

IMMUNITY AND PROTECTION AGAINST HUMAN METAPNEUMOVIRUS

By

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LIST OF ABBREVIATIONS

AMPV	Avian metapneumovirus
APC	Antigen-presenting cell
B7tg	B7 transgenic
CTLA-4	Cytotoxic T lymphocyte associated protein 4
DC	Dendritic cell
ERK	Extracellular signal-regulated kinase
FI-RSV	Formalin-inactivated respiratory syncytial virus
GAL-9	Galectin-9
HIV	Human immunodeficiency virus
HMPV	Human metapneumovirus
HPV	Human papillomavirus
IFNs	Interferons
IFNAR	Interferon alpha/beta receptor
ILC	Innate lymphoid cell
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
JAK1	Janus kinase-1
LAG-3	Lymphocyte activation gene-3
LCMV	Lymphocytic choriomeningitis virus
MDA-5	Melanoma differentiation-associated gene-5
MEK	Mitogen-activated or extracellular signal-regulated protein kinase
MyD88	Myeloid differentiation primary response protein 88
NK	Natural killer

PAMPs	Pathogen associated molecular patterns
PD-1	Programmed-death 1
PRRs	Pattern recognition receptors
PCR	Polymerase chain reaction
RIG-I	Retinoic acid inducible gene-I
RSV	Respiratory syncytial virus
SHIP	Src-homology 2 domain-containing inositol 5'phosphatase
SHP	Src homology 2 domain-containing phosphatase
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TIM-3	T cell immunoglobulin and mucin domain 3
TLRs	Toll-like receptors
TMG	TiterMax Gold
TRAIL	Tumor-necrosis factor-related apoptosis-inducing ligand
TYK2	Tyrosine kinase 2

CHAPTER I

INTRODUCTION

Thesis overview

This thesis provides an overview of human metapneumovirus (HMPV), the role of natural killer (NK) cells during infection, and the CD8⁺ T cell (T_{CD8}) immune response to an HMPV virus-like particle vaccine. In Chapter I, I provide background information about HMPV, its discovery, symptoms of infection, and animal models. I then review what is known about the innate and adaptive immune responses to HMPV, and how different cell types respond to infection. I summarize current knowledge about inhibitory receptors such as programmed death-1 (PD-1) and lymphocyte activation gene-3 (LAG-3) and their role in modulating the immune response. Lastly, I provide information about current treatments for HMPV, vaccine candidates that various groups of scientists have tested in animal models, and the advantages/disadvantages of these approaches. In Chapter II, I present data that even though NK cells respond to HMPV infection by degranulating and producing IFN γ , they do not affect the outcome of infection in mice, as NK cell depletion does not alter viral titer, lung histopathology, or the adaptive T cell immune response. In Chapter III, I explore whether virus-like particle (VLP) vaccination protects mice from HMPV challenge. I compare the intranasal and intraperitoneal routes of VLP administration with regards to viral titer after challenge, T_{CD8} response, and inhibitory receptor expression. Both routes of vaccination protected mice completely in the lungs and partially in the noses, and elicited functional lung T_{CD8}. However, after HMPV challenge, T_{CD8} in the lungs of vaccinated mice were no more functional than those in previously infected mice. In Chapter IV, I summarize my findings and offer future directions that address important questions in the field.

Human metapneumovirus background

Paramyxoviruses are enveloped, negative-sense, single-stranded RNA viruses that cause a number of diseases in humans and animals. This family of viruses includes measles, mumps, human parainfluenza viruses, respiratory syncytial virus (RSV), and human metapneumovirus (HMPV) (Figure 1-1). HMPV, the main focus of my studies, was identified in 2001 by a group in the Netherlands analyzing nasopharyngeal aspirate samples taken from children with respiratory tract infection of unknown cause (1). The virus was distinct from known respiratory viruses, since PCR and immunological assays using virus-specific antibodies failed to identify the agent. Using a technique called randomly primed PCR, the researchers obtained a limited genomic sequence that appeared to be closely related to that of avian metapneumovirus, and thus named the newly-discovered virus human metapneumovirus.

HMPV is a major cause of upper and lower respiratory tract infections in children and the immunocompromised worldwide (1-3). Young infants and those with compromised cardiac or pulmonary function as well as the elderly are at the greatest risk of severe disease. Like the other paramyxoviruses that cause respiratory tract infection, HMPV is implicated in a spectrum of illnesses due to upper airway infection (e.g. cough, rhinitis) and lower respiratory tract infection (e.g. bronchiolitis and pneumonia). Diseases caused by these viruses are indistinguishable on clinical grounds alone. HMPV is second only to RSV as a cause of bronchiolitis in infants and young children (4, 5). The incidence of HMPV-associated lower respiratory tract infections in young children varies by location and time of year, but is 5-15% in most studies (2, 6). HMPV has also been identified in children with acute otitis media. In one study, one-third of children with lower respiratory tract infections associated with HMPV were diagnosed with concomitant otitis media (2).

Order: Mononegavirales

Family: Paramyxoviridae

Subfamily: Paramyxovirinae

Genus: Respirovirus

Species: human parainfluenza types 1 and 3

Genus: Rubulavirus

Species: human parainfluenza types 2 and 4; mumps

Genus: Morbillivirus

Species: measles

Genus: Henipavirus

Species: Hendra virus; Nipah virus

Subfamily: Pneumovirinae

Genus: Pneumovirus

Species: respiratory syncytial virus

Subgroup: A and B

Genus: Metapneumovirus

Species: human metapneumovirus

Subgroup: A and B

Figure 1-1: HMPV classification. HMPV is in the Paramyxoviridae family, Pneumovirinae subfamily, Metapneumovirus genus. RSV, another pneumovirus, is the most closely related human pathogen to HMPV. Adapted from (7).

Genetically, HMPV is most closely related to avian metapneumovirus (AMPV), which causes rhinotracheitis in turkeys and swollen head syndrome in chickens (8, 9). The HMPV genome contains 8 genes, in the order 3'-N-P-M-F-M2-SH-G-L-5', encoding 9 proteins. There are three transmembrane glycoproteins on the virion surface: a small hydrophobic (SH) protein, of unknown function, a heavily-glycosylated putative attachment protein (G), and a fusion glycoprotein (F) that mediates viral fusion and entry. The matrix protein (M) lines the virion beneath the lipid bilayer, and, by analogy to RSV M protein, plays a role in viral assembly and budding (10). The nucleoprotein (N), phosphoprotein (P), and large RNA-dependent RNA polymerase protein (L), associate with the RNA genome to form the ribonucleoprotein complex (11). The M2 gene has two open reading frames, encoding M2-1 (transcription elongation factor) and M2-2 (which

controls the switch of the viral RNA polymerase from viral gene transcription to genome replication) (12), by analogy to their reported roles in RSV (13, 14) (Figure 1-2).

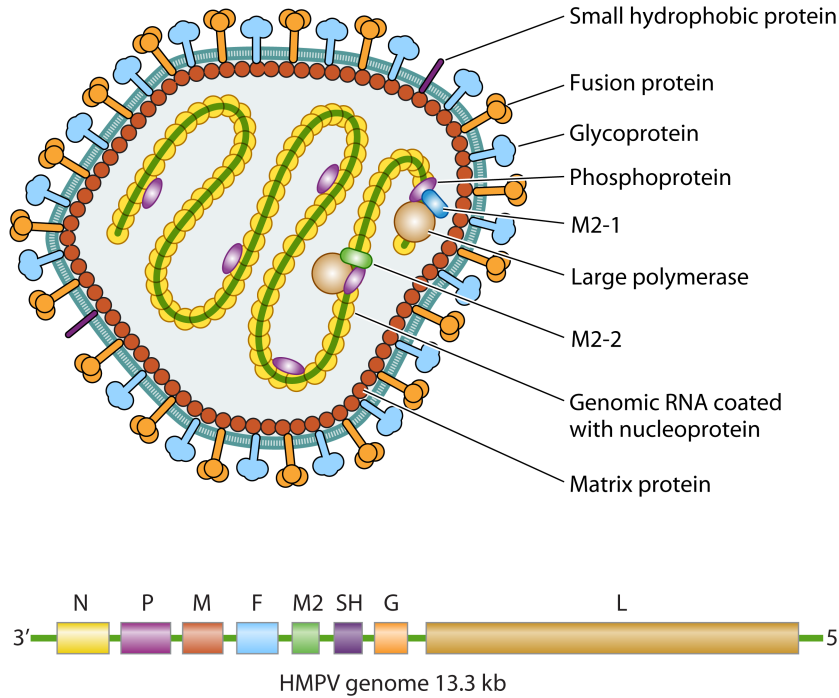


Figure 1-2: HMPV virion and genome. HMPV consists of an enveloped virion with three transmembrane proteins (F, G, SH), M protein coating the interior, a ribonucleoprotein complex (negative-sense RNA genome, N, P, L), and M2-1 and M2-2. The HMPV genome contains 8 genes, in the order 3'-N-P-M-F-M2-SH-G-L-5', encoding 9 proteins. Adapted from (CVI 2015, accepted with revision).

Based on genomic sequencing, there are four subtypes of HMPV that cause disease in humans: A1, A2, B1, and B2 (7). There is high amino acid sequence identity between most proteins in the different subtypes (>94% for N, M, F, M2-1, and L); the most divergent proteins are SH (59% identity) and G (37%) (14). In any given year, viruses of any genotype can circulate (or co-circulate), and the predominant strains vary from location to location and from year to year (7). While the virus was discovered in 2001, serological studies show that it had been circulating in the human population for at least 50 years, and almost all children have been infected by the time they reach five

years of age (1). Primary infections tend to cause the most severe disease, but reinfections occur throughout life.

Animal models of HMPV infection

Several animal models have been established to study HMPV pathology, kinetics of infection, and immune response. Since HMPV is a human pathogen, animals are semi-permissive for infection. Most commonly, rodents such as mice and cotton rats have been used, although other animals tested include hamsters, guinea pigs, ferrets, and non-human primates (15). Infection by intranasally inoculating anesthetized rodents with 10^5 pfu of HMPV leads to viral replication ranging from 10^2 pfu/gram of tissue (C3H mice) to 10^5 pfu/gram of tissue (hamster) in nasal turbinates, and from none detected (guinea pigs) to 10^5 pfu/gram tissue (cotton rats) in the lungs (16). Most infected animals do not show the signs of disease found in humans (rodents show no respiratory symptoms, while cynomolgus macaques exhibit mild rhinitis). Thus, in the absence of clinical disease, lung histopathology is often used as a measure of disease severity. Some groups have also used weight loss (17, 18), although our lab has generally not detected significant weight loss in wild-type rodent models. Therefore, while animals are models of replication and lung histopathology, they are not true disease models.

On the other hand, animal infections are useful to investigate antiviral immunity and evaluate vaccine candidates. HMPV lung infections in animals are accompanied by perivascular and peribronchiolar inflammatory cell infiltrates, including neutrophils, macrophages, NK cells, CD4+ and CD8+ T cells, and followed by the appearance of neutralizing antibodies. Autopsy evaluations of lung histopathology in patients who died from HMPV infection revealed peribronchiolar inflammation (19) with mostly mononuclear inflammatory cells (20). Rodent models are well established for other paramyxoviruses (e.g. measles virus and RSV), so differences in pathogenicity of these

viruses and the effectiveness of monoclonal antibodies and vaccine candidates can be studied using this model.

HMPV and innate immunity

Viruses contain pathogen-associated molecular patterns (PAMPs) that are recognized by pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs). In response to viral infections, TLRs 3, 4, 7, 8, and 9 are particularly important in the innate immune response (21). TLR3 recognizes double-stranded RNA produced during viral replication (22). The F protein of RSV activates TLR4 (23); TLR4 is also important for activation of the innate immune response following HMPV infection (24). TLRs 7 and 8 recognize single-stranded RNA (important in the immune response to RNA viruses), while TLR9 recognizes CpG DNA motifs (important in the response to DNA viruses). In addition to TLRs, two RNA helicases, retinoic acid-inducible gene (RIG)-I and melanoma differentiation-associated gene (MDA)-5, are important in the innate immune response to RNA viruses. HMPV infection induces RIG-I and MDA-5 expression; reduction of RIG-I expression enhances HMPV replication (25).

