# Defining the role of $CD4^+$ T cells during human metapneumovirus infection

by

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Human metapneumovirus (HMPV) is a leading cause of respiratory tract infection in pediatric, elderly, and immunocompromised populations. Clearance of respiratory viruses like HMPV rely primarily on the destruction of infected cells by cytotoxic $CD8^+$ T cells. However, signals provided by $CD4^+$ helper T cells significantly impact the magnitude and effectiveness of $CD8^+$ T cells. Despite HMPV being an important human pathogen, the role of $CD4^+$ helper T cells in the immune response to HMPV is largely unknown. Using a C57BL/6 mouse model of acute infection, we identified an immunodominant $CD4^+$ T cell epitope in the viral nucleoprotein and constructed the first MHC-II tetramer for HMPV. Analysis of pulmonary T cells revealed that virus-specific cells were most abundant on day 10 post-infection and were $T_H1$-skewed. Additionally, virus-specific $CD4^+$ T cells displayed phenotypic and functional markers of impairment, including inhibitory receptor co-expression and prolonged PD-1 upregulation. To determine the contribution of $CD4^+$ T cells to the $CD8^+$ T cell response, $CD4^+$ T cells were antibody-depleted prior to HMPV infection. Depletion of $CD4^+$ T cells led to delayed viral clearance and enhanced PD-1 expression on virus-specific $CD8^+$ T cells. We also investigated the importance of CD40/CD40L signaling as a mechanism of $CD4^+$ T cell help and demonstrate that either enhancing or blocking this pathway is detrimental in the context of HMPV infection. Further characterization of virus-specific $CD4^+$ helper T cells, their regulation by PD-1, and their role in $CD8^+$ T cell impairment will provide new insights that aid in the design of effective vaccines for HMPV.

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# 1.0 Introduction

## 1.1 Human metapneumovirus

Human metapneumovirus (HMPV) is a negative sense, single-stranded RNA virus belonging to the newly defined *Pneumoviridae* family (formerly a subfamily of the *Paramyxoviridae* family) [1]. In addition to HMPV, avian pneumovirus (APV), respiratory syncytial virus (RSV), and murine pneumonia virus (MPV) all belong to this new family. HMPV was discovered in 2001, but retrospective studies have detected antibodies in patient serum dating back to the 1950s, indicating that the virus has been circulating undetected in the human population for decades [2]. The genome is 13.3kb in length and comprises eight genes which encode nine proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), matrix-2 proteins (M2-1 and M2-2), small hydrophobic protein (SH), glycoprotein (G), and large polymerase protein (L) [2]. There are two main lineages of HMPV (A and B) which are further divided into subtypes A1, A2, B1, and B2 based on genetic diversity [3, 4]. There appears to be no dominant subtype, and multiple subtypes can be found co-circulating in a given year. HMPV shares over 80% homology with APV subgroup C, suggesting that HMPV evolved from this virus through cross-species transmission hundreds of years ago [5-7]. With respect to other human pathogens, HMPV is most closely related to RSV.

HMPV is the second leading cause of acute upper and lower respiratory tract infections in children, and most individuals become seropositive for HMPV by the age of five [2, 8]. Clinical presentation of infection is virtually indistinguishable from other respiratory pathogens, and ranges from asymptomatic to serious lower respiratory disease such as bronchiolitis or pneumonia [9]. In

addition to pediatric populations, HMPV is also a major concern in immunocompromised populations and older adults, with fatal HMPV infections reported in cancer patients and the elderly [10, 11]. As with most respiratory pathogens, HMPV infects with seasonal distribution and typically peaks during winter months. A 20-year retrospective study of upper respiratory tract infections in infants and children revealed that HMPV peaks in March and April, accounting for 5% of all infections occurring in March during the study [3]. Despite its immense clinical burden, there are currently no FDA-approved vaccines or effective therapeutics for HMPV. Treatment for infection is supportive and only moderately effective. Thus far, only the antiviral ribavirin and intravenous antibodies have been used to treat humans but many other potential treatments have been tested in animal models including fusion inhibitors, monoclonal antibodies, and RNAi [12]. Recurrent infections with HMPV are common throughout life, indicating that neutralizing antibodies generated during primary infection are not sufficient for protection and that immunological memory is impaired or dysfunctional.

Studies of HMPV pathogenesis and immunity are typically carried out using small animal models. Rodents such as mice, cotton rats, guinea pigs, and hamsters are all semi-permissive for HMPV [13]. Studies have also been conducted in non-human primates including chimpanzees, African Green monkeys, and cynomolgus macaques [14]. Due to the abundance of reagents and relatively low cost, most HMPV studies have been conducted in mice. Although mice do not exhibit overt symptoms of respiratory illness, they lose weight, experience immune cell infiltration into the lungs, and show signs of lung pathology when challenged with virulent strains of HMPV [15]. Much of the published work has used BALB/c mice but some researchers prefer to use the C57BL/6 strain due to its bias towards a stronger anti-viral immune response and the wider availability of genetically altered strains on the B6 background.

## 1.2 The immune response to HMPV

Although HMPV is a significant pathogen in children, mechanisms of immunity to the virus remain poorly understood. Upon infection of airway epithelial cells lining the respiratory tract, HMPV is recognized by multiple host pattern recognition receptors including Toll-Like Receptor 4 (TLR4), TLR7, retinoic acid inducible gene (RIG)-I, and mitochondrial antiviral signaling (MAVS) protein [16-19]. Engagement of these innate immune sensors induces an intracellular signaling cascade that results in the production of interferons (IFN) and the transcription of antiviral/anti-inflammatory genes. Importantly, IFN signaling leads to the activation of the signal transducer and activator of transcription (STAT) family of transcription factors and the induction of hundreds of interferon stimulated genes (ISGs) [20]. Type I IFN signaling is known to be important for controlling HMPV replication and inhibiting lung inflammation in mice [21]. However, most viruses have strategies to evade detection by the infected host to ensure survival and spread. We and others have shown that HMPV employs a variety of mechanisms to subvert the hosts innate immune system [22]. These include but are not limited to downregulation of type I IFN production [21, 23], inhibition of STAT1 phosphorylation [24], antagonism of TLR4 signaling [16], and the targeting of RIG-I and MAVS [25, 26]. Multiple groups have also demonstrated that HMPV is capable of infecting human and mouse dendritic cells (DCs) [27], a professional antigen presenting cell (APC) that plays a major role in both innate and adaptive immune responses. There are conflicting data, however, about the effects of DC infection on the immune response to HMPV *in vivo* [28-30]. Natural killer (NK) cells represent another cell type that is involved in the immune response to viral infection. Although important for controlling infections caused by DNA viruses, our lab demonstrated that depletion of NK cells

during HMPV infection had no effect on viral clearance, immunopathology, or T cell responses; indicating that the immune response to HMPV in mice occurs independently of NK cells [31].

Defense against respiratory virus infection primarily relies on a successful adaptive immune response, followed by the generation of long-lived memory cells and antibodies that protect from subsequent challenge. To initiate an adaptive immune response to respiratory pathogens, APCs carrying viral antigen from the lung activate naïve $CD8^+$ T cells in secondary lymphoid organs which then migrate back to the respiratory tract. Once there, virus-specific effector $CD8^+$ T cells function to kill infected epithelial cells displaying the proper peptide-MHC class I complex through the directed release of granules containing perforin and granzymes [32]. Activated $CD8^+$ T cells also secrete potent antiviral cytokines such as interferon gamma (IFN$\gamma$) and tumor-necrosis factor alpha (TNF-$\alpha$), which act through a variety of mechanisms to promote upregulation of MHC class I on nearby cells, enhance destruction of ingested pathogens by macrophages, and stimulate T cell-mediated killing [32]. Following acute infection, $CD8^+$ T cells enter a contraction phase and a small percentage of them adopt a memory phenotype, becoming poised to rapidly respond during reinfection. There are multiple subsets of memory $CD8^+$ T cells including central memory ($T_{CM}$), effector memory ($T_{EM}$), and resident memory ($T_{RM}$), which differ in their response time, tissue localization, and proliferative potential [33]. However, it was recently shown that the respiratory microenvironment impairs antiviral memory $CD8^+$ T cell generation [34]. Additionally, pulmonary (but not splenic) virus-specific $CD8^+$ T cells can become impaired during acute viral infection [35], which may serve as a natural mechanism to protect the lungs from immune-mediated damage at the expense of memory $CD8^+$ T cell development. Similar to T cell exhaustion in settings of chronic infection and cancer, T cell impairment following viral infection is characterized by a progressive loss in the ability to produce effector cytokines, altered

transcriptional programs, and the upregulated expression of multiple inhibitory receptors, including Programmed Cell Death-1 (PD-1) [36-38].

It is well known that the immune response to HMPV is dominated by $CD8^+$ T cell-mediated killing of infected cells [39]. However, our laboratory discovered that HMPV-specific $CD8^+$ T cells responding to both primary and secondary infection in C57BL/6 mice become impaired [35, 40, 41]. Virus-specific pulmonary $CD8^+$ T cells upregulate multiple inhibitory receptors including PD-1, LAG-3, and TIM-3, as well as exhibit decreased IFNγ production and reduced CD107a mobilization (an indicator of degranulation) compared to virus-specific splenic $CD8^+$ T cells from the same animal. Genetic ablation of PD-1 in mice rescued HMPV-induced $CD8^+$ T cell impairment at early time-points post-infection (p.i.), but failed to prevent impairment at later time-points. Antibody blockade of LAG-3 partially rescued PD-1 independent impairment of HMPV-specific $CD8^+$ T cells at later these later time-points but blocking TIM-3 had no effect on $CD8^+$ T cell functionality. However, lack of PD-1 signaling with or without combined blockade of LAG-3 came at the cost of increased lung immunopathology on day 10 p.i., supporting the notion that T cell inhibition may be a natural protective mechanism in the lung [41]. Even though virus is cleared in the lungs of mice by day 10 p.i., microarray analysis of immune genes in impaired $CD8^+$ T cells revealed that they are transcriptionally similar to $CD8^+$ T cells in the late stages of exhaustion [42]. Furthermore, both primary and secondary HMPV-specific $CD8^+$ T cells exhibit increased mRNA expression of TNF-related apoptosis inducing ligand (TRAIL) compared to splenic $CD8^+$ T cells [42].

Surprisingly, the role of $CD4^+$ helper T cells ($T_H$) in the immune response to HMPV infection is largely unknown. Studies examining bulk T cell responses revealed that $CD4^+$ T cells in bronchoalveolar lavage fluid peak at day 7 post-HMPV infection in BALB/c mice and are

primarily type 1 helper ($T_H1$)-skewed at early time-points p.i. [43]. Another study using BALB/c mice demonstrated that antibody-mediated depletion of $CD4^+$ T cells led to significantly less weight loss as well as reduced lung viral titers and immunopathology following infection compared to mice with an intact $CD4^+$ T cell compartment [39]. That study also noted complete protection from secondary challenge with HMPV in the absence of $CD4^+$ T cells, contradictory to many reports demonstrating the critical requirement for help in generating effective secondary responses. It should be noted, however, that the HMPV stock used in that study induced an unusual disease phenotype that has not been replicated by others in the field, including our lab. Although the importance of $T_H$ cells in the immune response to HMPV has not yet been fully determined, a recent study from our lab examined the role of $CD4^+$ regulatory T cells (Tregs) in HMPV infection [44]. We found that Tregs, which normally act as suppressors of other T cell subsets, play a temporal role during respiratory virus infection; depletion of Tregs before infection delayed viral clearance and reduced $CD8^+$ T cell numbers but Treg depletion at later time-points during infection improved virus-specific $CD8^+$ T cell functionality. Moreover, Treg depletion led to impaired migration of HMPV-specific $CD8^+$ T cells from mediastinal lymph nodes to the lungs, suggesting that Tregs are critical for the efficient priming of the $CD8^+$ T cell response.

