypothesis for the distinction between intravascular T cells and other subsets in the lungs. Large activated blast lymphocytes plug the proximal end of the lung blood capillary system, where they are readily stained by anti-CD45 antibody. These cells have to considerably deform before they can be pushed by hydrostatic pressure through the system. It is not clear, however, how long this process takes but it could be several hours. Once they encounter sites of infection, they pass out of the blood capillary and either cross the damaged alveolar epithelium or pass into the swollen interstitium. After encountering infected local macrophages, they release IFNγ, thus becoming negative upon staining for this cytokine. In addition, some express KLRG-1, needed for the cell to bind cadherins in the extracellular matrix, facilitating granuloma formation.

Memory Cells in Animals after Infection and Chemotherapy

Given the belief that memory immunity becomes established once the pathogen has been cleared and antigen is no longer available, early studies on memory immunity after *M. tuberculosis* infection consisted of initial infection then clearance of this infection by treating the animal with isoniazid chemotherapy. These showed (3) that the accelerated resistance conferred upon these animals was mediated by a long lived T cell population that was CD4-positive and not actively proliferating (prior to stimulation). In addition to acquired specific resistance, a component of non-specific resistance was also demonstrable in adoptive cell transfer studies. In keeping with the growing concept at the time (79) that secreted proteins rather than constitutive proteins of the bacillus drove initial protective immunity, triggering of the memory T cell response required the live organism [as indeed was shown earlier in a classical study on this topic (80)].

Memory Responses to Reinfection

For many years, "endogenous reinfection" was considered to be the primary cause of secondary disease or relapse in tuberculosis patients, and this formed the cornerstone of experimental models for some considerable time (81). Over the past decade, concomitant with the worsening epidemic in areas such as Southern Africa, it has become apparent that exogenous reinfection is probably the main cause of secondary disease (82), and in fact, it has been demonstrated that patients treated successfully for tuberculosis are at much higher risk of catching it again (83).

This latter result troubled us, because our animal model studies suggested that such people would be expected to be more resistant, not less. To revisit this, we infected mice with the virulent HN878 Beijing strain, and then rescued these mice by chemotherapy. When these animals were re-challenged, they were highly resistant and we observed the potent emergence of both TEM and TCM memory T cell subsets, both IFNγ-positive (6). However, 30–40 days later, the numbers of these cells progressively declined, and the mice developed a diffuse tuberculous pneumonitis, which was fatal. Examination of the declining T cell numbers showed that many were PD-1^{hi} [an expression we know can be reversed by chemotherapy (84)]. It is unclear if this represents exhausted cells—given the new information about PD-1 discussed above—but what it does illustrate is that memory immunity, thought to be long-lived and stable, may not be at all.

SPECIFIC TARGETING BY TB VACCINES

Because of the apparent dominance of TEM in peripheral non-lymphoid sites, it has been suggested (7) that a vaccine that elicits and maintains high-frequency TEM populations in the lungs would provide a more efficient degree of protection against *M. tuberculosis* infection. A further issue is the cytokine-secreting properties of the cells our vaccine induces, and to date, human studies have produced ambiguous results (85, 86). Similarly, it has been argued that IFNγ is of little importance in the expression of protective immunity in the mouse lung (77), although this stunning conclusion was drawn from an adoptive transfer study comparing very high numbers of bacteria in the lungs in which an "area under the curve" analysis was performed (the reader can draw their own conclusions as to whether this is an appropriate use of this test), and which of course is utterly contrary to classical studies using gene disrupted mice.

That aside, at the clinical trial level, there is no consensus as to whether a vaccine driving TH1 responses is essential, and indeed excessive T cell activation may be detrimental (86). In addition, humans are not mice and cannot be dissected, and a poor TH1 signal in the blood may simply reflect the fact that the cells expressing these are doing their job in lung lesions rather than recirculating. Moreover, some have also pointed out that IFNγ is not always essential for TM function (87), and we ourselves have recently suggested the idea (51) of an "rapid influx and weight of numbers" model that does not necessarily involve cytokines at all.

Evidence is starting to appear in favor of our own (65) argument that BCG is a poor vaccine because of lack of TCM generation, with reports that a new rBCG candidate that generates CXCR5 + TCM (68), plus the demonstration that boosting of BCG with the new fusion H56 generates expansion of Bcl-6 + CD4 cells (88), and data showing that the new live attenuated candidate SO2 also pushes TCM responses (89). Given our own published viewpoint, this is obviously encouraging. That said, the use of CXCR5 may be a confounding issue. CXCR5 may well be a marker of TCM, but it is also implicated as a marker of Tfh (90). CD4 cells expressing CXCR5 associate with "follicle-like" cell aggregates in the mouse granuloma (91), and express CD44^{hi}, ICOS, and PD-1 [in addition, about half express the orphan receptor ROR-γt (88)]. In fact, the presence of these cells might explain the mysterious arrival of B cell follicles into lung granulomas (92). Although CXCR5-gene KO mice still control *M. tuberculosis* infection in the lungs, they do so less efficiently, but in this regard, a more recent study (74) has questioned their importance. Moreover, in a malaria model (93), CXCR5^{+} cells have been implicated as precursors of both TCM and Tfh cells. As with other models, if the parasite is rapidly cleared, this favors TCM generation.