Downstream of TLR activation, intracellular signaling pathways induce inflammatory cytokines, chemokines, and type I interferons (IFNs). Type I IFNs then bind to their receptor, a heterodimer consisting of IFNAR1 and IFNAR2, which is associated with JAK1/TYK2 kinases. Downstream activation of STAT1 and STAT2 leads to formation of IFN-stimulated gene factor 3 complexes, which translocate to the nucleus and initiate gene transcription (26). With regards to HMPV, type I IFNs are important in controlling viral replication early after infection, although they do not shorten the time-course of infection (27). Also, HMPV inhibits the type I IFN signaling pathway by decreasing STAT1 phosphorylation.

HMPV interaction with dendritic cells

Dendritic cells (DCs) are specialized antigen-presenting cells that are key initiators of adaptive immunity. In the lung, they can protrude cellular extensions between respiratory epithelial cells to detect, capture, process, and present antigen, and provide key costimulatory signals to T cells (28). DCs acquire viral antigen by either becoming infected themselves (direct recognition), or by cross-presentation, a process whereby DCs phagocytose parts of infected cells, transfer antigen to the cytosol, then process epitopes and present them on MHC class I (29). Immature DCs recognize PAMPs through PRRs, migrate via the afferent lymphatics to the T cell paracortex of the lung-draining mediastinal lymph nodes, and activate naïve T lymphocytes (30). For epitope presentation to CD4⁺ T cells (T_{CD4}), MHC II $\alpha\beta$ heterodimers are loaded with peptide as they progress from early endosomes, late endosomes/lysosomes, and finally to the cell surface (31). For epitope presentation to CD8⁺ T cells (T_{CD8}), DCs process either cytosolic proteins (after infection by a virus) or engulfed proteins transported into the cytoplasm, and load peptides onto MHC class I in the endoplasmic reticulum.

HMPV can infect monocyte-derived DCs and plasmacytoid DCs, and trigger production of IFN α *in vitro* (32). However, infected DCs do not significantly up-regulate surface maturation markers CD83 and CD86, and cytokine expression remains low (33). HMPV infection induces moderate DC maturation, but the DCs are inefficient at activating naïve T_{CD4} , which show reduced proliferation, surface activation markers, and IL-2 secretion (34). There is also some evidence that HMPV M2-2 protein decreases DC production of cytokines and chemokines by interfering with MyD88-mediated antiviral signaling (35).

HMPV interaction with macrophages

Like DCs, macrophages engulf pathogens and present epitopes to T lymphocytes using MHC class I and II molecules. The major lung macrophages are alveolar macrophages, found at the luminal surface of alveoli. Other lung macrophages include pleural, interstitial, and intravascular macrophages (36). In the context of HMPV infection, macrophage cell numbers are increased in the alveoli after infection in both mouse (37) and cynomolgus macaque (38) models. BALB/c mice depleted of alveolar macrophages exhibit less lung inflammation, airway obstruction, and lung viral titers compared with alveolar macrophage-competent mice, suggesting that alveolar macrophages contribute to the pathogenesis of HMPV infection (39).

HMPV interaction with other innate immune cell types

There have been limited studies about how other innate cell types respond to HMPV infection. There is an increased number of neutrophils in the lungs of BALB/c mice (40) and cotton rats (17) during infection. Neutrophil numbers peak earlier in BALB/c mice than in cotton rats. There is no increase in the number of eosinophils in either animal model.

While NK cells are known to control viral infections and tumors, there were no in-depth studies analyzing their function during HMPV infection. Alvarez et al. suggested that NK cell depletion in BALB/c mice resulted in increased HMPV viral titer (by about 10-fold) (41). However, those results were presented as part of just one graph in their paper, and the researchers reported biphasic viral growth kinetics (with titers peaking on days 7 and 14, and replication continuing to 28 days) that have never since been replicated by any other group. In our lab and all other published studies, HMPV replication in the lungs decreases to below the limit of detection by day 10, suggesting that the NK cell depletion results published by Alvarez et al. are improbable.

The function of NK cells in infections by other paramyxoviruses (e.g. RSV) is not clear. Li et al. reported that NK cells increased in the lungs after RSV infection of BALB/c mice (42). These NK cells express a number of activating receptors and produce a large amount of IFN γ , which is responsible for lung injury. NK cell depletion using anti-asialo-GM1 antibody, as well as anti-IFN γ antibody treatment, attenuate lung inflammation. These results led the authors to conclude that NK cells are involved in the exacerbation of lung injury via IFN γ . In contrast, Kaiko et al. found that BALB/c mice depleted of NK cells using anti-asialo GM1 antibodies show an increased T_H2 response to RSV infection characterized by higher levels of IL-4, IL-13, and IgE, as well as higher numbers of eosinophils compared with wild-type controls (43). NK cell depleted mice also have greater T_H2 allergic responses to ovalbumin antigen. Furthermore, Hussell et al. reported that IL-12-activated NK cells express high levels of IFN γ and inhibit lung eosinophilia without causing illness (44). In addition, several clinical studies showed reduced NK cell numbers in infants with severe RSV infection (45, 46). These apparent contradictions in published reports, as well as the lack of knowledge about NK cell function during HMPV infection, led me to investigate this topic further.

Natural killer cell background

NK cells were first discovered in the 1970's as cells that have lytic activity against tumors without prior sensitization (i.e. having a "natural" ability to kill tumors) (47). As lymphocytes of the innate immune system, NK cells share a common lymphoid progenitor cell origin with T and B cells; however, they do not express rearranged antigen receptors. Instead, NK cells express a multitude of germline-encoded receptors, with overlapping subpopulations expressing the same receptors. During infection, NK

cells help the early innate control of pathogens during the period required for clonal expansion of antigen-specific T and B cells.

The “missing self” hypothesis first proposed by Karre et al. suggests that NK cells destroy cells that downregulate expression of self molecules such as MHC class I (e.g. some cancers and virus-infected cells) (48). However, NK cells can eliminate cells that maintain expression of MHC class I, as long as NK cell-activating receptors are activated. While it was originally thought that NK cells operate through a nonspecific response to inflammatory cytokines, NK cells express an activating receptor that recognizes a particular antigen in C57BL/6 mice: Ly49H, a C-type lectin-like receptor that is critical for clearing murine cytomegalovirus (MCMV) (49). Ly49H recognizes m157, a cell surface glycoprotein attached to the surface of MCMV-infected cells through a glycosylphosphatidylinositol (GPI) anchor (50). While m157 engages the activating receptor Ly49H in C57BL/6 mice, it also binds inhibitory Ly49 receptors in other mouse strains, including Ly49I in 129 mice. In different strains of MCMV, additional m157 variants are ligands for other inhibitory receptors of different mouse strains, suggesting that m157 is a decoy ligand that helps the virus escape from NK cell activation (51).

NK cell activation depends on a balance between activating and inhibitory receptors (Figure 1-3). While humans do not express Ly49 lectin-like receptors, functionally similar receptors such as killer immunoglobulin-like receptors (KIRs) and lectin-like heterodimers such as CD94/NKG2A recognize MHC class I molecules, while natural cytotoxicity receptors (e.g. NKp46, NKp44, and NKp30) recognize different virally derived products. In addition to MHC I and virally derived products, NK cells recognize stress-induced ligands via the NKG2 receptor family. TLR stimulation also plays an important role in NK cell activation (52-54).

Once the receptors engage their ligands, whether the effect is activating or inhibiting on the NK cell depends on their downstream signaling (Figure 1-3). Activating receptors are associated with signaling adaptor polypeptides (e.g. FcR γ and CD3 ζ), containing immunoreceptor tyrosine-based activation motifs (ITAMs). Activation recruits Src family protein tyrosine kinases, which phosphorylate ITAMs, leading to recruitment of Syk family protein tyrosine kinases (Syk and ZAP70). This recruitment activates downstream signaling pathways that result in activation of mitogen-activated or extracellular signal-regulated protein kinase (MEK) and extracellular signal-regulated kinase (ERK). A different activating receptor, NKG2D, is associated with DAP10, a transmembrane adaptor polypeptide that signals independently of Syk-family protein tyrosine kinases (55). Upon stimulation of inhibitory receptors, immunoreceptor tyrosine-based inhibition motifs (ITIMs) on the receptors' cytosolic domains become phosphorylated by Src kinases. These phosphorylation events lead to the activation of phosphatases SHP-1, SHP-2, and SHIP-1, which dephosphorylate downstream molecules in the activation signaling pathway. NK cells can also be activated by cytokines including type I IFNs, IL-12, IL-15, and IL-18 produced by infected cells or activated APCs. IL-2 promotes NK cell proliferation and cytotoxicity (56).

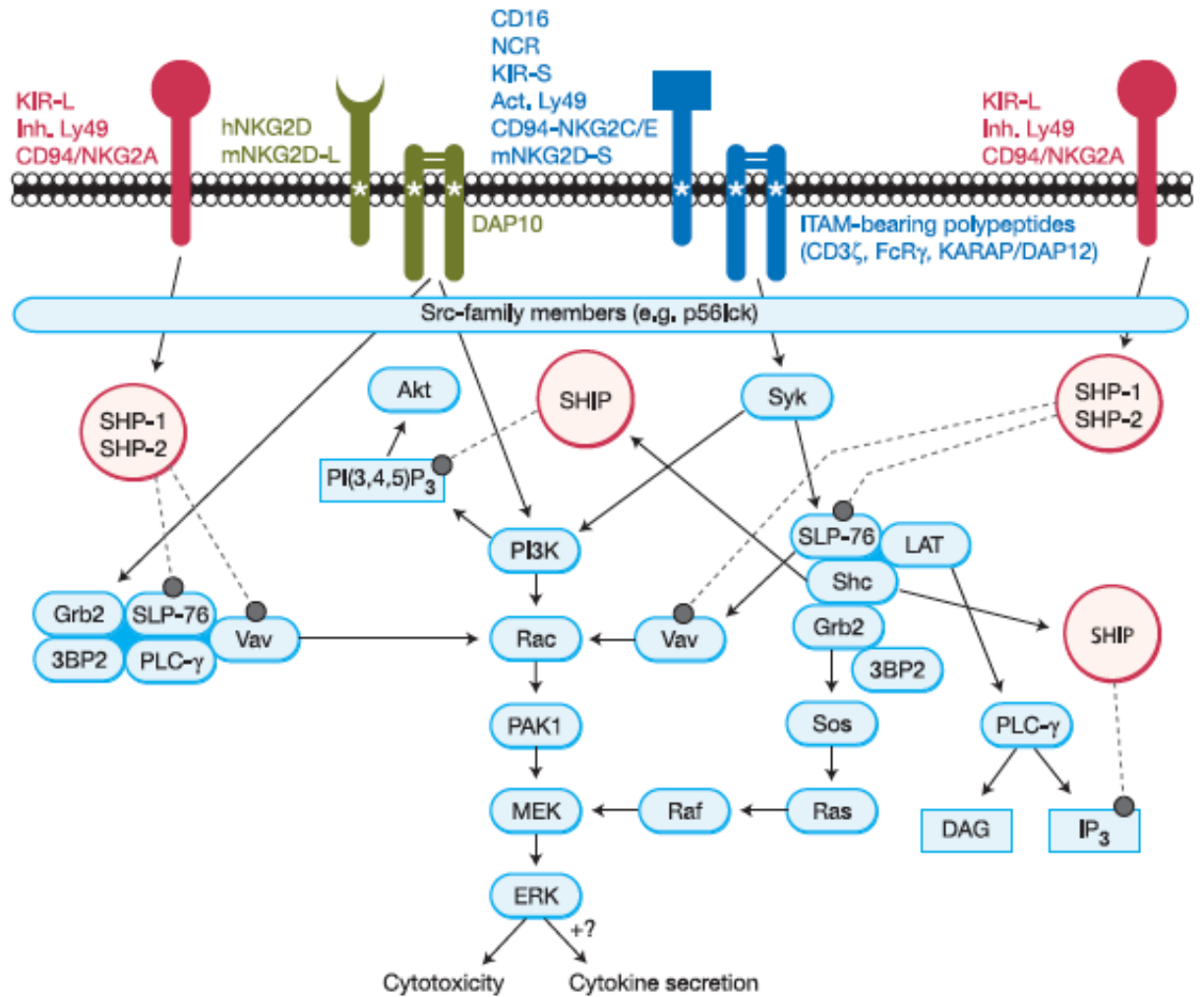


Figure 1-3: NK cell receptor signaling. NK cell activity depends on the balance in downstream signaling of activating and inhibitory receptors. Activating complexes are formed by transmembrane ligand-binding polypeptides associating non-covalently with signaling adaptor polypeptides. Most signaling adaptor polypeptides contain ITAMs, whereas DAP10 contains a YxxM motif, where x is any amino acid. In contrast, inhibitory receptors express intracytoplasmic ITIMs. Adapted from (55).