Neutralizing antibodies against HMPV are important for preventing re-infection. Although every individual is seropositive for HMPV by age five, antibodies alone are not sufficient to protect against reinfection throughout life. Serological studies in non-human primates showed that neutralizing antibodies towards HMPV wane over time and have variable cross-reactivity to different subtypes and strains of HMPV, supporting the notion that immunity towards the virus is transient [14]. Rodents generate protective antibody responses and are only semi-permissive hosts; thus, they cannot be productively re-infected with HMPV [13]. The F protein is the main target for

antibodies and is highly immunogenic [45]. Antibodies against F are also cross-protective due to the high level of conservation between F proteins from different HMPV subtypes. However, antibodies targeting the other outer membrane proteins SH and G are not immunogenic or protective [45].

Numerous HMPV vaccine candidates have been tested in small animal models of infection. Vaccines such as virus-like particles (VLPs) and protein subunits have incorporated portions of the F protein due to its immunogenicity [46-48]. Multiple groups have reported the induction of neutralizing antibodies following subunit or VLP vaccination of small animals, but in many studies the antibodies were not long-lasting. A VLP vaccine containing the F and G proteins induced both B and T cell responses in a mouse model of vaccination, although virus-specific $CD8^+$ T cells still became impaired following vaccination [49]. Additionally, classical vaccine preparations including live-attenuated viruses, chimeric viruses, and inactivated viruses have been widely investigated and many have shown promise in pre-clinical models [12]. A live-attenuated HMPV vaccine was recently tested in a phase I clinical trial in adults and children, but the experimental vaccine was overattenuated in seronegative children and therefore not sufficiently immunogenic in the target population [50]. While significant progress has been made in understanding HMPV since its discovery less than 20 years ago, much more work is needed to uncover the complexities of the immune response to this pathogen.

### 1.3 Mechanisms of $CD4^+$ T cell help

$T_H$ cells play an important role in combating viral infections by coordinating the actions of multiple types of immune cells including $CD8^+$ T cells and B cells. Moreover, $T_H1$ cells produce

large quantities of IFNγ and TNFα, which promote antiviral responses and inflammation [51]. The requirement for $CD4^+$ T cell help during primary $CD8^+$ T cell responses depends on a variety of factors including the antigen, size of inoculum, route of infection, and virulence [52]. The ability of a pathogen to induce type I interferons, and thus the ability to activate APCs, also greatly impacts the need for $CD4^+$ T cell help during $CD8^+$ T cell priming [53, 54]. Non-inflammatory pathogens are thought to require $CD4^+$ T cell help to aid in $CD8^+$ T cell activation while $CD8^+$ T cell responses to highly inflammatory agents are believed to be $CD4^+$ T cell help independent. However, conflicting data have been published regarding the necessity of help during primary $CD8^+$ T cell responses for multiple intracellular pathogens including influenza virus, vaccinia virus, lymphocyte choriomeningitis virus (LCMV), and *Listeria monocytogenes* [52, 55].

It is well-established that $T_H$ cells are critical to the development of effective memory $CD8^+$ T cells following immunization and infection [56-58]. Numerous studies using mouse models of viral or bacterial infection have demonstrated that $CD8^+$ T cells primed in the absence of $CD4^+$ T cells (unhelped $CD8^+$ T cells) are impaired in their ability to protect against reinfection [56, 57, 59-61]. These T cells are poor cytokine producers, upregulate the inhibitory receptor PD-1, and fail to proliferate in response to secondary challenge [59]. Furthermore, unhelped $CD8^+$ T cells upregulate expression of TRAIL and undergo activation induced cell death upon re-stimulation [62]. Several studies have demonstrated that impairment of unhelped $CD8^+$ T cells can be overcome by addition of exogenous cytokines or costimulatory signals normally provided by activated DCs and $T_H$ cells [63, 64].

The best characterized mechanism of $CD4^+$ T cell help for $CD8^+$ T cells is through the licensing of DCs via interactions between CD40 and CD40L **(Fig. 1)** [65, 66]. Induction of this signaling pathway enhances the capacity for antigen presentation and promotes the upregulation

of costimulatory molecules by the DC including CD80/86 and CD70, stimulating robust $CD8^+$ T cell activation. Signaling through CD70 and CD27 on $CD8^+$ T cells has been implicated as the critical pathway connecting $CD4^+$ T cell help signals to $CD8^+$ T cell function [67, 68]. In addition to the upregulation of costimulatory molecules, licensed DCs secrete CCL3 and CCL4 which promote the recruitment of naïve $CD8^+$ T cells to the site of DC-$CD4^+$ T cell clusters, increasing the likelihood of naïve $CD8^+$ T cells encountering their cognate antigen [69]. Licensed DCs also produce multiple cytokines such as IL-12 and IL-15, which have been shown to be critical for $CD8^+$ T cell differentiation, proliferation, and survival. Besides DC licensing, $CD4^+$ T cells can directly stimulate $CD8^+$ T cell responses through the production of their own activating cytokines including IL-2, IFNγ, and IL-21 [55]. Importantly, IFNγ produced by $CD4^+$ T cells was shown to control the migration of pathogen-specific $CD8^+$ T cells into mucosal tissues during viral infection [70]. Recent transcriptomic data has also revealed that $CD4^+$ T cell help induces downregulation of inhibitory receptors on effector $CD8^+$ T cells [67]. Finally, $CD4^+$ T cells have been shown to control expression of the high affinity IL-2 receptor alpha chain CD25 on pathogen-specific $CD8^+$ T cells, which plays a prominent role in the formation of short-lived effector $CD8^+$ T cells [71].

$CD4^+$ T cells are also key contributors to successful B cell and antibody responses generated towards invading pathogens. A subset of $CD4^+$ T cells known as T follicular helper ($T_{FH}$) cells are the primary helpers for B cell responses within germinal centers. These cells act through a variety of mechanisms to promote survival, proliferation, differentiation, somatic hypermutation, immunoglobulin class switching, attraction, and adhesion [72]. Mechanisms include direct interactions between CD40L and CD40 on B cells, engagement of costimulatory molecules, and production of specialized chemokines and cytokines by $T_{FH}$ including IL-21 and IL-4 [72].

The complexities surrounding the requirement for and mechanisms of $CD4^+$ T cell help have remained a challenge for the field. To date, a comprehensive analysis of the mechanisms of $CD4^+$ T cell help for both $CD8^+$ T cell and B cell responses during HMPV infection has not been conducted.

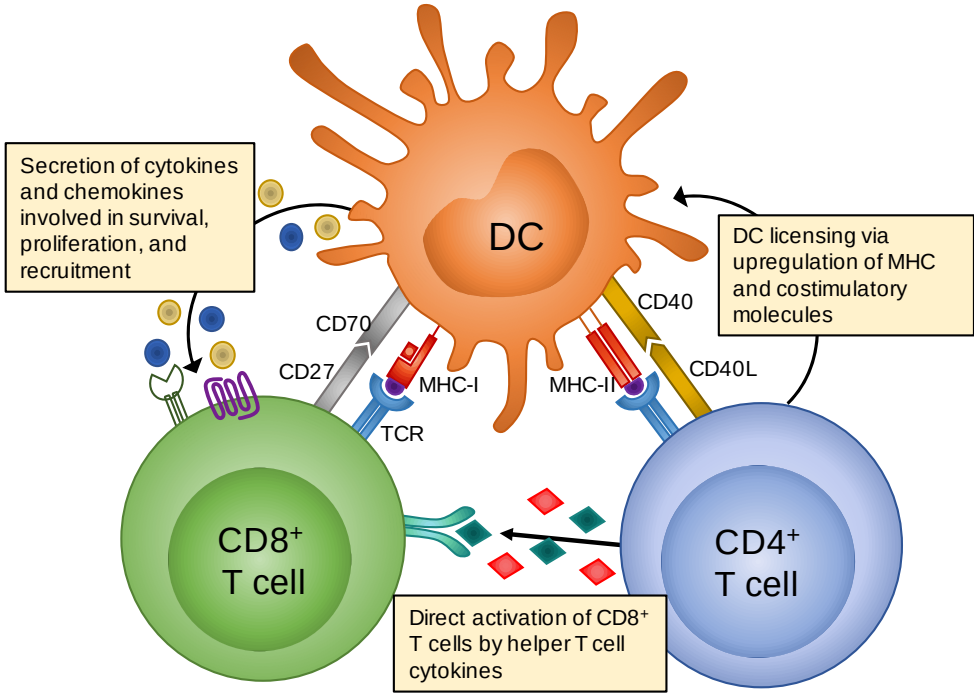


**Figure 1: Mechanisms of $CD4^+$ T cell help.**

$CD4^+$ T cells can provide help to $CD8^+$ T cells by licensing DCs via interactions between CD40 and CD40L. Induction of this signaling pathway enhances antigen presentation by increasing surface expression of MHC molecules and promotes the upregulation of costimulatory molecules on the DC. Licensed DCs also produce multiple cytokines which are critical for $CD8^+$ T cell differentiation, proliferation, and survival and secrete chemokines that promote the recruitment of naïve $CD8^+$ T cells to the site of the DC, increasing the probability of naïve $CD8^+$ T cells encountering their cognate antigen. $CD4^+$ T cells can directly stimulate $CD8^+$ T cell responses through the production of their own activating cytokines including IL-2, IFNγ, and IL-21. $CD4^+$ T cells have also been shown to control expression of CD25 on pathogen-specific $CD8^+$ T cells.

## 2.0 Identification and characterization of HMPV-specific $CD4^+$ T cells

### 2.1 Introduction

Human metapneumovirus (HMPV) is a leading cause of respiratory tract infection in children worldwide [3]. HMPV also causes significant morbidity and mortality in immunocompromised populations including premature infants, cancer patients, transplant recipients, and the elderly [8, 10, 73, 74]. All individuals experience primary HMPV infection by the age of five, but re-infections occur throughout life, indicating that immunological memory is incomplete or dysfunctional. Despite its widespread clinical burden, there are currently no FDA approved vaccines or effective therapeutics for HMPV.

Antibodies alone are insufficient at protecting humans against reinfection, as symptomatic infections can occur despite having detectable HMPV antibody titers [75]. Cytotoxic $CD8^+$ T cells play a vital role in combating respiratory virus infection, but it has recently been determined that HMPV-specific $CD8^+$ T cells become impaired following both primary and secondary infection in mice [35, 40]. Although $CD8^+$ T cells are the primary mediators of viral immunity, $CD4^+$ helper T cells play a prominent role in promoting and sustaining $CD8^+$ T cell responses. To date, the contribution of virus-specific $CD4^+$ T cells to the immune response to HMPV is completely unknown. Here, we identified an immunodominant MHC-II restricted HMPV epitope recognized by $CD4^+$ T cells from WT C57BL/6 mice and constructed the first MHC-II tetramer specific for an HMPV epitope. We found that virus-specific $CD4^+$ T cells are primarily $T_H1$ cells and display phenotypic and functional markers of impairment during acute HMPV infection.