Regarding CD4 cells, we have shown that these are the predominant subset in the lungs of BCG vaccinated mice, and >90% are of the TEM phenotype (5). However, it is unknown if these are truly resident TRM or are slowly recirculating through the lung lymphoid tissues. This idea has been addressed elsewhere by Gebhardt et al. (94) who have predicted that this subset would be slowly lost over time, an idea in keeping with our own hypothesis (65) regarding the gradual disappearance of BCG mediated protection in children/young adults.

Can BCG or *M. tuberculosis* directly induce TRM? In the case of BCG, it appears so, but also seems to depend on the route by which the vaccine is given. As noted above, very recently Perdomo et al. (67) demonstrated that T cells induced in the lungs after the vaccine was injected into the trachea expressed CXCR3 (promoting ingress into these tissues), produced IFNγ, and stained CD103^{+}CD69^{+}. While some concern remains as to whether CD103 is a consistent marker of TRM [30], these are interesting findings, consistent with the hypothesis that BCG

induces TRM, and hopefully will be further confirmed in the near future if they can be shown to have the distinct transcriptional signature that appears to specifically identify this subset, which distinguishes these from other CD44^{hi} CD62L^{lo} memory subsets. Clearly, expression of the CD103 integrin indicates an intention to "stay put," and these cells are also CCR7^{lo} and S1PR1^{lo}, molecules needed for tissue exit.

Generating TRM is a reasonable starting point, but would amplifying this subset rather than others result in local immunity *but* no immunity to bacteria that we know disseminate *via* the blood and lymphatics? We know that subcutaneous BCG generates a strong TEM response in the lungs, and in fact, it has been proposed (7) that because exposure to *M. tuberculosis* involves prolonged antigen stimulation and considerable inflammation, the generation of high-frequency TEM could help counteract the immune-evasion properties of the bacillus. Our own experience indicates that BCG does indeed do this in the mouse model, but since the infection is "controlled and contained" but not eradicated, then, this is only a pyrrhic victory.

The ultimate ambition here is to find a vaccine of some sort that generates a form of immunity that will recognize the presence of the bacillus before it gets into the interstitial space (from whence macrophages/dendritic cells will carry them off to the lymph nodes) (95). While from an immunological point of view one might speculate that this could be achieved, unfortunately, the anatomy of the lung precludes this. Alveolar macrophages prowl the lung epithelial surface through a sea of surfactant, and probably most of the time, kill ingested *M. tuberculosis*, but when they fail to do so, no sort of back-up protective mechanism seems to exist.

Recently, the existence of non-classical lymphocyte-like cell populations has been identified in gut mucosa, and there appear to be complimentary populations existing in the lungs. Among these, a subset that occurs in relatively high frequency are mucosal-associated invariant T cells. These possess a semi-invariant MHC alpha chain, indicating a restricted recognition pattern, and can secrete protective cytokines (96). In general, these cells are perceived as part of a "first line innate" system, and there is no evidence they can be manipulated or differentiated into a memory cell population by vaccination (53). One can make a similar argument for other tissue-resident innate cells, such as NK cells, NKT cells, and innate lymphoid cells.

CONCLUDING REMARKS

There is no avoiding the fact that TB vaccine development has been glacial. Only one candidate has been fully evaluated in clinical trials (as a BCG boosting vaccine), and this candidate failed (97, 98). The reaction to this has ranged from the very unhelpful "never should have been tried in the first place" to a more sober explanation from our laboratory that argued that the vaccine was tested in a region where the *M. tuberculosis* strains were low fitness, making BCG boosting impossible (99). Several other candidates exist and are apparently moving slowly through the pipeline, but calls from some of us to test them jointly in head to head evaluations in different animal models in laboratories that have no vested interest themselves have been ignored for over a decade.

If there is room for optimism, it reflects the fact that we are starting to consider if different subsets of memory T cells could be specifically targeted; as noted above, BCG given *via* the trachea generates a much stronger TRM response. This translated into improved protection after aerosol challenge, although whether this actually reflects local "nonspecific resistance" due to the presence of BCG in the lungs (absent in mice vaccinated subcutaneously) remains unclear.

As noted above, to find ways to stop the TB bacillus dead in its tracks will require a paradigm shift in our thinking. The presence of the bacillus is not even recognized until it uses its ESX system to break through the alveolar epithelium and into the interstitium, and even then, there is a favorable lag period while some bacilli are transported to draining lymph nodes to sensitize T cells, then, a further delay before these enter the blood and find their way back to the sites of bacterial implantation. By this time, as necrosis-prone animal models such as the guinea pig illustrate, the damage has already been done.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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