NK cells use several strategies to kill virally infected cells: release of cytolytic granules, death receptor-mediated cytotoxicity, and production of cytokines (especially IFN γ) (Figure 1-4). Target cell recognition induces the release of perforin and granzymes from granules. Perforin forms pores in the cell membrane, while granzymes are serine proteases that cleave caspases, resulting in cell death (57). In addition, NK cells can use

FasL and tumor-necrosis factor-related apoptosis-inducing ligand (TRAIL) for NK cell-mediated cytotoxicity (58). NK cells can also mediate antibody-dependent cell-mediated cytotoxicity (ADCC) through the interaction of CD16 with IgG. NK cells are major producers of cytokines such as $\text{IFN}\gamma$, $\text{TNF}\alpha$, and IL-10, as well as chemokines, including CCL2, CCL3, CCL4, CCL5, and CCL8 (IL-8) (59).

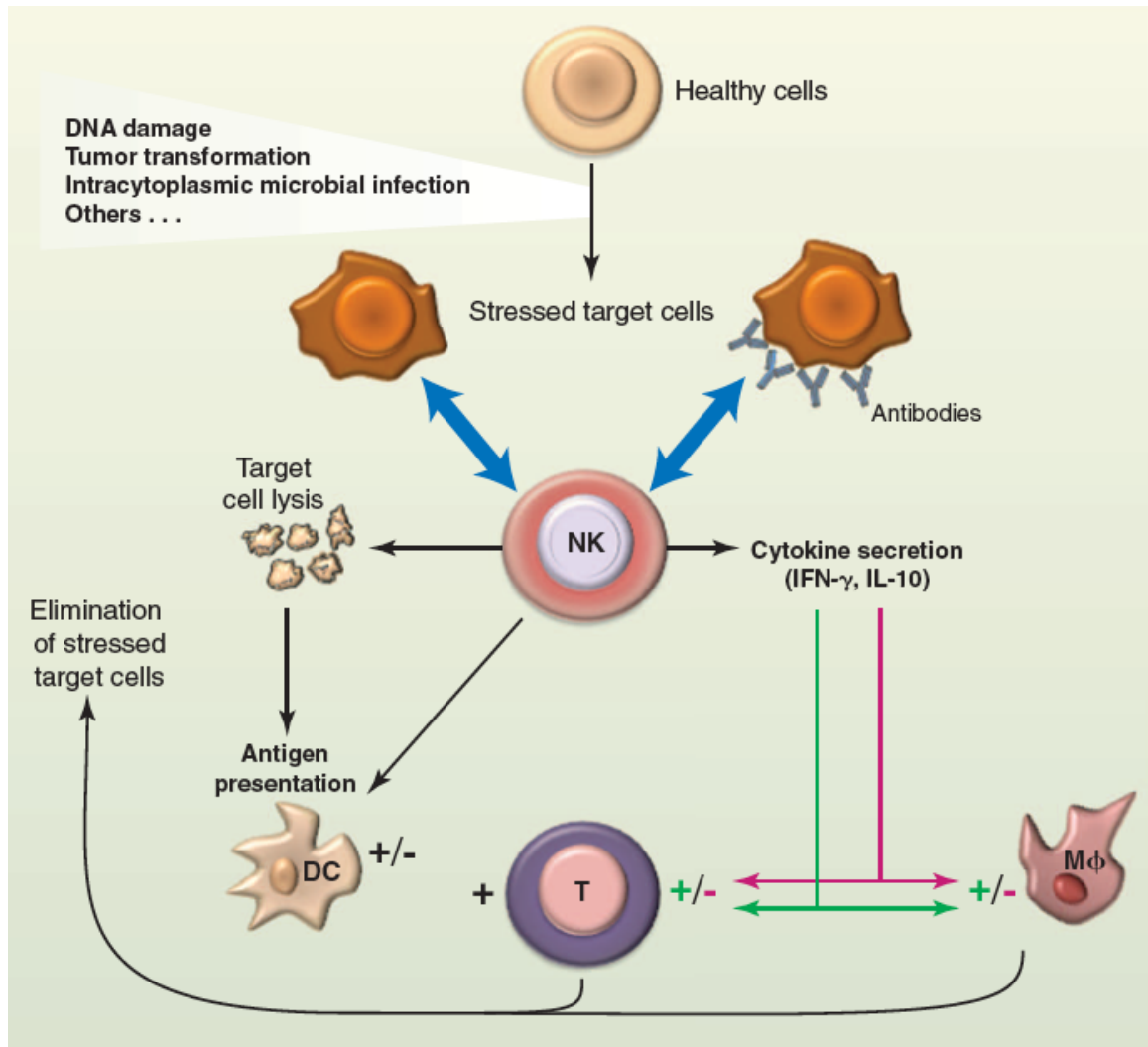


Figure 1-4: NK cell functions. NK cells recognize stressed cells in the presence or absence of antibodies. Activation can lead to the lysis of target cells and to the production of various cytokines and chemokines. NK cells can also kill immature DCs, or promote DC maturation, which leads to enhanced antigen presentation to T cells. Killing of target cells can lead to DC cross-presentation of antigens from apoptotic cells. NK cells also boost or dampen macrophage responses through $\text{IFN}\gamma$ or IL-10. Adapted from (59).

In addition to target cell killing and cytokine production, NK cells act as regulatory cells to influence other cell types. NK cells can kill immature DCs (60, 61), or promote DC maturation via secretion of IFN γ and TNF α . NK cells induce antigen-specific adaptive immune responses by killing target cells and increasing the cross-presentation of antigens by DCs. IFN γ secretion by NK cells can promote the development of T_H1 T_{CD4}. NK cells can also act to control immunopathology. By controlling early MCMV infection, NK cells dampen early type I IFN-dependent immunopathology induced by uncontrolled virus dissemination (62).

Several years ago, it was discovered that NK cells can exhibit memory-like properties. Mice devoid of T and B cells demonstrate contact hypersensitivity responses to haptens to which they were previously sensitized (63). Mice that lack all lymphocytes (including NK cells) could acquire contact hypersensitivity responses after adoptive transfer of natural killer cells from sensitized donor livers. Additionally, in a mouse model of cytomegalovirus infection, NK cells bearing the virus-specific Ly49H receptor reside in lymphoid and non-lymphoid organs for several months after the contraction phase (64). These self-renewing NK cells rapidly degranulate and produce cytokines upon reactivation, and adoptive transfer of these NK cells into naïve mice results in protective immunity.

Natural killer cells and viral infections

Several research groups have studied NK cell functions during viral infections. I have already mentioned RSV in a previous paragraph; here, I will briefly discuss what is known about NK cell functions against other viruses. *In vivo* studies using mice suggest that NK cells are required for clearance of influenza virus (65-68). Jansen et al. found that decreased NK cell activation may be one mechanism associated with enhanced

pathogenicity of highly pathogenic avian influenza (69). However, Monticelli et al. did not find a difference in influenza viral titer in NK cell-depleted mice compared with control mice (70). As for other viruses, NK cell-depleted mice infected with MCMV have increased hepatocyte degeneration, spleen necrosis, and mortality rate as compared to controls (71). On the other hand, NK cell depletion has no effect on viral titers in the early stages of acute LCMV infection or during persistent LCMV infection (72), suggesting that the importance of NK cells varies depending on the virus.

The effects of NK cells in humans are also a matter of debate. Patients with NK cell deficiencies are predisposed to severe, recurrent viral infections, especially those of the herpesvirus and papillomavirus families (73). Additionally, patients with severe influenza infection have diminished NK cell frequencies in the blood (74, 75) and a near absence of pulmonary NK cells (46). On the other hand, some patients with NK- severe combined immunodeficiency who undergo allogeneic hematopoietic transplantation or IL2RG gene therapy have very few circulating NK cells, but do not develop sensitivity to viruses or tumors (76).

It is unclear why there are such discrepancies in the literature. One possibility is that asialo-GM1 is expressed on T cells as well as NK cells (77). The anti-asialo GM1 antibody also mediates basophil depletion (78). Thus, the anti-asialo-GM1 antibody treatment of mice used in many published reports might have depleted other cell types in addition to NK cells. Furthermore, in several of the human studies, NK cells were not the only cell type affected (46). Thus, the disease phenotype may have been at least partially caused by the absence of other cell types. Another possibility is that there is redundancy in the immune system; perhaps the consequences of NK cell deficiency might be exacerbated if there are two or more simultaneous assaults on the immune system. Furthermore, NK cells may play important roles in some microbial infections that have disappeared or been rendered rare due to hygiene development or vaccines.

The role of NK cells in HMPV infections was unclear. Their cytotoxic ability may help the host clear virus during the early stages of infection, thus decreasing viral titers. NK cell production of IFN γ might enhance lung pathology just like in RSV infection (42). On the other hand, IFN γ might protect against T_H2-mediated inflammation. Furthermore, due to the known effects of NK cells on the adaptive immune response, it is possible that NK cells enhance the T cell response to HMPV. Another possibility is that early control of viral titers by NK cells decreases T cell functions during the adaptive immune response. These are the questions I sought to examine in Chapter II.

HMPV and the adaptive immune system

While the innate response is a first line of defense against infection, vertebrates can also mount an adaptive immune response that is highly specific to the particular pathogen. There are two broad categories of adaptive immune responses – antibody responses and cell-mediated responses, carried out by B cells and T cells, respectively. I will now summarize what is known about how the adaptive immune system responds to HMPV infection.

Humoral response

In their original article describing the discovery of HMPV, van den Hoogen et al. reported the results of retrospective serologic studies, demonstrating the presence of HMPV antibodies in humans more than 50 years earlier. While almost all children are infected with HMPV by the age of five, repeated infections with homotypic and heterotypic strains from the two different subgroups occur throughout life despite high rates of seroprevalence (79). Lusebrink et al. analyzed 2000 randomly selected serum samples collected from the archives of the Institute of Virology at the University Hospital

Bonn and demonstrated that neutralizing capacities to HMPV remain high in all age groups with the exception of patients younger than two years of age (80). This led the authors to hypothesize that T cell immunity is more important to clearing HMPV infection. There is some evidence from mouse and human studies that aging decreases neutralizing antibody titer (80, 81).

While Alvarez et al. reported HMPV persistence in BALB/c mice up to day 60 post-infection (p.i.) despite the presence of neutralizing antibodies (41), our group and others have found that wild-type mice clear virus by day 10 p.i. and cannot be re-infected. On the other hand, antibody-deficient μ MT mice can be re-infected. This suggests that mice, as semi-permissive hosts, are protected from HMPV re-infection to a greater extent than are humans. Indeed, passive antibody transfer can protect mice from HMPV infection (82), and a neutralizing monoclonal antibody to F protein confers protection against challenge (83). However, the same is not true for the transfer of anti-avian metapneumovirus antibodies to turkeys (natural hosts for AMPV) (84). Likewise, infection of macaques with HMPV induces only transient protective immunity, as animals could be re-infected eight months after primary infection (85). Healthy adults who have experienced natural RSV infection can be productively re-infected experimentally with the same RSV strain, as early as two months after the first infection (86). Thus, there is limited protection against reinfection, even with antigenically identical viruses.