## 2.2 Methods

### Mice and viruses

C57BL/6 were purchased from The Jackson Laboratory (Bar Harbor, Maine). PD-$1^{-/-}$ mice were obtained with permission from Tasuku Honjo (Kyoto University, Kyoto, Japan) and kindly provided by Karen Haas (Wake Forest University). Animals were bred and maintained in specific pathogen-free conditions in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee. Six- to twelve-week-old age- and sex-matched mice were used in all experiments. HMPV (clinical strain C2-202, subtype B1) was grown and titered in LLC-MK2 cells as previously described [13]. For all experiments, mice were anesthetized via isoflurane inhalation and inoculated intratracheally with $2.5x10^5$ PFU of HMPV or sucrose-purified cell lysate in a 100μl-volume.

### Peptides

We used both predictopes and overlapping peptide approaches to identify MHC-II H2-$IA^b$-restricted HMPV epitopes. For initial screening, 15-mer peptides overlapping by 9 amino acids covering the entire HMPV matrix (M), nucleoprotein (N), fusion (F), and glycoprotein (G) open reading frames were synthesized (GenScript). Predicted H2-$IA^b$-restricted epitopes for each HMPV protein were generated using the Immune Epitope Database (IEDB) (http://www.iedb.org) [76, 77]. 15-mer predictopes were constructed when the percentile rank was lower than 0.2 (GenScript).

**IFNγ ELISPOT**

Murine IFNγ Single-Color Enzymatic ELISPOT Assays (ImmunoSpot) were performed according to the manufacturer's instructions with slight modifications. $CD8^+$ T cells were magnetically removed from a single cell suspension of lung lymphocytes (EasySep Mouse CD8a Positive Selection Kit II, Stemcell Technologies) prior to plating $1x10^5$-$2x10^5$ cells/well in duplicate. For initial screens, dead cells were also removed prior to removal of $CD8^+$ T cells (EasySep Dead Cell Removal Kit, Stemcell Technologies). The mitogen concanavalin A (ConA, Sigma-Aldrich) was used as a positive control, while stimulation with media alone or an irrelevant peptide was used as a negative control. Additionally, wells containing the $CD8^+$ T cell epitope H2-$K^b/N_{11\text{-}19}$ were included to account for $CD8^+$ T cell contamination. For all screens, peptides were plated at a final concentration of 10μM. Subsequent screens from top hits only included one peptide per well. The average number of spots counted from the $CD8^+$ T cell contamination well was subtracted from the average number of spots in each of the HMPV epitope wells, and data were expressed as spot-forming cells (SFC) per $10^6$ lung $CD4^+$ T cells.

**MHC-II tetramers**

The H2-$IA^b$/HMPV $N_{218\text{-}229}$ (VYYRSLFIEYGK) tetramer was generously provided by Dr. Timothy Hand (University of Pittsburgh) and constructed as previously described [78]. The H2-$IA^b$/influenza A $NP_{311\text{-}325}$ (QVYSLIRPNENPAHK) tetramer was provided by the NIH Tetramer Core. Tetramers were conjugated to the fluorophore allophycocyanin.

**Flow cytometry**

Cells were isolated from lungs and spleens of animals as previously described [35]. Lymphocytes were stained with LIVE/DEAD Fixable Violet Dead Cell Stain (Life Technologies), anti-mouse CD16/CD32 (Fc Shield) (Clone 2.4G2, Tonbo Biosciences) and antibodies (all from BioLegend unless otherwise noted) for CD3 (Clone 145-2C1, BD Biosciences), CD19 (Clone 6D5), CD4 (Clone RM4-5), CD44 (Clone IM7), PD-1 (Clone RMP1-30), TIM3 (Clone RMT3-23), LAG3 (Clone C9B7W), and APC-labeled tetramers. For transcription factor analysis, cells were first stained for viability, CD19, and CD4. Cells were fixed and permeabilized with TrueNuclear Fixation/Permeabilization solution (BioLegend) before staining for T-bet (Clone 4B10, BioLegend), GATA3 (Clone 16E10A23, BioLegend), RORγT (Clone Q31-378, BD Biosciences), and FoxP3 (Clone FJK-16s, BioLegend). Flow cytometry data were collected using a BD LSRII cytometer (BD Biosciences) and analyzed via FlowJo software (FlowJo). MFI refers to geometric mean fluorescence intensity.

**Intracellular cytokine staining**

Lung and spleen lymphocytes isolated from infected animals were re-stimulated *ex vivo* for 5 hours at 37°C with either N218 synthetic peptide (10μM final concentration) or PMA/ionomycin (50 ng/ml PMA plus 2 μg/μl ionomycin, Sigma-Aldrich) in the presence of monensin (BD Biosciences). After stimulation, cells were stained for viability with LIVE/DEAD Fixable Violet Dead Cell Stain (Life Technologies), CD3, CD4, IFNγ (Clone XMG1.2, BD Biosciences), and IL-17a (Clone TC11-18H10.1).

### Statistics

Data anaylsis was performed using Prism version 7.0 (GraphPad Software). Comparisons between two groups were performed using unpaired, two-tailed Student *t*-tests, and significance is noted by asterisks (*). Error bars on each graph represent SD.

## 2.3 Results

### Identification of MHC-II H2-IA$^b$-restricted HMPV epitopes in C57BL/6 mice

It was previously reported that the early CD4$^+$ T cell response to HMPV infection in mice is predominately $T_H$1-like [43]. Therefore, we used IFNγ ELISPOT assays to identify immunodominant MHC-II H2-IA$^b$-restricted HMPV epitopes. Lung lymphocytes were isolated from HMPV-infected mice on day 7 p.i. and plated in duplicate with overlapping peptides and predictopes. However, because HMPV-specific CD8$^+$ T cells are also key producers of IFNγ [35], CD8$^+$ T cells were magnetically removed prior to plating lymphocytes to reduce false-positive results. Initial screens with overlapping peptide pools spanning four of the nine viral proteins identified seven pools that induced robust IFNγ production above background **(Table 1)**. Further analysis of peptide sequences revealed that two of the overlapping peptide pools, N1-27 and M73-105, contained previously identified MHC-I restricted HMPV epitopes [35, 49] and were excluded from future screens. Remaining overlapping peptides were rescreened individually along with predictopes obtained from the IEDB online algorithm software. Three peptides consistently induced a potent IFNγ response in lung CD4$^+$ T cells **(Fig. 2 and Table 2)**, suggesting that they are true MHC-II H2-IA$^b$ restricted HMPV epitopes. Interestingly, CD4$^+$ T cells recognizing the

M217 epitope consistently produced larger, but fewer spots compared to the N217 peptide **(Fig. 2B)**.

**Table 1: Overlapping HMPV peptide pools recognized by lung CD4$^+$ T cells.**

| Peptide pool name | # of overlapping peptides in pool | Avg # of spots per $10^6$ CD4$^+$ T cells | Overlapping Peptide Sequences |
|---|---|---|---|
| N1-27 | 3 | 1476.3 | 1- MSLQGIHLSDLSYKH<br>2- HLSDLSYKHAILKES<br>3- YKHAILKESQYTIKR |
| N217-243 | 3 | 2786.35 | 1- **KVYYRSLFIEYGKAL**<br>2- LFIEYGKALGSSSTG<br>3- KALGSSSTGSKAESL |
| M73-105 | 3 | 1881.95 | 1- NASAQGAAMSVLPKK<br>2- AAMSVLPKKFEVNAT<br>3- PKKFEVNATVALDEY |
| M199-231 | 4 | 231 | 1- GLIMIMTMNNPKGIF<br>2- TMNNPKGIFKKLGAG<br>3- GIFKKLGAGTQVIVE<br>4- **GAGTQVIVELGAYVQ** |
| F225-251 | 4 | 300.3 | 1- AELARAVSNMPTSAG<br>2- RAVSNMPTSAGQIKL<br>3- NMPTSAGQIKLMLEN<br>4- SAGQIKLMLENRAMV |
| F373-395 | 3 | 2448 .6 | 1-VALSPLGALVACYKG<br>2- PLGALVACYKGVSCS<br>3- **LVACYKGVSCSIGSN** |
| F385-407 | 3 | 808.5 | 1- YKGVSCSIGSNRVGI<br>2- SCSIGSNRVGIIKQL<br>3- GSNRVGIIKQLNKGC |

**Table 2: Individual HMPV peptides and predictopes recognized by lung CD4$^+$ T cells.**

| Peptide Name | Protein | Position | Peptide Sequence | IEDB percentile rank |
|---|---|---|---|---|
| N217 | N | 217-231 | KVYYRSLFIEYGKAL | 34.44 |
| M36 | M | 36-50 | FPLFQANTPPAVLLD | 0.14 |
| M217 | M | 217-231 | GAGTQVIVELGAYVQ | 64.19 |
| F229 | F | 229-243 | RAVSYMPTSAGQIKL | 2.96 |
| F381 | F | 381-395 | LVACYKGVSCSIGSN | 36.05 |

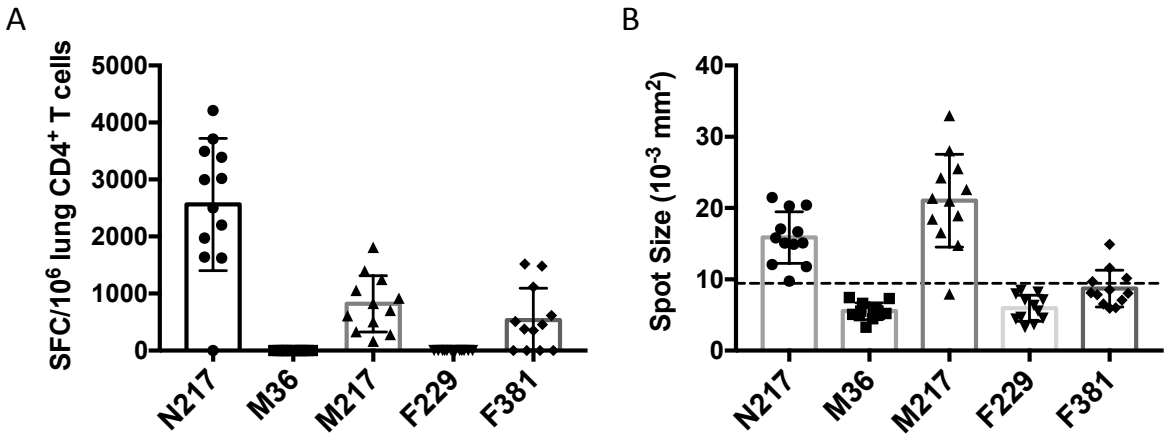


**Figure 2: HMPV peptides are recognized by lung CD4$^+$ T cells.**

HMPV peptides are recognized by IFNγ-producing CD4$^+$ T cells in WT mice. Lymphocytes were isolated from the lungs of HMPV-infected mice on day 10 p.i. and CD8$^+$ T cells were magnetically removed. Isolated CD4$^+$ T cells were stimulated with 10μM of HMPV peptide or predictope for 20hr at 37°C. IFNγ-spots were counted and SFC/10$^6$ lung CD4$^+$ T cells calculated and corrected by subtracting SFC from well containing a known CD8$^+$ T cell epitope (N11) (A). Average spot size for each peptide (B). Dotted line represents the average size of spots from N11 wells. Data are combined from three independent experiments with n=4 per group. *p<0.05, Student *t*-test.

## Enumeration of HMPV-specific CD4$^+$ T cells using MHC-II tetramers

To determine if CD4$^+$ T cells from infected WT mice can directly bind HMPV epitopes *ex vivo*, we utilized MHC-II tetramers. MHC-II tetramers specific for N217 were constructed since stimulation with this antigenic epitope induced the largest IFNγ response **(Fig. 2A)**. Since the core epitope was determined to begin at position 218, the tetramer will herein be referred to as N218 and is defined as shown in **(Fig. 3A)**. C57BL/6 mice were infected with HMPV and their lungs and spleens were harvested at various timepoints post-infection to assess the kinetics of the HMPV-specific CD4$^+$ T cell response. The frequency of N218 positive cells in the lungs ranged from 0.65% to 11.4% of CD4$^+$ T cells and peaked on day 10 p.i. **(Fig. 3B)**. In the spleen, tetramer-

specific cells ranged from 0.01% to 0.45% of $CD4^+$ T cells and peaked on day 13 p.i. **(Fig. 3C)**. As HMPV replication is restricted to the lungs, the low frequency of N218 positive cells in the spleen was expected. These results indicate that N218 is a highly immunodominant MHC-II epitope and establishes the N218 tetramer as the first known tool to study HMPV-specific $CD4^+$ T cells.

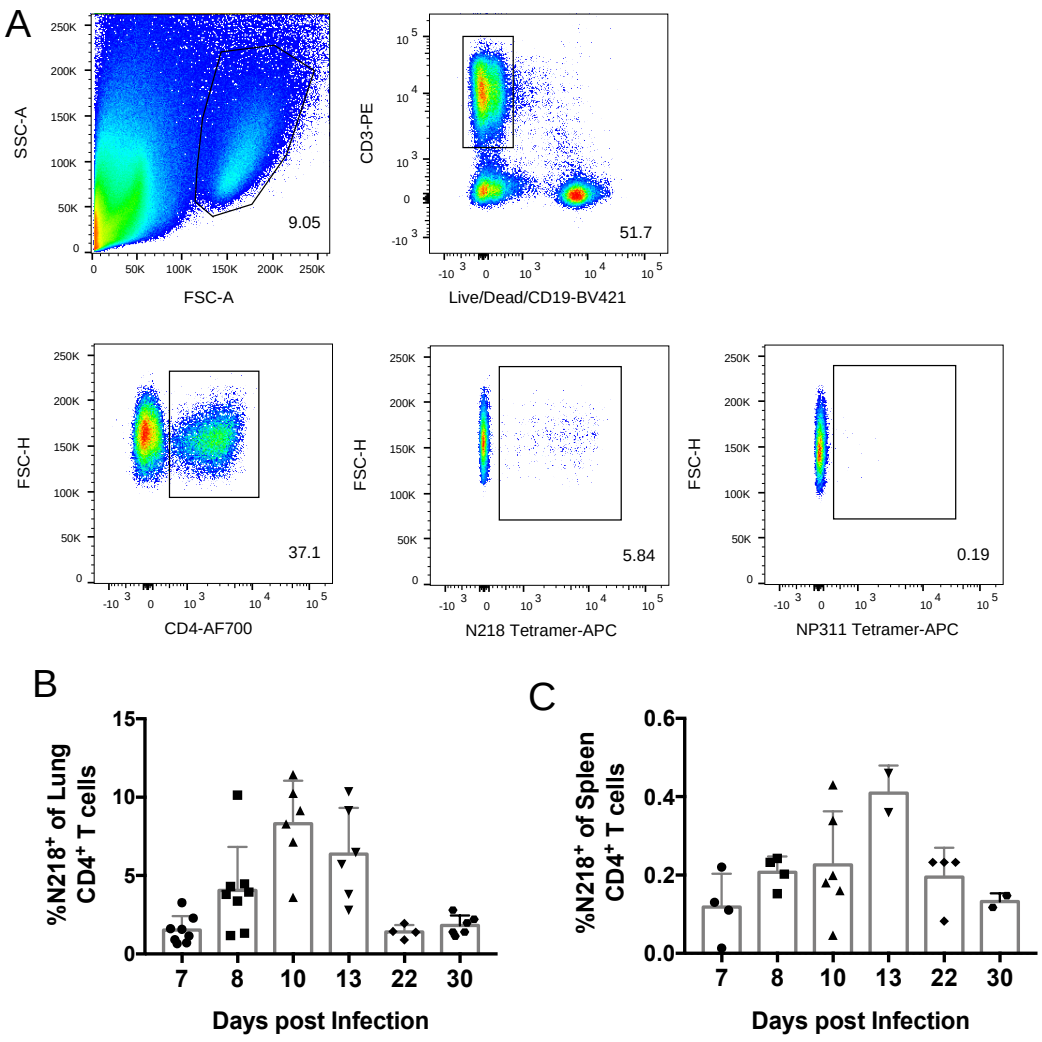


**Figure 3: Identification of HMPV-specific CD4$^+$ T cells using an MHC-II tetramer.**

Lungs and spleens were harvested at various time-points p.i. from WT mice infected with HMPV. Representative flow plot of gating strategy used to enumerate lung virus-specific T cells with N218 tetramer, day 13 p.i. Staining with influenza A NP311 tetramer was used as negative control for all experiments (A). Kinetic analysis of N218-specific CD4$^+$ T cells in lung (B) and spleen (C). Data are combined from 1-3 independent experiments, n=2-4 mice per time-point.

**Characterization of HMPV-specific CD4$^+$ T cells**

To identify which type of helper T cell recognizes the N218 epitope, we assessed the expression of subset-defining transcription factors in N218-specific CD4$^+$ T cells from the lungs of HMPV-infected mice on day 10 p.i. We found that about 80% of tetramer positive cells expressed T-bet, the master transcription factor of $T_H1$ cells **(Fig. 4A)**. Additionally, we observed variable expression of ROR$_\gamma$T in N218-specific cells suggesting that some HMPV-specific CD4$^+$ T cells skew towards a $T_H17$ phenotype. Besides transcription factor expression, CD4$^+$ helper T cell subsets are also distinguished by their cytokine profile [79]. Although we had identified the N218 epitope using an IFNγ-based assay, we stimulated pulmonary lymphocytes harvested from HMPV-infected mice on day 7 or 10 p.i. with PMA/ionomycin and performed intracellular cytokine staining (ICS) to determine if HMPV-specific CD4$^+$ T cells produce additional cytokines. Consistent with transcription factor staining and previous results from our lab and others [39, 43], our analysis indicated that IFNγ was the dominant cytokine produced by lung CD4$^+$ T cells during HMPV infection **(Fig. 4B)**. Interestingly, we observed a small but consistent population of IL-17a producing cells **(Fig. 4B)** but no detectable IL-4 or IL-5 (data not shown). Together, these data suggest that N218-specific CD4$^+$ T cells in the lung are predominately $T_H1$ cells, but that some may be $T_H17$-skewed.

HMPV-specific CD8$^+$ T cells in the lungs become impaired following infection and exhibit sustained upregulation of multiple inhibitory receptors [40, 41]. To determine if virus-specific CD4$^+$ T cells display characteristics similar to impaired CD8$^+$ T cells, we analyzed the expression of PD-1 on N218-specific T cells throughout HMPV infection. Surprisingly, the majority of N218-specific cells in the lung expressed PD-1 by day 7 p.i. and the frequency of PD-1 positive cells remained above 85% through day 30 p.i. **(Fig. 5A)**. Conversely, the frequency of non-tetramer

specific lung $CD4^+$ T cells expressing PD-1 never surpassed 80% and declined to about 20% by day 30 p.i. While most N218-specific $CD4^+$ T cells still express PD-1 by day 30 p.i., PD-1 expression on a per cell basis was reduced by almost 50% compared to day 7 p.i. **(Fig. 5B)**. In the spleen, the frequency of PD-1 positive N218-specific $CD4^+$ T cells ranged from 40-80% on day 7 p.i. and peaked at over 90% on day 10. However, PD-1 expression dropped dramatically by day 13 p.i. before increasing again at later time-points **(Fig. 5C)**. Within the lungs, PD-1 positivity is significantly higher on N218-specific lung $CD4^+$ T cells compared to non-tetramer positive $CD4^+$ T cells on day 10 p.i., suggesting that PD-1 upregulation is virus-induced **(Fig. 5D)**. Although PD-1 is often used as a marker of impairment, PD-1 expression alone is not sufficient to conclusively indicate T cell impairment or exhaustion [80]. Therefore, we assessed the expression of two other common inhibitory receptors, TIM-3 and LAG-3, on N218-specific $CD4^+$ T cells. Of the N218-specific cells that expressed PD-1, 40% co-expressed PD-1 and TIM-3, and nearly 7% expressed all three inhibitory receptors examined **(Fig. 5E)**. In contrast, only 23% of $CD4^+$ T cells that were not specific for N218 co-expressed PD-1 and TIM-3. This suggests that $CD4^+$ T cells recognizing the HMPV N218 epitope phenotypically resemble impaired T cells during acute respiratory virus infection.

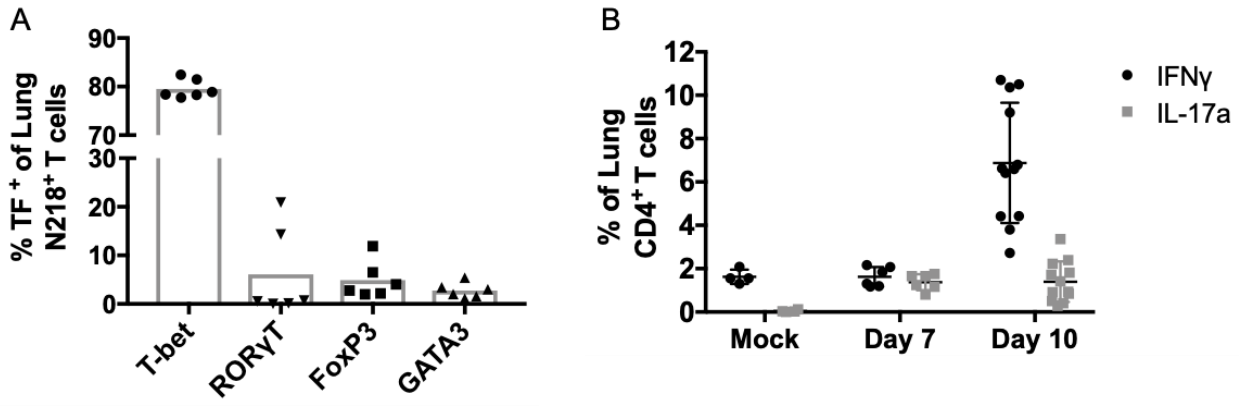


**Figure 4: N218-specific CD4$^+$ T cells are predominately $T_H$1 cells.**

Lungs were harvested from HMPV-infected WT mice on day 10 p.i. and stained for common helper T cell subset transcription factors (A). Lung lymphocytes isolated from mock or HMPV-infected animals were subjected to *ex vivo* stimulation with the mitogens PMA and ionomycin followed by ICS for common helper T cell subset cytokines (B). For (A), data are combined from 2 independent experiments with n=2-4 mice and for (B), data are combined from 1-3 independent experiments with n=2-4 animals per group.