Cellular immunity

Since reinfection occurs despite virtually all humans being seropositive to HMPV, it is thought that antibody-mediated protection is not sufficient to prevent disease (11, 79). Reports of severe and fatal infections in immunocompromised patients suggest that T cell immunity is important for viral clearance and resolution of illness (20, 87, 88). For

example, HMPV infection is more severe in immunodeficient HIV-positive patients (89, 90).

In BALB/c mice, T cell depletion results in higher viral titers but also reduced lung pathology after HMPV infection (18). In the absence of T_{CD4}, mice have impaired generation of neutralizing anti-HMPV antibodies. Depletion of both T_{CD4} and T_{CD8} prevents airway obstruction and hyper-reactivity, suggesting that T lymphocytes contribute to antiviral immunity, but also to pathogenesis. Since MHC class II-restricted epitopes have not yet been defined for HMPV, little is known about the HMPV-specific T_{CD4} response. On the other hand, murine MHC class I-restricted epitopes for HMPV have been described in both BALB/c and C57BL/6 mice (91-93), making it possible to examine HMPV-specific T_{CD8} responses. Passive transfer of HMPV-specific T_{CD8} partially protects mice against HMPV challenge.

A previous graduate student in the Williams lab, John Erickson, found that while HMPV-specific T_{CD8} are found in the lungs of C57BL/6 mice on day 5 p.i., by day 7 p.i., a substantial percentage of them were impaired (i.e. do not degranulate or produce IFN γ) (93). He showed that this functional impairment was due to signaling through the programmed death-1/programmed death ligand-1 (PD-1/PD-L1) pathway. While T_{CD8} impairment was previously associated with chronic infections and cancer, more recent reports have found that impairment also occurs in acute infections in mice with viruses such as influenza, parainfluenza, RSV, and HMPV (94-97). While it is known that PD-1 is upregulated in humans chronically infected with HIV and hepatitis viruses, its role in acute infections in humans is less well understood. However, it is known that PD-1 and PD-L1 are expressed in the lower airways of patients who died from severe acute respiratory tract infections, suggesting that the PD-1/PD-L pathway may also contribute to infection severity in humans (93).

CD8+ T cells and inhibitory receptors

T_{CD8} provide immunity against viruses, intracellular bacteria, and tumors. DCs are efficient antigen presenting cells that prime naïve T_{CD8} in secondary lymphoid organs (such as the lymph node or spleen) by presenting peptides in the context of MHC class I molecules. T_{CD8} recognize the peptide:MHC I complex via their T cell receptor (TCR). DCs also express costimulatory ligands CD80 (B7-1) and CD86 (B7-2) that interact with CD28 on the T cell surface. Inflammatory cytokines provide the third signal to fully differentiate and expand T_{CD8} . Downstream of TCR/CD28 activation, signaling through the PI3K/Akt pathway activate T_{CD8} . Activated T_{CD8} migrate to sites of infection, where they recognize peptide:MHC I on the surface of infected cells. Like NK cells, T_{CD8} possess lytic granules containing perforin and granzyme that are released to kill infected cells. T_{CD8} can also secrete cytokines such as $IFN\gamma$, $TNF\alpha$, and IL-2.

PD-1 and its ligands, PD-L1 and PD-L2, deliver inhibitory signals that regulate the balance between T cell activation, tolerance, and immunopathology (98). First identified as a gene upregulated in a T-cell hybridoma undergoing cell death (99), PD-1 is a type I transmembrane protein composed of an immunoglobulin superfamily domain, a transmembrane domain, and an intracellular domain. PD-1 is encoded by the *Pdcd1* gene on chromosome 1 in mice and chromosome 2 in humans. It is expressed on activated T cells, B cells, NKT cells, monocytes, and dendritic cells (Figure 1-5). PD-1 is induced by TCR or BCR signaling and remains high in the setting of persistent antigen stimulation (100). The common γ chain cytokines IL-2, IL-7, IL-15, and IL-21, can also induce PD-1 expression on T cells (101). Furthermore, type I interferons can induce PD-1 upregulation in macrophages.

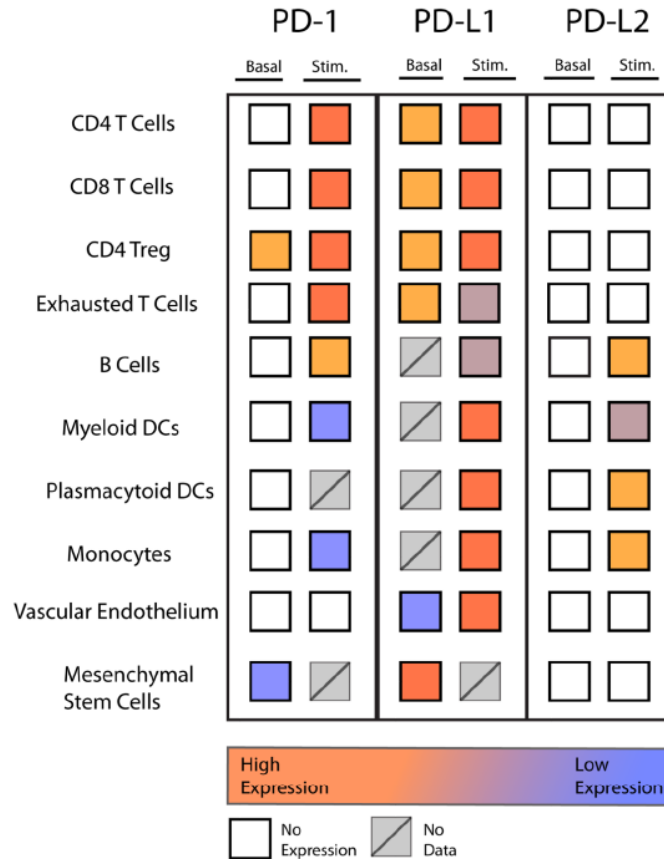


Figure 1-5: PD-1, PD-L1, and PD-L2 expression. PD-1 is expressed during thymic development and is induced in hematopoietic cells by antigen receptor (TCR and BCR) signaling and common gamma chain cytokines. PD-1 is highly expressed on impaired T cells in the setting of viral infections and cancers. PD-L1 is constitutively expressed on hematopoietic cells and inducibly on various nonhematopoietic cells. PD-L2 is also inducibly expressed on various hematopoietic cells, although its expression is more restricted than that of PD-L1. Cytokines are potent stimuli for PD-L1 and PD-L2 expression. Adapted from (100).

PD-L1 is constitutively expressed on T cells, B cells, DCs, macrophages, and mast cells, and further upregulated upon activation. PD-L1 can also be expressed on nonhematopoietic cells such as vascular endothelial cells, respiratory epithelial cells, pancreatic islet cells, and neurons. PD-L2 is inducibly expressed on several types of hematopoietic cells, although its expression is more restricted than that of PD-L1. Both type I and type II interferons as well as the common γ chain cytokines upregulate PD-L1 and PD-L2 (101).

The intracellular domain of PD-1 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM). Upon ligand engagement, PD-1 is phosphorylated on its intracellular tyrosines, and then binds SHP-1 and SHP-2 phosphatases that downregulate antigen receptor signaling through dephosphorylation of signaling intermediates CD3 ζ , ZAP70, and PKC θ , inhibiting PI3K activity and downstream activation of Akt (102) (Figure 1-6). The result is reduced cytokine production, cytolytic function, proliferation, and survival. PD-1 also inhibits the expression of transcription factors associated with effector cell function, including GATA-3, Tbet, and Eomes (103).

The PD-1 pathway has important roles in regulating the balance between effective immune defenses and immune-mediated tissue damage. *Pdcd1*^{-/-} mice clear adenovirus infection more rapidly than wild-type mice, but develop more severe hepatocellular injury (104). Also, John Erickson showed that blocking PD-1 signaling using anti-PD-L antibodies results in increased breath distension (as measured by pulse oximetry) as compared to control mice (93). In humans with hepatitis B virus infection, high T_{CD8} PD-1 levels correlate with positive clinical outcome, presumably due to reduced liver damage (105). These studies suggest that the PD-1:PD-L pathway limits the detrimental consequences of over-active effector cells. On the other hand, microorganisms can leverage this system to establish persistent infection by rendering pathogen-specific T cells nonfunctional. Blocking PD-1 signaling leads to substantial reductions in viral burdens with respiratory viruses and LCMV (93, 106), demonstrating that this could be a novel strategy for treating infections.

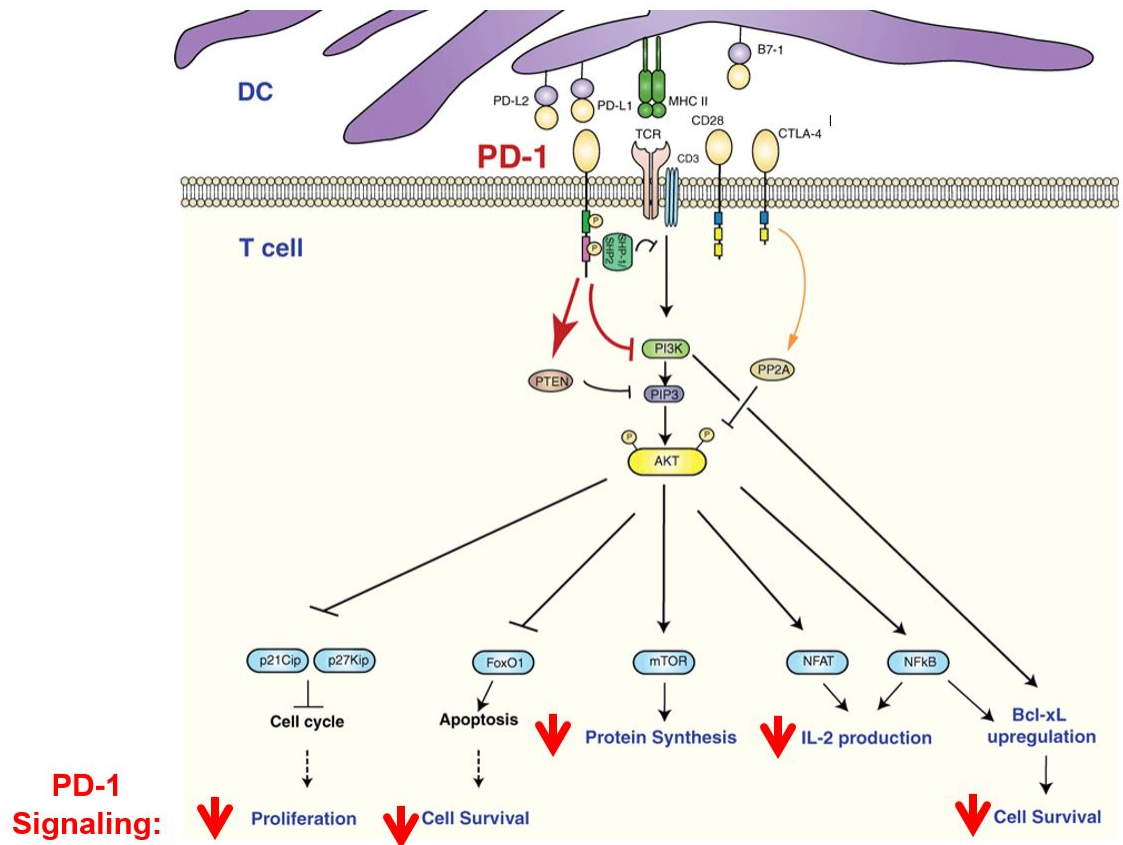


Figure 1-6: PD-1 signaling. Binding of the PD-1 ITSM by SHP-1 or SHP-2 results in the dephosphorylation of downstream TCR signaling molecules (CD3 ζ and ZAP-70) and augmentation of PTEN expression, which blocks PIP₃. PD-1 signaling attenuates the activation of PI3K/Akt and results in decreased T cell proliferation, survival, protein synthesis and IL-2 production. Adapted from (100).

Other inhibitory receptors

In addition to PD-1, several other inhibitory receptors contribute to functional T_{CD8} impairment during infection, including TIM-3, LAG-3, CTLA-4, and 2B4 (CD244) (107, 108). Wherry et al. examined the molecular signature of T_{CD8} exhaustion by comparing the gene-expression profiles of exhausted LCMV-specific T_{CD8} during chronic infection to those of functional LCMV-specific T_{CD8} during acute infection. In addition to expressing multiple cell-surface inhibitory receptors, exhausted T_{CD8} downregulated transcription of genes encoding molecules downstream of TCR and cytokine receptor signaling (109).