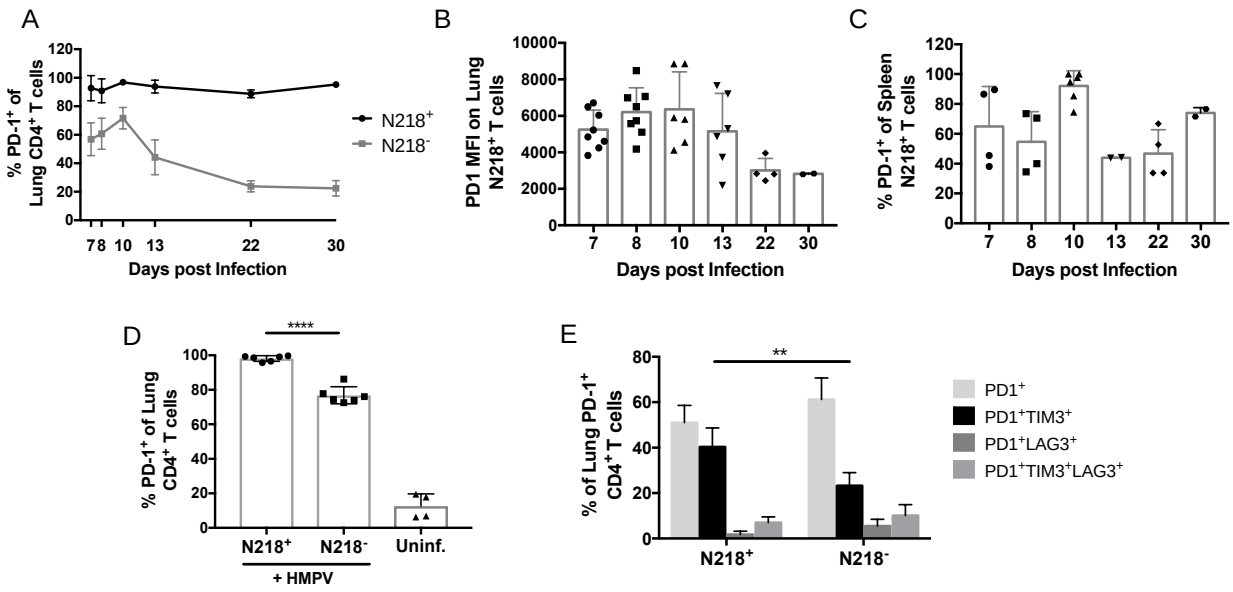


**Figure 5: N218-specific CD4$^+$ T cells display phenotypic markers of impairment during HMPV infection.**

PD-1 expression on N218-specific and non-N218-specific CD4$^+$ T cells in lung was quantified at multiple time-points post-HMPV infection of WT mice (A). PD-1 MFI (mean fluorescnce intensity) on N218-specific CD4$^+$ T cells (B). PD-1 expression on N218-specific CD4$^+$ T cells in the spleen was quantified at multiple time-points post-HMPV infection (C). Frequency of PD-1 expression on lung N218-specific cells isolated from HMPV-infected mice day 10 p.i., non-N218-specific lung CD4$^+$ T cells from HMPV-infected mice day 10 p.i., and bulk CD4$^+$ T cells from uninfected mice (D). Inhibitory receptor expression on PD-1 positive cells from lung N218-specific and non-N218-specific lung CD4$^+$ T cells from HMPV-infected mice day 10 p.i. (E). For (A-C), data combined from 2-3 independent experiments, n=2-4 mice per time-point. For (D-E), data are combined from 2 independent experiments, n=2-4 mice per group. ****p<0.0001, **p<0.01, Student *t*-test.

**PD-1 signaling does not contribute to lung N218-specific $CD4^+$ T cell impairment**

Multiple inhibitory receptors are upregulated on N218-specific $CD4^+$ T cells from the lungs of HMPV-infected mice and almost all virus-specific cells expressed PD-1 by day 10 p.i. **(Fig. 5)**. To determine if there was a functional consequence of this expression, we infected either WT or global PD-$1^{-/-}$ mice with HMPV and analyzed virus-specific $CD4^+$ T cell functionality on day 10 p.i. This was determined using two separate assays performed in parallel: N218 tetramer staining to quantify HMPV-specific $CD4^+$ T cells and *ex vivo* peptide stimulation with N218 followed by ICS for IFNγ to assess functional abilities. Lymphocytes isolated from both the lungs and spleens of WT animals produced very little IFNγ in response to stimulation with N218 peptide, suggesting that N218-specific cells are functionally impaired **(Fig. 6A and B)**. While the frequency of N218-specific $CD4^+$ T cells was significantly reduced in the lungs of PD-$1^{-/-}$ mice, we did not observe any improvement in IFNγ production by cells from the global knockouts compared to WT animals **(Fig. 6A)**. However, cells isolated from the spleens of PD-$1^{-/-}$ animals produced significantly more IFNγ following peptide stimulation compared to WT mice despite there being no difference in the frequency of N218-specific cells between groups **(Fig. 6B)**. Importantly, similar trends were observed when total numbers of virus-specific and IFNγ-producing cells were calculated for both lung and spleen **(Fig. 6C and D)**. These data suggest that while PD-1 signaling may limit IFNγ production by virus-specific $CD4^+$ T cells in a non-inflammatory environment such as the spleen, other mechanisms or inhibitory pathways may limit the functionality of HMPV-specific $CD4^+$ T cells in the lung.

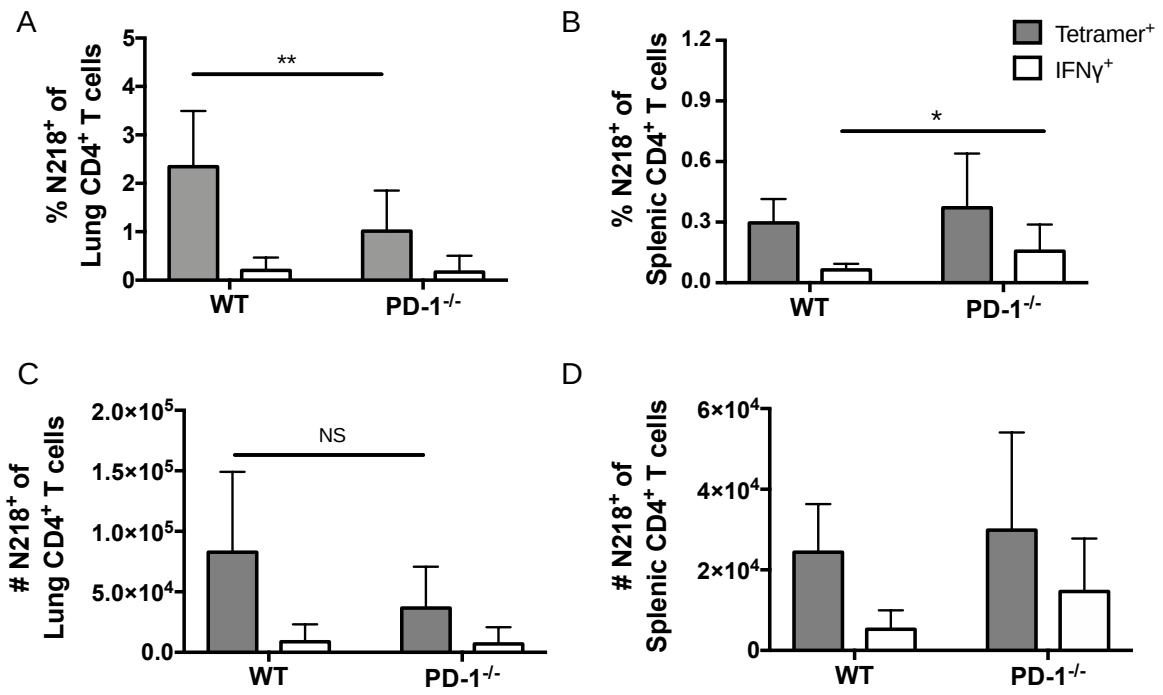


**Figure 6: N218-specific CD4$^+$ T cell impairment in the lung is not controlled by PD-1 signaling.**

Lungs and spleens were harvested from HMPV-infected mice on day 10 p.i. for N218 tetramer staining and *ex vivo* peptide stimulation followed by ICS for IFNγ. Frequencies (A and B) and total numbers (C and D) of N218-specific CD4$^+$ T cells and IFNγ-producing cells were quantified. Data combined from 3 independent experiments with 3-4 mice per group. **p<0.01, *p<0.05, Student *t*-test.

## 2.4 Discussion and Future Directions

HMPV remains a significant human pathogen in pediatric and immunocompromised populations. Recent efforts to design safe and effective vaccines for HMPV have shown promise, but a lack of understanding regarding certain aspects of the immune response to infection has hindered many candidates from moving forward. Furthermore, tools to study HMPV-specific $CD4^+$ T cells have not been available until now. Here we have identified, constructed, and characterized the first MHC-II tetramer specific for an HMPV epitope in C57BL/6 mice.

Our study began with ELISPOT assays testing IFNγ production in response to stimulation with viral peptides. In this manner, we identified three epitopes that led to consistent IFNγ production. MHC-II tetramers specific for the N217 epitope were constructed because of its ability to induce the most robust IFNγ production. While the N217 epitope induced the most spots, stimulation with the M217 epitope led to the formation of larger spots. This may indicate that $CD4^+$ T cells recognizing the M217 epitope are more functional, or that TCR: peptide-MHC complex interactions in those cells are stronger. Without having MHC-II tetramers specific for the M217 peptide, studies designed to test these possibilities are currently not feasible. In addition to testing overlapping peptides for four viral proteins, we tested a predicted epitope (M36) derived from online algorithms provided by The Immune Epitope Database. Unfortunately, predictive methods for MHC-II epitopes are often less accurate than for MHC-I epitopes due to variability in the length of peptides that can fit into MHC-II grooves [81]. Thus, despite HMPV M36 being ranked very highly for predicted binding affinity, the epitope was not recognized *in vivo*. Future HMPV MHC-II epitope discovery projects should explore additional algorithms because there are many other platforms available for predicting MHC-II binding.

After validating the specificity of the N218 tetramer, we performed a kinetic analysis of the virus-specific $CD4^+$ T cell response in HMPV-infected mice. N218-positive cells peaked in the lungs on day 10 p.i. and on day 13 p.i. in the spleen. Epitope-specific $CD4^+$ T cells are often found at lower frequencies than epitope-specific $CD8^+$ T cells and bind with lower avidity than immunodominant class-I epitopes, which may hinder MHC-II tetramer efficacy [82, 83]. Thus, the robustness of the N218-specific response in the lung following primary HMPV infection without artificially boosting or enriching for any population of cells was quite surprising. We think the delayed accumulation of virus-specific cells in the spleen is likely due to the migration of early-stage central memory cells to the spleen from the lungs following resolution of infection. Analysis of memory markers including CD62L and CD44 could confirm if splenic N218-specific $CD4^+$ T cells at later time-points are in fact memory or memory precursor cells.

As expected, N218-specific $CD4^+$ T cells are predominantly $T_H1$ skewed. $T_H1$ cells are known to play an important role during the immune response to intracellular pathogens by aiding $CD8^+$ T cell-mediated killing of infected cells [55]. We also observed a small population of RORγT positive and IL-17a producing cells, suggesting that some HMPV-specific $CD4^+$ T cells are $T_H17$-skewed. $T_H17$ cells are primarily associated with defense against bacterial and fungal infections, and so the presence of N218-specific $T_H17$ cells was unexpected. However, IL-17 was shown to play pathogenic role in a mouse model of RSV infection as blockade of IL-17 decreased viral load, reduced lung inflammation, and increased the number RSV-specific $CD8^+$ T cells [84]. Additionally, IL-17 has been shown to synergize with antiviral signaling to promote excessive inflammation during infection of human cells with several respiratory viruses [85]. HMPV induces the upregulation of numerous proinflammatory cytokines both *in vivo* and *in vitro* (unpublished observations), but the role of IL-17 during infection has not been studied. Our data support the

possibility that IL-17-producing virus-specific $CD4^+$ T cells may play a previously unrecognized role in the immune response to HMPV.

Recent data demonstrating that HMPV-specific $CD8^+$ T cells become impaired during infection led us to ask whether the same is true for HMPV-specific $CD4^+$ T cells. Assessment of PD-1 expression on N218-specific $CD4^+$ T cells from the lungs and spleens throughout primary HMPV infection indicated that lung N218-specific $CD4^+$ T cells exhibit sustained upregulation of PD-1 (over 90%) long after virus has been cleared. Furthermore, over half of the N218-specific $CD4^+$ T cells expressing PD-1 also expressed at least one other inhibitory receptor. PD-1/TIM-3 co-expression was the most common combination expressed on both N218-specific and non-N218-specific lung $CD4^+$ T cells. Interestingly, PD-1/TIM-3 co-expression was also the most common inhibitory receptor combination observed on HMPV-specific $CD8^+$ T cells following both primary and secondary infection of mice [40, 41]. Despite the observation that almost all N218-specific $CD4^+$ T cells expressed PD-1, lack of PD-1 signaling did not improve the functionality of these cells in the lungs. Given the small percentage of IFNγ-positive cells in either experimental group, alternative strategies to measure IFNγ production or additional assays to quantify other $T_H1$ cytokines including IL-2 and TNF-β may be required to determine if PD-1 truly does not contribute to N218-specific $CD4^+$ T cell impairment. It is worth noting that the mice used in these experiments were global knockouts for PD-1 and that lack of PD-1 signaling on other cells could have contributed to poor IFNγ production in our assay. Future studies plan to use $CD4^+$ T cell-specific PD-1 knockout mice to conclusively determine the role of PD-1 signaling on HMPV-specific $CD4^+$ T cells during infection.

The identification of an immunodominant MHC-II restricted HMPV epitope has opened the doors for new studies exploring the immune response to HMPV. In the future, it will be

important to determine the biological impact that HMPV-specific $CD4^+$ T cells have on $CD8^+$ T cell responses during both primary and secondary infection. Hopefully, the N218 MHC-II tetramer will be a useful tool for other HMPV researchers that aids in the continued investigation of the immune response to this important pathogen.

## 3.0 Defining the mechanism(s) of $CD4^+$ T cell help during HMPV infection

### 3.1 Introduction

$T_H$ cells play a prominent role in promoting and sustaining $CD8^+$ T cell responses during viral infection. Depending on the virus, $CD4^+$ T cells provide help to $CD8^+$ T cells by aiding in the activation of APCs and/or by directly influencing $CD8^+$ T cell survival and proliferation [52]. Furthermore, many groups have demonstrated that memory $CD8^+$ T cells primed in the absence of $CD4^+$ T cell help are functionally impaired, establishing a critical role for $T_H$ cells in memory $CD8^+$ T cell formation and protection against rechallenge [56, 57, 60, 86].

While the importance of $CD4^+$ T cell help has been observed across many viral infection models, the role of $CD4^+$ T cells in the immune response to HMPV infection is largely unknown. Our laboratory has discovered that HMPV-specific pulmonary $CD8^+$ T cells become impaired following both primary and secondary infection of WT mice [35, 40]. This impairment is characterized by reduced cytotoxic effector molecule release, decreased cytokine production, and the upregulation of multiple inhibitory receptors, including PD-1. HMPV-induced $CD8^+$ T cell impairment occurs even in the presence of $T_H$ cells, and virus-specific $CD8^+$ T cells display features suggestive of limited $T_H$ cell help. Here, we examined the importance of $CD4^+$ T cells during primary HMPV infection and the contribution of CD40/CD40L signaling to $CD8^+$ T cell responses.

## 3.2 Methods

### Mice and viruses

C57BL/6 were purchased from The Jackson Laboratory (Bar Harbor, Maine). Animals were bred and maintained in specific pathogen-free conditions in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee. Six- to twelve-week-old age- and sex-matched mice were used in all experiments. HMPV (clinical strain C2-202, subtype B1) was grown and titered in LLC-MK2 cells as previously described [13]. For all experiments, mice were anesthetized via isoflurane inhalation and inoculated intratracheally with $2.5x10^5$ PFU of HMPV in a 100μl-volume. Lung viral titers were quantified by plaque titration as described previously [13].

### Flow cytometry

Cells were isolated from lungs and spleens of animals and tetramer staining was performed as previously described [35]. Lymphocytes were stained with LIVE/DEAD Fixable Violet Dead Cell Stain (Life Technologies), anti-mouse CD16/CD32 (Fc Shield) (Clone 2.4G2, Tonbo Biosciences) and antibodies for CD19 (Clone 6D5, BioLegend), CD3 (Clone 145-2C1, BD Biosciences), CD8 (Clone 53-6.7, BD Biosciences), CD44 (Clone IM7, BioLegend), PD-1 (Clone RMP1-30, BioLegend), and APC-labeled MHC class I tetramers ($H2\text{-}K^b/N_{11\text{-}19}$) (NIH Tetramer Core). Flow cytometry data were collected using a BD LSRII cytometer (BD Biosciences) and analyzed via FlowJo software (FlowJo). MFI refers to geometric mean fluorescence intensity.

**Intracellular cytokine staining**

Lung and spleen lymphocytes isolated from infected animals were restimulated *ex vivo* for 5 hours at 37°C with HMPV N11 peptide (10μM final concentration) in the presence of brefeldin A and monensin (both from BD Biosciences). Stimulation with PMA/ionomycin (50 ng/ml PMA plus 2 μg/μl ionomycin, Sigma-Aldrich) served as a positive control. After stimulation, cells were stained for viability with LIVE/DEAD Fixable Violet Dead Cell Stain (Life Technologies), CD19, CD3, CD8, CD107a (Clone 1D4B), and IFNγ (Clone XMG1.2, both from BD Biosciences).

**$CD4^+$ T cell depletion**

C57BL/6 mice were injected i.p. with 300μg of anti-mouse CD4 (Clone GK1.5) or Rat IgG2b control (Clone LTF-2) (both from Bio X Cell) in 200μl PBS one day prior to intratracheal inoculation with HMPV. Animals were boosted with 150μg of antibody on day 3 of infection.

**Agonistic CD40 treatment**

C57BL/6 mice were injected i.p. with 100μg of anti-mouse CD40 (Clone FGK4.5) or Rat IgG2a control (Clone 2A3) (both from Bio X Cell) in 200μl PBS on day 1 of HMPV infection.

**CD40L blockade**

C57BL/6 mice were injected i.p. with 250μg of anti-mouse CD40L (Clone MR-1) or Armenian Hamster IgG control (both from Bio X Cell) in 200μl PBS on days 0, 2, and 4 of HMPV infection.

**Statistics**

Data anaylsis was performed using Prism version 7.0 (GraphPad Software). Comparisons between two groups were performed using unpaired, two-tailed Student *t*-tests, and significance is noted by asterisks (*). Error bars on each graph represent SD.

## 3.3 Results

**Effect of $CD4^+$ T cell depletion during primary HMPV infection**

To determine the requirement for $CD4^+$ T cell help during primary HMPV infection, we antibody-depleted $CD4^+$ T cells one day prior to infection of WT mice with $5x10^5$ PFU of HMPV. Animals were boosted with antibody on day three p.i. to ensure $CD4^+$ T cell depletion was maintained throughout the experiment. Contrary to what has been published previously in a BALB/c model of HMPV infection [39], depletion of $CD4^+$ T cells had no impact on body weight loss in our model **(Fig. 7A)**. Animals treated with the depletion antibody displayed higher levels of virus in the lungs compared to IgG control antibody treated animals on day 7 p.i. **(Fig. 7B)**, but virus was completely cleared in both treatment groups by day 10 p.i. **(Fig. 7C)**. Peak viral titers on day 5 p.i. were no different between treatment groups (data not shown).

Analysis of virus-specific $CD8^+$ T cells using MHC-I tetramers of the HMPV epitope $N_{11-19}$ revealed a significant decrease in the frequency of cells in the lungs of $CD4^+$ T cell-depleted mice on day 10 p.i., but there was no difference in the functional capacity of these cells **(Fig. 7D)**. There was not a significant difference in the frequency of HMPV-specific $CD8^+$ T cells isolated from the spleen and these cells were not impaired in either treatment group (data not shown). $CD4^+$

T cell help has been shown to promote downregulation of inhibitory receptors on effector $CD8^+$ T cells [67], so we examined PD-1 expression on virus-specific $CD8^+$ T cells next. We observed a significant increase in the frequency of HMPV-specific $CD8^+$ T cells expressing PD-1 in the lungs of $CD4^+$ T cell depleted animals compared to control mice on day 10 p.i. **(Fig. 7E)**, as well as increased PD-1 expression on a per cell basis **(Fig. 7F)**. Similar results were seen on day 7 p.i. (data not shown). These data indicate that help provided by $CD4^+$ T cells during primary HMPV infection contributes to viral clearance and reduces inhibitory receptor expression on virus-specific $CD8^+$ T cells. Additionally, $CD4^+$ T cell help may be important for effector $CD8^+$ T cell survival or migration during infection.