Similarly, John Erickson found that TIM-3, LAG-3, and to a lesser extent, 2B4 were upregulated on T_{CD8} during HMPV infections (unpublished data).

T cell immunoglobulin and mucin domain 3 (TIM-3) is a type I transmembrane protein. Signaling pathways downstream of TIM-3 binding to its ligand, galectin-9, have not yet been elucidated. Although TIM-3 contains multiple tyrosine residues in its cytoplasmic tail, the sequences around these tyrosines do not conform to known inhibitory signaling motifs (e.g. ITIM or ITSM). These tyrosines can be phosphorylated, although the identity of the kinases has not yet been shown *in vivo*. Exhausted virus-specific T_{CD8} upregulate TIM-3 during LCMV, HIV, and hepatitis C infection, and blocking the TIM-3/GAL-9 pathway resulted in superior T_{CD8} responses during infection by several different viruses, including influenza (110) and HIV (111). Similarly to PD-1, TIM-3 upregulation severely impairs the ability of T_{CD8} to secrete cytokines and other effector molecules.

The lymphocyte activation gene-3 (LAG-3) receptor is a member of the immunoglobulin superfamily related to CD4. Although LAG-3 and CD4 are related, LAG-3 binds to MHC class II with higher affinity and is mainly expressed after lymphocyte activation. At the cell surface, LAG-3 is expressed as a dimer. It is primarily expressed in activated T_{CD4} and T_{CD8} in inflammatory conditions, and can function as a negative regulator of T cell function after binding to MHC class II. Cross-linking of LAG-3 on T cells impairs TCR-mediated activation, resulting in decreased calcium influx and cytokine expression (112).

Since 2010, antibody-based therapies that block T cell inhibitory receptors have been widely studied for cancer immunotherapy. Anti-CTLA-4 antibodies prolonged survival in approximately 20% of patients with advanced metastatic melanoma in one study (113), and PD-1 blocking antibodies also showed excellent responses in cancer patients after peptide vaccination (114). Since blocking many of these inhibitory

pathways decreases viral titer during several viral infections in mouse models, this suggests that altering signaling through these receptors can represent future approaches to improve responses to vaccines against viruses.

Treatments for HMPV infections

There is no vaccine for HMPV licensed for use in humans, and treatment is mainly supportive (i.e. fluids and oxygen). Patients with severe HMPV infections have been treated with ribavirin and intravenous immunoglobulins (IVIG) (115, 116). Ribavirin is a nucleoside analog that has efficacy against HMPV both *in vitro* (117) and in BALB/c mice (118). However, adverse effects can be serious (119). The use of ribavirin in the clinic remains controversial, since there have been no human trials performed to study its efficacy in treating HMPV infection (117). IVIG has antiviral activity against HMPV *in vitro* (117), although it requires infusions of large fluid volumes and is associated with adverse events in children with congenital heart disease (79).

Other potential treatments for HMPV infection have been tested *in vitro* and in animal models. Several monoclonal antibodies that bind F protein were effective against HMPV in rodent models (120), including one that neutralized both HMPV and RSV in DBA/2 mice. Other potential treatments include fusion inhibitors, siRNAs targeting mRNAs of various HMPV proteins, and NMSO₃, a sulfated sialyl lipid, which had antiviral activity against HMPV and RSV *in vitro* (121). No antiviral drugs have advanced beyond very early preclinical studies, and thus vaccines remain the most promising approach to prevent severe HMPV disease. However, the development of safe and effective HMPV vaccines may be challenging.

Paramyxovirus vaccines and enhanced disease

In the 1960s, a clinical trial of a formalin-inactivated alum-precipitated respiratory syncytial virus (FI-RSV) vaccine in human infants resulted in enhanced disease, elevated hospitalization rate, and in some cases, death, when the infants were naturally infected (122, 123). Despite the presence of serum neutralizing antibody, vaccinated infants who acquired RSV infection exhibited severe pneumonia, bronchiolitis, and bronchitis. 80% of vaccinees required hospitalization at the time of infection, as compared to 5% of the control group. Post-mortem examination of the infants who died revealed extensive bronchopneumonia and atelectasis, with peribronchiolar monocytic infiltration and excess eosinophils on histologic examination.

The enhanced disease elicited by FI-RSV was later attributed to non-neutralizing antibodies that formed immune complexes and activated complement, leading to tissue damage (124). Additional evidence attributed the atypical disease following FI-RSV vaccination and subsequent infection to a lack of antibody affinity maturation due to poor Toll-like receptor stimulation (125), and the tendency of formaldehyde to generate carbonyl groups on treated antigens, leading to increased T helper type 2 (T_H2) responses, which could be reversed by reduction of the carbonyl groups (126).

Similarly to the FI-RSV vaccine, a formalin-inactivated measles vaccine (FI-MV) was tested in a clinical trial during the 1960's (127, 128). While FI-MV elicited neutralizing antibodies, several immunized children subsequently infected with measles developed atypical measles, a severe disease characterized by high fever, morbilliform rash, and pneumonitis requiring hospitalization (129). Several hypotheses were proposed to explain the pathogenesis, including an imbalance in the antibody response to hemagglutinin and fusion proteins, immune complex deposition, disruption of the fusion protein during formalin inactivation, and T_H2 polarization of the immune response.

Neither the FI-RSV nor FI-MV vaccines elicited a detectable virus-specific T_{CD8} response.

Vaccine candidates for HMPV

The adverse outcomes of the formalin-inactivated RSV vaccine raised concerns about the safety of new vaccine candidates against paramyxoviruses. Indeed, a formalin-inactivated HMPV (FI-HMPV) vaccine increased lung pathology characterized by interstitial pneumonitis and alveolitis in cotton rats after HMPV challenge (130), even though the animals did develop serum neutralizing antibodies and were protected from viral replication upon HMPV challenge. In addition, FI-HMPV vaccination of macaques followed by HMPV challenge resulted in enhanced pulmonary disease characterized by eosinophilic bronchitis and bronchiolitis (131). In this study, vaccinated animals were not protected from HMPV challenge. Furthermore, a heat-inactivated HMPV vaccine resulted in eosinophilic infiltration in the lungs and increased levels of T_{H2} cytokines in BALB/c mice (37). These examples suggest that alternative vaccine strategies must be considered to avoid vaccine-mediated enhanced disease.

In addition to the inactivated vaccines mentioned above, several other candidates have been tested in animal models, including subunit proteins, live-attenuated and chimeric viruses, T_{CD8} epitopes, and virus-like particles. Since there is currently no HMPV vaccine licensed for use in humans, I was interested in studying potential vaccine candidates, particularly virus-like particles (described in more detail below).

Subunit proteins

Viral coat proteins are prime targets for neutralizing antibodies. While HMPV has three membrane proteins F, G, and SH, studies show that only the highly-conserved F protein elicits a neutralizing antibody response (132). Paramyxovirus fusion proteins are class I transmembrane glycoproteins synthesized as an inactive precursor that is cleaved by host proteases, resulting in two polypeptides linked by disulfide bonds (33). In cotton rats, a soluble HMPV F protein lacking the transmembrane domain elicited neutralizing antibodies after viral challenge (133). There was a high level of protection in the lungs, although protection in the upper respiratory tract (i.e. nasal turbinates), was more limited, with modest but significant reduction in titers. There was no evidence of a skewed T_H2 response, enhanced alveolitis, or eosinophilic infiltrates in immunized animals. F subunit vaccines combined with various adjuvants also induced protective antibody levels against both homologous and heterologous HMPV infections in Syrian golden hamsters (134). Following a study that showed HMPV M protein activated dendritic cell maturation and cytokine production *in vitro* (135), Aerts et al. immunized BALB/c mice with F protein with or without M protein and alum adjuvant (136). Mice immunized with F+M had lower pulmonary viral titers post-challenge than mice immunized with F only, and had a lower T_H2/T_H1 cytokine ratio, suggesting that the addition of M protein to an HMPV vaccine modulates immune responses to subsequent infection. Since F protein is well conserved across HMPV strains, F protein immunization protects against subsequent challenge with a heterologous strain of HMPV.

Unlike live attenuated vaccines, subunit proteins do not carry the risk of mutating to a virulent form of the virus. On the other hand, they are typically less immunogenic than live attenuated vaccines, and do not efficiently activate T_{CD8} (137, 138). This suggests that subunit proteins may not be as effective in humans as in animal models, since humans can be re-infected with HMPV despite having serum antibodies.

Live attenuated and chimeric virus vaccines

Live-attenuated vaccines have the advantage of mimicking natural infection and inducing a broad immune response, including mucosal IgA (139). Furthermore, these vaccines elicit both T and B cell responses, and fewer doses are typically required than for non-replicating vaccines. No enhanced disease has been observed in animal studies with HMPV or RSV live attenuated vaccines (140). On the other hand, the challenge of using a live attenuated vaccine is to generate a sufficient level of attenuation and still maintain immunogenicity. Live attenuated vaccines also have the potential to revert to a wild-type phenotype and cause disease in vaccinated individuals, making this approach a safety risk (141).

Several live-attenuated HMPV vaccine candidates have been tested in animal models. Repeated passage of HMPV at low temperatures resulted in the accumulation of mutations in the genome and the generation of cold-passage temperature-sensitive HMPV strains (140). Replication of these viruses was reduced in the respiratory tract of hamsters, but the viruses induced high titers of HMPV-specific antibodies and protected the animals from challenge infection.

Recombinant HMPVs that lack the SH and/or G proteins (Δ SH, Δ G, and Δ SH/G) generated by a reverse genetics system replicated efficiently *in vitro*, but while Δ G and Δ SH/G were attenuated in the respiratory tract of hamsters, Δ SH did not show attenuation *in vivo* (142). Both Δ G and Δ SH/G induced high titers of neutralizing serum antibodies and conferred complete protection against HMPV challenge in the lungs. A recombinant Δ M2-2 virus also induced high titer HMPV neutralizing antibodies in hamsters and protected against wild-type HMPV challenge (12); however, a Δ M2-1 virus did not induce neutralizing antibodies or protect against HMPV challenge. In a separate study, a live-attenuated vaccine candidate was generated by removing one N-linked

carbohydrate in the F protein (143). Immunization of BALB/c mice with this vaccine produced a high titer of neutralizing antibodies against homologous and heterologous virus.

Several groups also generated live-attenuated chimeric HMPV vaccines. HMPV F expressed in a chimeric, live-attenuated parainfluenza (PIV) vaccine containing the F and HN genes was protective in hamsters and African green monkeys (144). In addition, a chimeric vaccine formed by replacing the nucleoprotein (N) and phosphoprotein (P) of HMPV with those of avian metapneumovirus elicited high levels of neutralizing antibodies following vaccination and challenge (145).

Cytotoxic T lymphocyte epitope vaccines

Since a high anti-HMPV serum antibody titer is insufficient to prevent reinfection (146), it has been suggested that both humoral and cellular immunity contribute to protection (18, 147). To test the efficacy of T_{CD8} epitopes as potential vaccines, Herd et al. vaccinated BALB/c mice with identified HMPV epitopes (91). They found that peptide vaccination reduced viral load and immunopathology of mice, while enhancing T_{H1} cytokine levels. In another study, vaccination of C57BL/6 mice with an identified HMPV-specific T_{CD8} epitope + anti-CD40 Ab + polyI:C (TriVax) reduced lung viral titers when the mice were later challenged with HMPV (147). However, in both of these studies, protection was incomplete, as there was still a high lung viral titer after HMPV challenge.

Virus-like particles

Virus-like particles (VLPs) are promising vaccine candidates because, based on previous studies on FDA-approved VLP vaccines, they are known to elicit both antibody and T cell responses (148-151). Furthermore, their noninfectious nature alleviates safety concerns associated with live-attenuated vaccines. VLPs are formed by the assembly of

viral structural proteins and lack viral genome (152). They mimic virus structure and present antigens in a repetitive and ordered fashion, a characteristic that strongly triggers B cell responses by enhancing the cross-linking of immunoglobulins (153). In addition, VLP size facilitates their uptake by DCs for cross-presentation onto MHC class I. VLPs can be designed to incorporate only the immunogenic viral proteins, thus focusing the immune response on protective antigens. Adjuvants can be given with VLPs or directly incorporated into VLPs. For example, membrane-anchored flagellin that was incorporated into an influenza virus-like particle vaccine enhanced cross-protective heterosubtypic immune responses (154).

The human papillomavirus and hepatitis B vaccines are VLP vaccines licensed for use in humans (155). Several VLP vaccines have been tested in animal models, including VLPs for influenza, hepatitis C, and RSV (156-158). Additional VLP vaccine candidates are in clinical trials (159).

A previous graduate student in the Williams lab, Reagan Cox, found that VLPs containing F and M proteins generated a neutralizing antibody response that was enhanced with the addition of adjuvant (141). Reagan also discovered that two weeks after VLP vaccination, mice were completely protected from HMPV challenge in the lungs and had restricted HMPV replication in nasal tissues. Levy et al. also analyzed retrovirus-based VLPs as a potential vaccine strategy for HMPV. They found that VLPs displaying both F and G HMPV glycoproteins (F/G-VLPs), or F protein alone (F-VLPs), induced neutralizing antibodies in mice, whereas VLPs containing G protein alone did not (160). Mice immunized with F/G-VLPs did not have a higher yield of neutralizing antibodies as compared to those immunized with F-VLPs. This finding is not surprising considering that HMPV G protein does not elicit protective immunity (161). Mice immunized with F-VLP or F/G-VLP had reduced viral titers in the lungs after challenge with either homo- or heterosubtypic HMPV strains.

While both the above studies focused on the antibody response to HMPV VLP vaccines, I was more interested in the T_{CD8} response. As mentioned above, it is thought that both antibodies and T cells are important in the immune response to HMPV in humans. Indeed, human patients with previous HMPV respiratory disease were shown to have T_{CD8} responses to HMPV proteins (162). Many of these epitopes are conserved across HMPV subtypes, illustrating the potential for T_{CD8} epitopes in vaccine development.

It is known from the work of John Erickson and other scientists that following HMPV infection, IFN γ -producing T_{CD8} accumulate in the lungs of mice (163). However, is VLP vaccination alone sufficient to elicit HMPV-specific T_{CD8}, or is viral challenge necessary? Furthermore, given what is already known in the field about inhibitory receptors and T_{CD8} impairment after viral infection, do the T_{CD8} elicited by VLP vaccination express similar levels of impairment?

In my work on VLP vaccination (see Chapter III), I sought to determine whether vaccination protects mice from viral challenge over a longer time frame than the two weeks Reagan used in her protocol. This would allow me to determine the efficiency of HMPV VLPs in long-term protection of the host. Furthermore, I sought to determine whether there are any differences in routes of vaccine administration (i.e. intranasal vs. intraperitoneal) in terms of host protection, T_{CD8} response, and inhibitory receptor expression.

Several papers in the cancer field have found increased PD-1 expression on T_{CD8} responding to tumors. Combining inhibitory receptor blockade with therapeutic vaccination improved tumor rejection in mice by increasing effector T_{CD8} activity (164-166). Similarly, a small clinical trial in humans combining tumor vaccines with inhibitory receptor blockade found increased inflammatory infiltrates and tumor regression (113).

Currently, antibodies targeting two inhibitory receptors (PD-1 and CTLA-4) have been approved by the FDA for therapy in patients with cancer (167).

In the infectious diseases field, most of the research on the immune response post-vaccination emphasizes antibodies rather than T cells. Pinto et al. evaluated cell-mediated immune responses (lymphoproliferation and cytokine production) in peripheral blood mononuclear cells of patients receiving human papillomavirus VLP vaccine; however, they did not analyze T cell inhibitory receptor expression post-vaccination (148). Martinez Salazar et al. found that T_{CD8} in mice vaccinated with leishmania lipophosphoglycan increased their PD-1 expression (168). Inhibitory receptor blockade improved cellular immune responses in mice after therapeutic LCMV vaccination (169), and vaccination with a dendritic cell-directed lentiviral vector encoding HIV-1 Gag protein, but these authors did not investigate the inhibitory receptor profile after vaccination alone (170). Since VLP vaccines are known to elicit T cell responses, I was interested in determining whether vaccination alone induces inhibitory receptor expression on vaccine-specific T cells.

In summary, my thesis is concerned with two major cell types with known roles in the immune response against viral infections: NK cells and T_{CD8} cells. Both cells fight infections by releasing perforin and granzyme from intracellular granules, and by secreting cytokines such as $IFN\gamma$. In Chapter II, I present data showing the innate NK cell response to HMPV infection, and in Chapter III I examine the T_{CD8} response to VLP vaccination. These results will help elucidate the innate and adaptive responses to HMPV infection and vaccination.

CHAPTER II

CLEARANCE OF HUMAN METAPNEUMOVIRUS OCCURS INDEPENDENTLY OF NATURAL KILLER CELLS

Introduction

Natural killer cells are lymphocytes of the innate immune response that play an important role in controlling many viral infections. In this study, I sought to elucidate the role of NK cells in HMPV infection. As NK cells in the lung make up a high percentage of the total lymphocyte population, display a mature phenotype, and are rapidly activated in response to infection (171, 172), I hypothesized that NK cells help the host immune response by controlling viral replication at early time points and by enhancing the T cell response. I found that while lung NK cell numbers increase post-HMPV infection, NK cell depletion had minimal effects on HMPV titer, lung histopathology, and cytokine production despite the NK cells having an activated phenotype. Furthermore, depletion of NK cells had no significant influence on the adaptive T_{CD8} and T_{CD4} responses. These results indicate that NK cells are not essential in the host response to all viruses, and are in fact expendable during the course of HMPV infection.

I acknowledge Pavlo Gilchuk for making the tetramers used in my experiments, Moni Johnson for her expertise in RT PCR, and Sharon Tollefson for her help with viral plaque assays. I also acknowledge Kelli Boyd for her work analyzing lung histopathology, and Bryan Shepherd for his contribution to statistical analysis.

Materials and Methods

Mice and virus

C57BL/6 (B6) mice were purchased from the Jackson Laboratory. CD1d^{-/-} mice (B6 background) were a gift from Luc Van Kaer (Vanderbilt University, Nashville, TN). HLA B7.2 transgenic (B7tg) mice were a gift from Alessandro Sette (La Jolla Institute for Allergy and Immunology, La Jolla, CA) and Francois Lemonnier (Institut Pasteur, Paris, France). Animals were bred and maintained in specific pathogen-free conditions. Six- to ten-week-old mice were used in all experiments. Experimental procedures were performed in accordance with the Vanderbilt University Animal Care and Use Committee guidelines. HMPV (clinical strain TN/94-49, genotype A2) was propagated in LLC-MK2 cells, as previously described (16). UV-inactivated HMPV was generated using a Stratalinker UV Crosslinker; inactivation of virus was confirmed by plaque titration. For all experiments, mice were anesthetized intraperitoneally (i.p.) with ketamine/xylazine and inoculated intranasally (i.n.) with 6×10^5 pfu HMPV, mock (LLC-MK2 cell lysate), or UV-inactivated HMPV in a 100- μ l volume.

Flow cytometry and intracellular cytokine staining

Lungs were minced and incubated for 1 hour in 2 ml medium (RPMI-1640 with 10% FBS, 2 mM glutamine, 50 μ g/ml gentamicin, 2.5 μ g/ml amphotericin B, and β -mercaptoethanol) at 37°C with 2 mg/ml collagenase A and 20 μ g/ml DNase (both from Roche). Single-cell suspensions were obtained by pushing digested lung tissue through metal screens, and passing over nylon cell strainers (BD Falcon). Erythrocytes were lysed with red blood cell lysis buffer (Sigma-Aldrich), and lymphocytes were counted with a hemocytometer. The number of cells in each subset population was calculated based on the % determined by flow cytometry. The data were analyzed with FlowJo software

(Tree Star, Ashland, OR). All antibodies were purchased from BD Biosciences unless otherwise indicated.

NK cell studies. Lung lymphocytes were restimulated *in vitro* with 50 ng/ml PMA and 2 µg/ml ionomycin (Sigma-Aldrich) for 6 hours at 37°C in the presence of anti-CD107a (clone 1D4B). Brefeldin A and monensin (BD Biosciences) were added for the last 4 hours of stimulation. After staining with live/dead cell stain (Invitrogen) and blocking Fc receptors with anti-CD16/CD32, lymphocytes were incubated with FITC-anti-CD49b (clone DX5) and APC-Cy7-anti-CD3e (clone 145-2C11). CD69 surface staining was performed with (Brilliant Violet 605) anti-CD69 (clone H1.2F3, BioLegend). After extracellular markers were stained, cells were fixed, permeabilized (BD Cytotfix/Cytoperm), and stained for intracellular IFN γ (clone XMG1.2) before flow cytometric analysis (LSR II, BD Biosciences).

T cell studies. After staining with live/dead dye (Invitrogen) and blocking Fc receptors with anti-CD16/CD32, lymphocytes were incubated with APC-labeled tetramers (0.1-1 µg/ml), anti-CD19 (clone 6D5, iCyt), anti-CD8 α (clone 53-6.7), and anti-PD-1 (clone J43) or isotype control antibody (hamster IgG2 κ) in FACS buffer (PBS/1% fetal bovine serum) containing 50 nM dasatinib (LC Laboratories) for 1 hour at room temperature. Background staining levels were determined by staining with a tetramer loaded with irrelevant (vaccinia virus derived) peptide. Tetramers were generated for HMPV epitope HLA-B*0702/M₁₉₅₋₂₀₃ (APYAGLIMI) and vaccinia epitope HLA-B*0702/A34R₈₂₋₉₀ (LPRPDTRHL) as previously described (93). For intracellular cytokine staining, lung lymphocytes were restimulated *in vitro* with 10 µM M₁₉₅₋₂₀₃ peptide (APYAGLIMI) for 6 hours at 37°C in the presence of anti-CD107a (clone 1D4B). Brefeldin A and monensin

(BD Biosciences) were added for the last 4 hours of stimulation. Restimulation with an irrelevant (vaccinia) peptide served as a negative control; stimulation with 50 ng/ml PMA and 2 μ g/ml ionomycin (Sigma-Aldrich) served as a positive control. Lymphocytes were then stained with anti-CD3 ϵ (clone 145-2C11), anti-CD19 (clone 6D5, iCyt), and anti-CD8 α (clone 53-6.7). After extracellular markers were stained, cells were fixed, permeabilized, and stained for intracellular IFN γ (clone XMG1.2) before flow cytometric analysis. For CD4 $^+$ T cell experiments, lymphocytes were stimulated with PMA/ionomycin and stained with anti-CD3 ϵ , anti-CD19, and anti-CD4 (clone RM4-5) followed by staining for intracellular IFN γ .

NK cell depletion

Mice were injected i.p. with 200 μ g anti-NK1.1 antibody (clone PK136) or isotype control antibody (both from BioXCell) five days before infection and with 100 μ g of antibody on the day of infection. For analysis of the adaptive T cell response, mice were injected as above and then again five days post-infection. NK cell depletion was confirmed in both spleens and lungs by flow cytometry.

Viral titration

Lung tissues were collected and pulverized in glass homogenizers before centrifugation at 1200 rpm at 4°C for 10 min. Supernatants were collected, aliquoted into cryovials, and snap-frozen in dry ice-ethanol for storage at -80°C until further use. Viral titers were quantified by plaque titration as previously described (16).

Lung histopathology

Lungs were inflated with 10% formalin and fixed for 24 hours, embedded in paraffin, and cut into 5- μ m-thick sections before being placed on slides. The paraffin was removed, and the sections were stained with hematoxylin and eosin. Slides were evaluated by an experienced veterinary pathologist (KLB) blinded to the composition of the groups. Lung pathology was scored in a semi-quantitative manner in airways, peribronchiolar spaces, and perivascular spaces from 0-4 with 4 representing the maximum inflammation (93, 173).