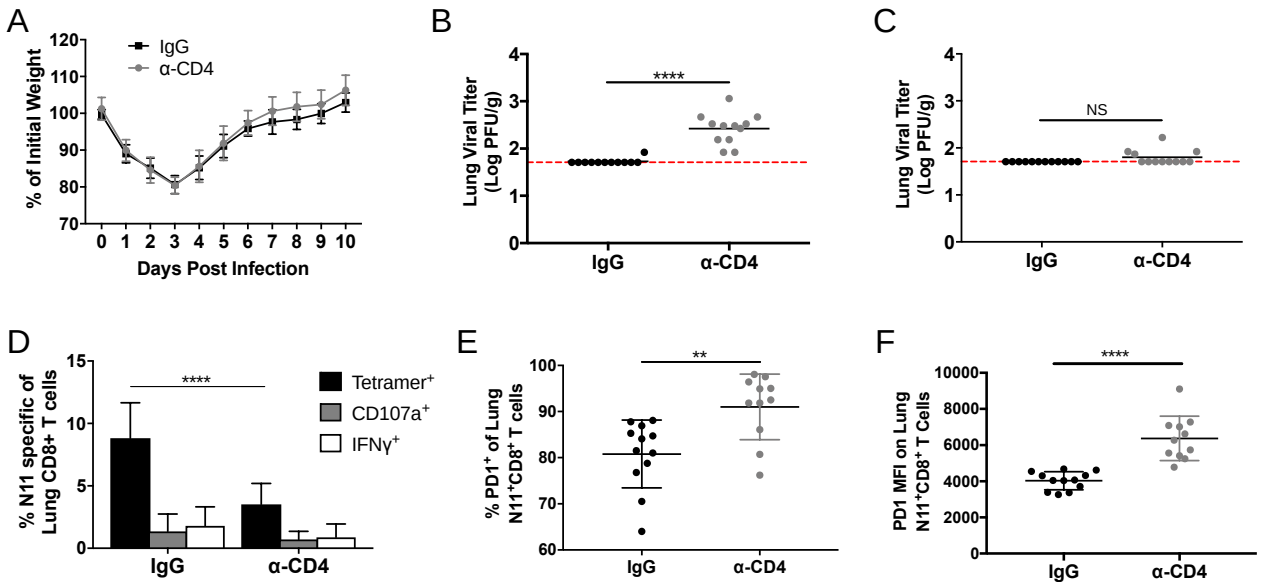


**Figure 7: CD4$^+$ T cell depletion delays viral clearance and reduces frequency of HMPV-specific CD8$^+$ T cells.**

WT mice were i.p. injected with 300μg of α-CD4 or IgG control antibody on day -1 and 150μg of antibody on day 3 of HMPV infection. Mice were weighed daily (A). Lung viral titers were quantified on days 7 (B) or 10 (C) p.i. HMPV-specific CD8$^+$ T cell responses (D), PD-1 expression (E), and PD-1 MFI (F) were quantified at day 10 p.i. Data are combined from three independent experiments, n=3-4 per group. **p<0.01, ****p<0.0001, Student *t*-test.

**Agonistic CD40 negatively impacts the immune response to HMPV**

Multiple reports have demonstrated that the addition of exogenous CD40 can overcome a lack of $CD4^+$ T cell help to directly stimulate antigen presenting cells [65, 66]. To test the hypothesis that existing $CD4^+$ T cells are dysfunctional during HMPV infection, we injected mice with agonistic CD40 antibody or an isotype-matched control antibody one day after inoculation with HMPV. Mice treated with the agonistic antibody lost significantly more weight than their isotype control counterparts beginning on day 4 p.i. and continued to lose weight until euthanasia, indicative of worse disease **(Fig. 8A)**. However, these animals displayed reduced lung viral titers on day 7 p.i. compared to IgG control mice **(Fig. 8B)**. Analysis of immune cells revealed a significant increase in the total number of lymphocytes in both the lungs and spleens of animals treated with agonistic CD40 on day 7 p.i. **(Fig. 8C)**, as well as a significant increase in the frequency of activated $CD8^+$ T cells **(Fig. 8D)**. We did not see a difference between groups in the frequency of pulmonary HMPV-specific cells expressing PD-1 on day 7 p.i., but there were significantly more bulk $CD8^+$ T cells expressing PD-1 in the mice treated with agonistic CD40 **(Fig. 8E)**. Although most widely known for its role as an inhibitory receptor, PD-1 is also an indicator of T cell activation at early time points of infection [87]. Unexpectedly, we observed a significant reduction in the frequency of HMPV-specific $CD8^+$ T cells in the lungs of CD40 treated animals on day 7 p.i. **(Fig. 8F)**. These data indicate that addition of exogenous CD40 during HMPV infection nonspecifically over-activates adaptive immune cells which may be detrimental to the HMPV-specific $CD8^+$ T cell response.

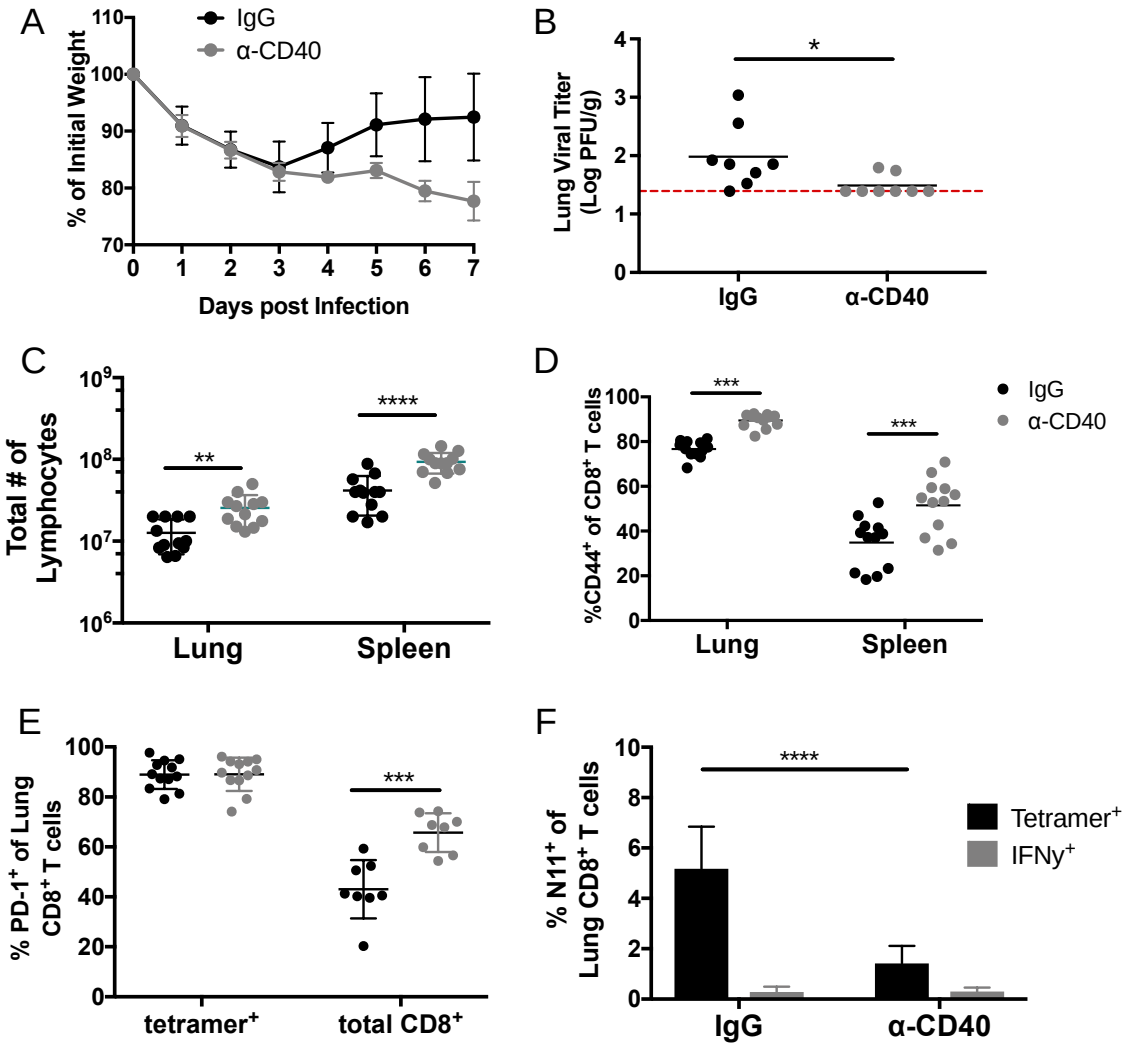


**Figure 8: Agonistic CD40 negatively impacts the immune response to HMPV.**

WT mice were injected i.p. with 100μg of α-CD40 or IgG control antibody on day 1 of HMPV infection. Mice were weighed daily (A) and lung viral titers were quantified on day 7 p.i. (B). Total lymphocyte numbers (C), activated $CD8^+$ T cell frequencies (D), PD-1 expression (E), and functionality (F) of HMPV-specific $CD8^+$ T cells was quantified on day 7 p.i. Data combined from 2-3 independent experiments, n=4 per group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, Student *t*-test.

**CD40L-blockade inhibits $CD8^+$ T cell activation during HMPV infection**

To test whether CD40/CD40L signaling is an important mechanism of $CD4^+$ T cell help for $CD8^+$ T cells during HMPV infection, mice were injected with an α-CD40L blocking antibody or isotype control antibody throughout primary HMPV infection. CD40L blockade had no impact on body weight loss **(Fig. 9A)**, but did prevent virus from being completely cleared from the lungs by day 7 p.i. **(Fig. 9B)**. Contrary to what was observed with the agonistic CD40 treatment, there was a significant reduction in the total number of lymphocytes **(Fig. 9C)** and $CD8^+$ T cells **(Fig. 9D)** in the lungs of animals treated with α-CD40L. Additionally, virus-specific $CD8^+$ T cell frequencies in the lungs were significantly lower in the CD40L-blockade group compared to isotype controls on day 7 p.i. **(Fig. 9E)**. HMPV-specific pulmonary $CD8^+$ T cells isolated from α-CD40L treated mice also expressed significantly less PD-1 on day 7 p.i. than virus-specific $CD8^+$ T cells from control animals **(Fig. 9F)**. These data indicate that CD40 blockade inhibits $CD8^+$ T cell activation during HMPV infection and leads to delayed viral clearance, suggesting that CD40/CD40L signaling between $CD4^+$ T cells and APCs is necessary for a successful $CD8^+$ T cell response.