Real-time RT-PCR

200 μ l of lung homogenate was mixed with 400 μ l RLT lysis buffer (QIAGEN) and frozen at -80°C until further use. RNA was extracted using an RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. Real-time RT-PCR was performed using 5 μ l of extracted RNA and 20 μ l of reaction mixtures on an ABI StepOnePlus Real-Time PCR system (Life Technologies/Applied Biosystems) using the AgPath-ID One-Step RT-PCR kit (Applied Biosystems/Ambion). All values were normalized to the housekeeping gene Hprt, and reported as fold change compared to mice that were treated with isotype control antibody, using the $\Delta\Delta$ Ct method.

ELISA

96-well flat-bottom Immulon plates (Thermo) were coated with 1 μ g/ml capture anti-IFN γ antibody (clone R4-6A2, Biolegend) and incubated overnight at 4°C. Wells were washed with PBS/Tween and blocked with 1% BSA for 1 hour. 100 μ l of lung homogenate or diluted IFN γ standard (BioLegend) was added to the plate and incubated for 3 hours at room temperature. Plates were incubated with 2 μ g/ml biotin-labeled detection antibody

(BioLegend) for 1 hour before addition of avidin-horseradish peroxidase (SouthernBiotech). After 30 min, TMB substrate was added for 20 min before the color reaction was stopped with 1 N H₂SO₄. The optical density for each well was read with a microplate reader set to 450 nm.

Statistical analysis

For Fig. 2-1, a multiple linear model was fit including experiment, day post-infection, and group (mock, UV-inactivated, or HMPV). The association between day and outcome was modeled using a quadratic term (i.e. day + day²) and an interaction between group and day (group*day + group*day²). To test the overall effect of group on outcome trajectories, a likelihood ratio test was performed, where the model was fit with the exclusion of group. Pairwise analyses were performed (i.e. by comparing the trajectories for mock vs UV-inactivated, mock vs HMPV, and UV-inactivated vs HMPV). The analysis for T cells was performed the same way, except without the quadratic term. For all other experiments, student's *t* tests were used for statistical analyses. The data were expressed as mean ± standard error of the mean and were the combination of the results of two to five independent experiments (indicated in the Figure legends). P < 0.05 was considered significant by convention.

Results

Lung NK cell numbers increase after HMPV infection and are functionally competent.

Since NK cell kinetics had not been previously described in the context of HMPV infection, I first examined NK cells by flow cytometry in B6 mice infected with 6×10^5 pfu HMPV (Figures 2-1A and B). Lung NK cell numbers in HMPV-infected animals increased significantly by day 1 post-infection and reached a peak on day 3 before decreasing; however, there was no increase of NK cells in mice inoculated with mock cell lysate or UV-inactivated HMPV (Figure 2-1B). To measure NK cell functionality, I performed intracellular cytokine staining for IFN γ , a major cytokine produced by NK cells (174), and surface staining for CD107a, a marker for cytotoxic granule release (175). There was a significantly higher number of IFN γ + and CD107a+ NK cells in HMPV-infected mice as compared to those in mock and UV-inactivated groups, indicating that NK cells in infected animals were functional (Figures 2-1C and D). There was also a higher number of CD3+ T cells in the HMPV-infected group as compared to the other two groups (Figure 2-1E). Together, these results suggest that HMPV replication results in an increase in both lung NK and T cells.

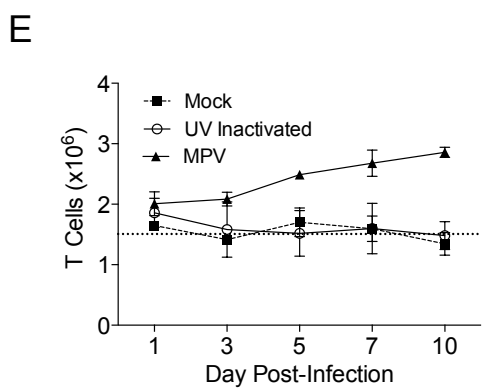
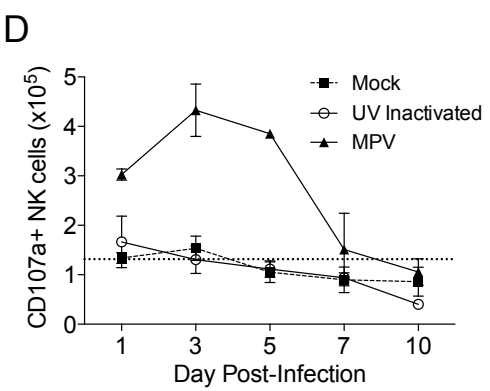
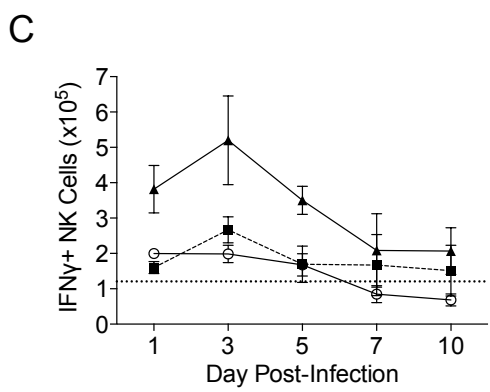
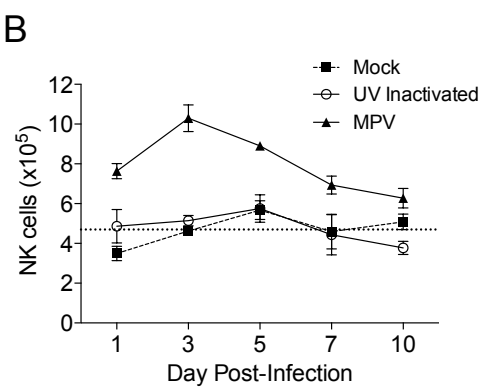
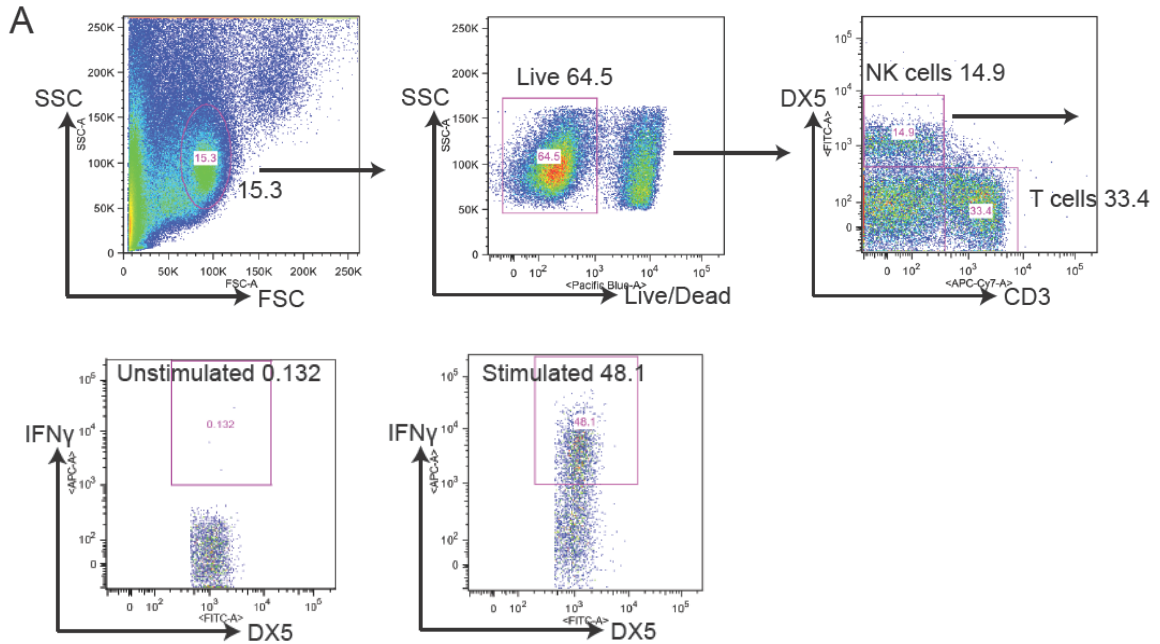


Figure 2-1: Lung NK cell numbers increase after HMPV infection and are functionally competent. B6 mice were inoculated intranasally with mock cell lysate, UV-inactivated HMPV, or HMPV and the lung NK cell response was assessed. Lymphocytes were counted with a hemocytometer, and stained for flow cytometry.

(A) NK cells were defined as the DX5+CD3⁻ population; several experiments were confirmed with NKp46 as the NK cell marker (not shown). Several samples were stimulated with PMA and ionomycin. **(B)** The number of NK cells, **(C)** NK cells producing IFN γ or **(D)** degranulating, and **(E)** T cells were quantified. Each symbol represents the mean of two independent experiments with three individual mice per group for each time point and experiment \pm standard error of the mean (SEM). Dotted lines represent the numbers at baseline (i.e. in uninfected mice). For all graphs shown in **B-E**, the data for the HMPV group are significantly higher than the other two groups, with $p < 0.005$.

Lung viral titers remain unchanged with NK cell depletion.

To test whether NK cells are required to clear HMPV, B6 mice were injected i.p. with either anti-NK1.1 or isotype control antibody five days before and on the day of infection (Figure 2-2A). This injection protocol depleted >95% of NK cells until at least day 7 post-infection, as confirmed by flow cytometry on splenocytes and lung lymphocytes (Figure 2-2B and data not shown). Mice that were to be euthanized on day 10 were given another antibody injection on day 5 post-infection. As expected, isotype-control mice did not lose weight post-HMPV infection, as previously described in other animal models (176); NK cell-depleted mice did not differ significantly from isotype-control mice in weight (Figure 2-2C). Surprisingly, NK cell-depleted mice had similar lung viral titers as control mice on all of the days tested, and were able to clear virus as quickly as control mice (Figure 2-2D). The trend towards higher titer in NK cell-depleted mice on day 3 did not reach statistical significance, and was less than $0.5 \log_{10}$.

Since NK1.1 is expressed on the surface of both NK and NKT cells, the anti-NK1.1 antibody used in the above experiments potentially depleted both types of cells (177). To address this issue, I used CD1d^{-/-} mice, which were on a B6 background and lacked NK1.1+ T cells but had normal numbers of NK cells, B cells, and conventional T_{CD4} and T_{CD8} (178). HMPV-infected CD1d^{-/-} mice had similar weights and viral titers as NK cell-depleted and isotype control B6 mice (data not shown and Figure 2-2E). These results indicate that neither NK nor NKT cells help the host clear HMPV.