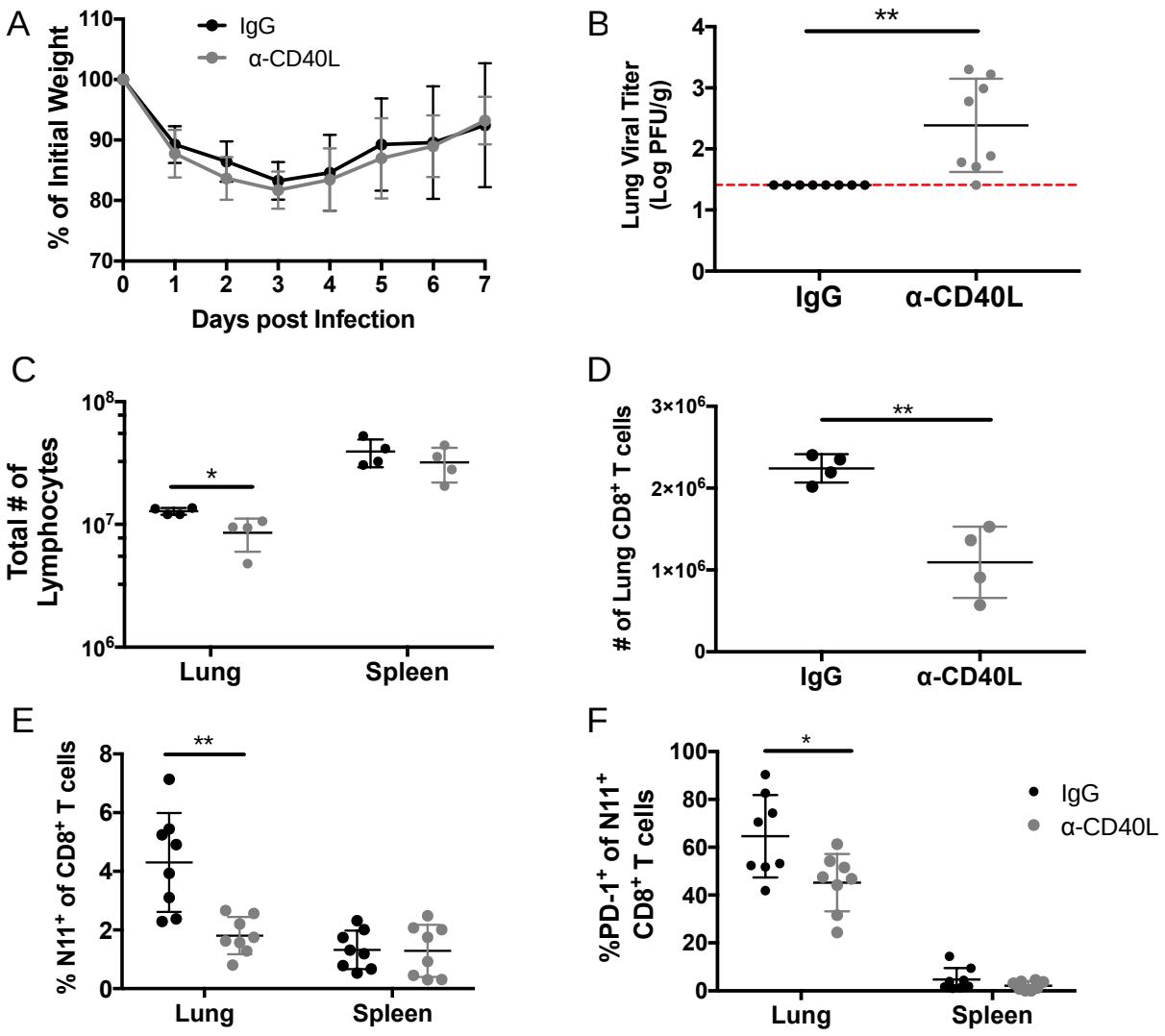


**Figure 9: CD40L-blockade inhibits CD8$^+$ T cell activation during HMPV infection.**

WT mice were injected i.p. with 250μg of α-CD40L or IgG control antibody on days 0, 2, and 4 of HMPV infection. Mice were weighed daily (A) and lung viral titers were quantified on day 7 p.i. (B). Total lymphocyte numbers (C), total CD8$^+$ T cell numbers (D), HMPV-specific CD8$^+$ T cell frequencies (E), and PD-1 expression (F) was quantified on day 7 p.i. Data are combined from 1-2 independent experiments, n=4-8 per group. *p<0.05, **p<0.01, Student *t*-test.

### 3.4 Discussion and Future Directions

The immune response to acute viral infection is dominated by $CD8^+$ T cell-mediated killing of infected cells. However, this process requires the coordinated actions of multiple types of immune cells that aid in $CD8^+$ T cell activation, function, and survival. These experiments sought to define the mechanism(s) by which $CD4^+$ T cells provide help to effector $CD8^+$ T cells during HMPV infection. We first examined the requirement for $CD4^+$ T cell help during primary HMPV infection by antibody-depleting $CD4^+$ T cells prior to infection. Although depletion antibodies are the most widely used method to investigate mechanisms of $CD4^+$ T cell help, all $CD4^+$ T cells, including regulatory T cells (Tregs), are depleted in these experiments. Recent findings from our lab show that Tregs are critical for efficient priming of $CD8^+$ T cells during HMPV infection, as Treg depletion throughout infection decreased virus-specific $CD8^+$ T cell numbers, delayed viral clearance, and impaired migration of HMPV-specific $CD8^+$ T cells [44]. Therefore, we are currently unable to determine if the phenotypes we observed during our $CD4^+$ T cell depletion experiments were due to a lack of $CD4^+$ T cell help or the lack of Tregs. To overcome this problem, I recommend developing a novel mouse model in which the diphtheria toxin receptor (DTR) is expressed under the control of the CD4 promotor. Then, animals could be treated with diphtheria toxin to selectively delete $CD4^+$ T cells, followed by adoptive transfer of Tregs isolated from FoxP3-GFP mice to generate CD4-DTR+Treg mice. In such a system, one could then probe the importance of $CD4^+$ helper T cells while keeping the Treg compartment completely intact. This system could also be beneficial for studies examining both primary and secondary $CD8^+$ T cell responses in the absence of $CD4^+$ T cell help. Additionally, it is possible that antibody and memory $CD8^+$ T cell responses are weakened by the lack of proper help from $CD4^+$ helper T cells. Future studies should examine the timing of $CD4^+$ T cell help to determine when help is needed most.

Results from these experiments could help inform vaccine design to improve the longevity of the immune response to HMPV vaccination.

Engagement of CD40 on DCs by CD40L on activated $CD4^+$ T cells is a key mechanism of $CD4^+$ T cell help [65, 66]. CD40 is a costimulatory receptor belonging to the TNFR family of costimulatory molecules [88]. It is primarily expressed on APCs and B cells and engages with its ligand CD40L to induce positive costimulatory functions. CD40L is primarily expressed by activated T cells, but under inflammatory conditions such as a viral infection, can be transiently upregulated on other immune cells including NK cells, mast cells, monocytic cells, and basophils [88]. Given its potent costimulatory abilities, CD40 is occasionally used as a vaccine adjuvant to boost T cell responses and has been successfully used in HMPV vaccines tested in mice [40, 89, 90]. To determine if CD40/CD40L signaling is one of the major mechanisms of $CD4^+$ T cell help during HMPV infection, we provided either agonistic CD40 or blocked CD40 signaling *in vivo* using monoclonal antibodies. Interestingly, treatment with agonistic CD40 antibody significantly reduced the frequency of HMPV-specific $CD8^+$ T cells in the lungs of infected mice but enhance viral clearance. Similar results were reported in an LCMV infection model where following anti-CD40 treatment, virus-specific $CD8^+$ T cells initially proliferated normally but were lost at an increased rate compared to those in untreated mice [91]. In that report, the premature collapse of the virus-specific $CD8^+$ T cell compartment depended partly on Fas expression. It would be worthwhile to explore this possibility in the context of HMPV infection. As expected, blocking CD40 signaling severely impaired $CD8^+$ T cell activation and led to delayed viral clearance, indicating that CD40 signaling is important for the induction of a successful $CD8^+$ T cell response during HMPV infection. Although this work is still preliminary, these results also suggest that CD40/CD40L signaling is not impaired during HMPV infection since we see profound differences

between our CD40L-blockade and control groups. Moreover, this works highlights the importance of considering CD40 adjuvant dose during vaccine design, as too much co-stimulation is more harmful than helpful in the context of HMPV infection.

Future studies should explore additional mechanisms of $CD4^+$ T cell help including IL-2, $T_H1$-specific IFNγ, and DC activation. It is likely that $CD4^+$ T cells contribute to multiple aspects of the $CD8^+$ T cell response to HMPV infection through both direct and indirect mechanisms. Unraveling the mechanisms of $CD4^+$ T cell help will provide new insights that may aid in the development of effective vaccines or therapies targeting HMPV. As many potential therapies for HMPV fail due to the inability to generate long-lived memory $CD8^+$ T cells in preclinical models, a greater comprehension of how $CD4^+$ T cells aid in the $CD8^+$ T cell response is essential.

## 4.0 Concluding Remarks

HMPV remains an important human pathogen in pediatric and immunocompromised populations. Despite significant progress being made in understanding the pathogenesis of and immunity to HMPV since its discovery almost 20 years ago, there are still no FDA approved vaccines or targeted therapeutics to prevent and treat infection. One of the many reasons why vaccine development has proved challenging is due to the inability of preclinical models to induce long-term protection following vaccination. Therefore, it is imperative that the HMPV community continues to investigate the basic mechanisms of immunity.

This project set out to determine the contribution of $CD4^+$ helper T cells to the immune response to HMPV through two specific aims; by determining the HMPV-specific $CD4^+$ T cell response and by defining the mechanisms by which $CD4^+$ T cells contribute to $CD8^+$ T cell functionality. Through the combined efforts of many individuals, we successfully identified immunodominant $IA^b$-restricted HMPV epitopes and constructed the first MHC-II tetramer specific for an HMPV peptide. We used this tetramer to demonstrate that HMPV-specific $CD4^+$ T cells display phenotypic and functional markers of T cell impairment during infection. While we have not yet determined the mechanism(s) of impairment, there is evidence suggesting that the inhibitory receptors PD-1 and TIM-3 may play a prominent role in limiting $CD4^+$ T cell functionality in the lung. I hope that future studies will continue to probe the mechanisms of $CD4^+$ T cell impairment, and that information gained from those studies will help enhance our understanding of the immune response to HMPV.

Next, we investigated the role of $CD4^+$ T cells as helpers of the $CD8^+$ T cell response during primary HMPV infection. Through a series of depletion experiments, we learned that $CD4^+$ T cells

contribute to viral clearance and potentially to $CD8^+$ T cell survival and/or recruitment. Unfortunately, results obtained from these experiments were clouded by the fact that $CD4^+$ regulatory T cells were also depleted using conventional antibody-mediated depletion strategies. Future studies aim to overcome this issue through the development of a novel murine model that is only lacking $CD4^+$ helper T cells. In this aim, we also examined the importance of CD40/CD40L signaling as a mechanism of $CD4^+$ T cell help for $CD8^+$ T cells. Through the addition of either agonistic CD40 or CD40L blocking antibodies, we discovered that this signaling pathway plays an important role in promoting $CD8^+$ T cell responses. Addition of agonistic CD40 enhanced viral clearance but led to a reduced frequency of HMPV-specific $CD8^+$ T cells in the lungs of infected animals. However, blocking CD40 signaling completely led to reduced $CD8^+$ T cell activation and delayed viral clearance. Although CD40/CD40L signaling often occurs between $CD4^+$ T cells and APCs to activate the APC, we were unable to determine if our results were due to alterations in the activation or efficiency of APCs. Future studies should explore this possibility to determine if CD40/CD40L signaling is a primary component of the $CD4^+$ T cell help signature during HMPV infection.

Collectively, this project has uncovered important new information about the contribution of $CD4^+$ helper T cells in the immune response to HMPV. Further defining the role of $CD4^+$ T cell help during infection may provide new insights that aid in the development of effective vaccines or therapies targeting HMPV. Moreover, additional studies could greatly enhance our understanding of the underlying mechanisms of $CD8^+$ T cell impairment during acute respiratory virus infection.

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