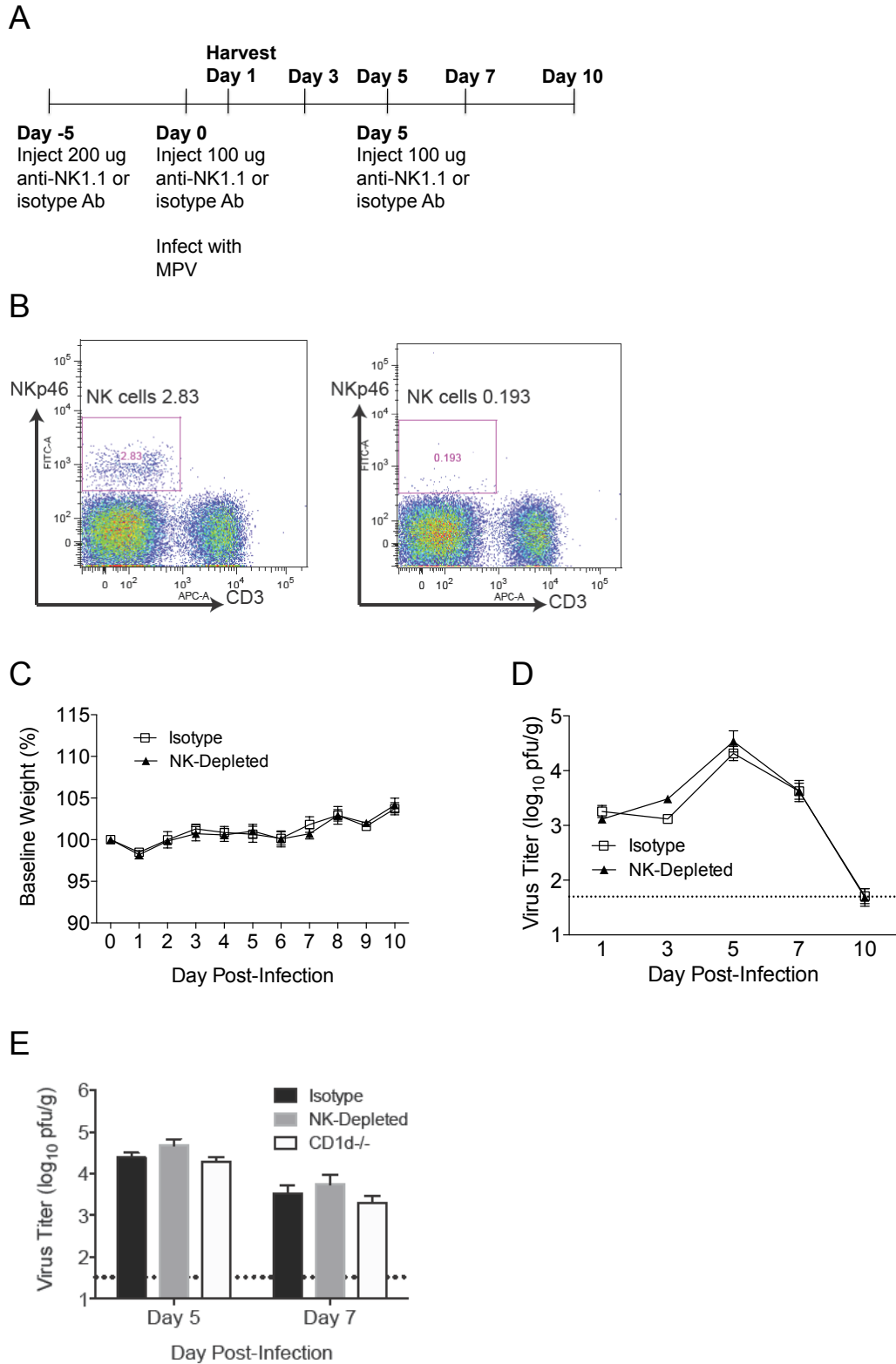


Figure 2-2: Lung viral titers remain unchanged with NK cell depletion. (A) B6 mice were injected with either isotype (control) antibody or anti-NK1.1 antibody and infected

i.n. with HMPV. **(B)** NK cell depletion was confirmed by flow cytometry on splenocytes from isotype-treated animals (left) and anti-NK1.1-treated animals (right). **(C)** Mice were weighed daily from the day of infection until euthanization. **(D)** Viral titers were quantified in pfu per gram of lung tissue. **(E)** Additional experiments were performed to compare viral titers in B6 mice and CD1d^{-/-} mice. Dotted lines represent the limit of viral detection. Data in **C** are combined from five independent experiments; data in **D** are combined from two to five independent experiments, and data in **E** are combined from two independent experiments with at least three mice per group for each time point and experiment \pm SEM.

NK cells do not alleviate or exacerbate lung histopathology.

Since NK cells were previously described to contribute to inflammation in a model of RSV infection (42), I next examined whether NK cells affected lung inflammation post-HMPV infection. Histology samples were analyzed qualitatively and quantitatively by an expert pathologist blinded to the conditions of the experiment. In contrast to the RSV studies, NK cell depletion did not alter the degree of lung inflammatory cell infiltration post-HMPV infection (Figures 2-3 A-C). As NK cells were reported to regulate macrophage cell number (56, 179), lung sections were also analyzed for macrophages by F4/80 staining; no differences were found (Figure 2-3D). In both isotype treated and NK cell-depleted groups, inflammation was predominately perivascular on days 3 and 5 (data not shown and Figure 2-3E), although there was some peribronchiolar inflammation on day 7 (Figure 2-3F). These results indicate that NK cells do not affect the degree of lung inflammation during acute HMPV infection.

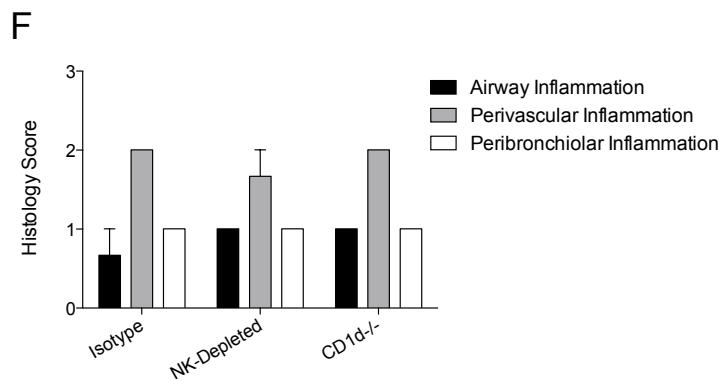
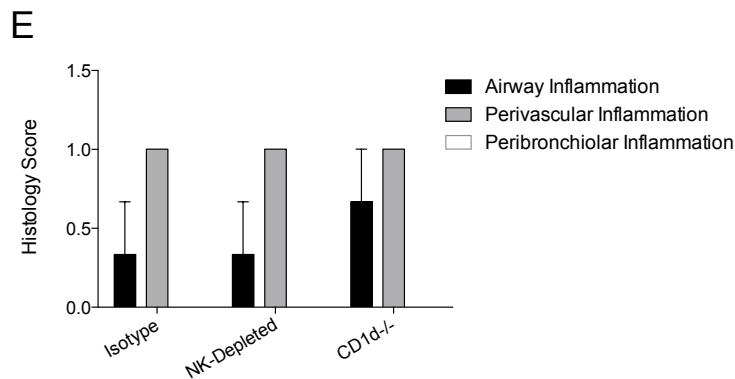
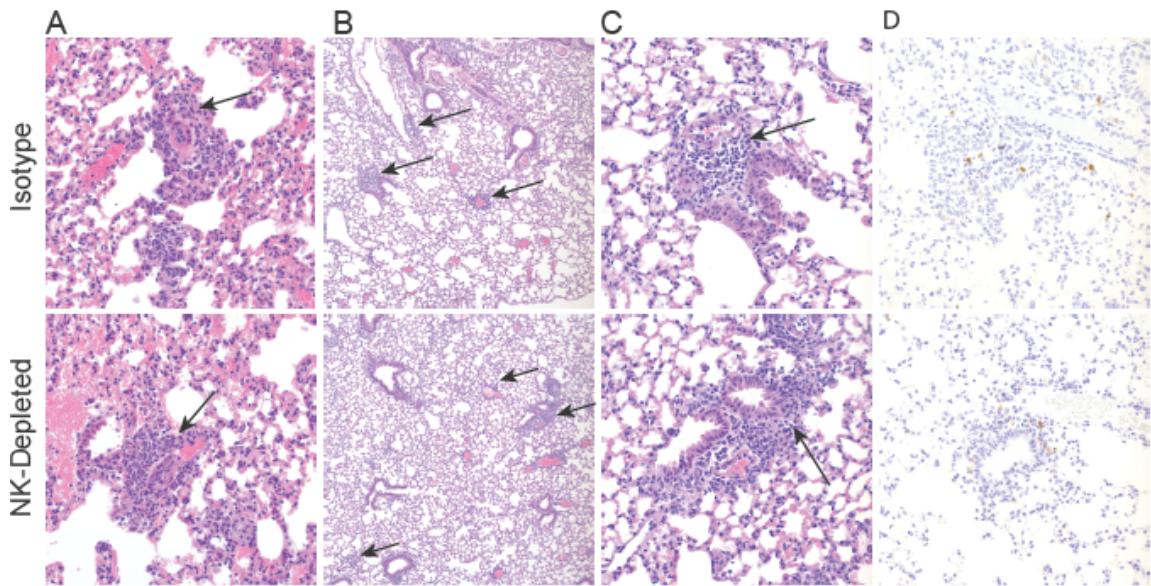
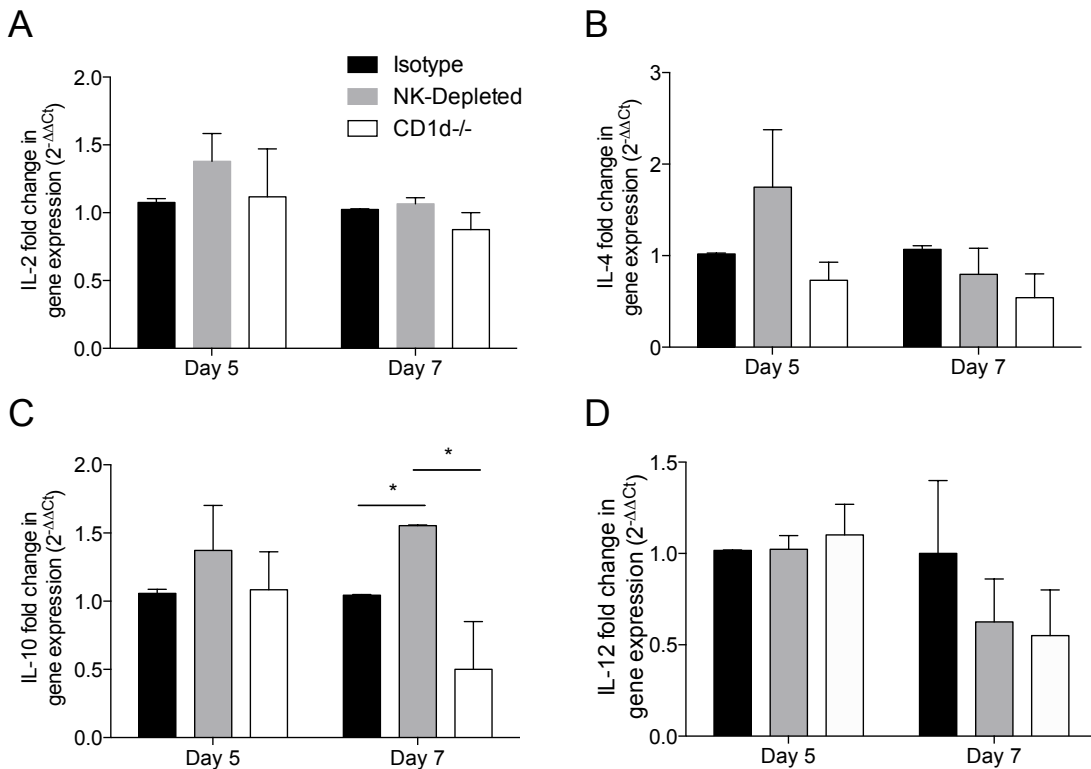


Figure 2-3: NK cells do not alleviate or exacerbate lung histopathology. Mice were infected with HMPV and whole lungs were excised for histology. Slides were stained with (A-C) H&E or (D) F4/80 and representative images are shown for (A) day 5 (400x magnification), (B) day 7 (100x magnification), (C) day 7 (400x magnification), and (D) day 7 (400x magnification). Arrows point to areas of inflammation. Inflammation was scored by a blinded expert pathologist on a scale from 0 to 4, with 4 being the maximum level of inflammation, on (E) day 5 and (F) day 7. Error bars indicate standard deviation.

Lung cytokine production is maintained despite the absence of NK cells.

As NK and NKT cells produce a variety of cytokines (56, 180) and can affect the secretion of cytokines by other leukocytes (175), I examined lung cytokine levels in isotype control, NK cell-depleted, and CD1d^{-/-} animals post-infection. Overall, there were similar levels of IL-2, IL-4, IL-10, IL-12, and IFN γ gene expression in all three groups of mice on days 5 and 7 post-infection (Figures 2-4 A-E). Differences in IL-10 levels did reach significance on day 7 post-infection, although the fold changes were not large (Figure 2-4C). IFN γ protein production was verified by ELISA and corroborated these findings (Figure 2-4F). These results indicate that the absence of neither NK nor NKT cells affects cytokine production to a substantial degree in the acute phase of HMPV infection.



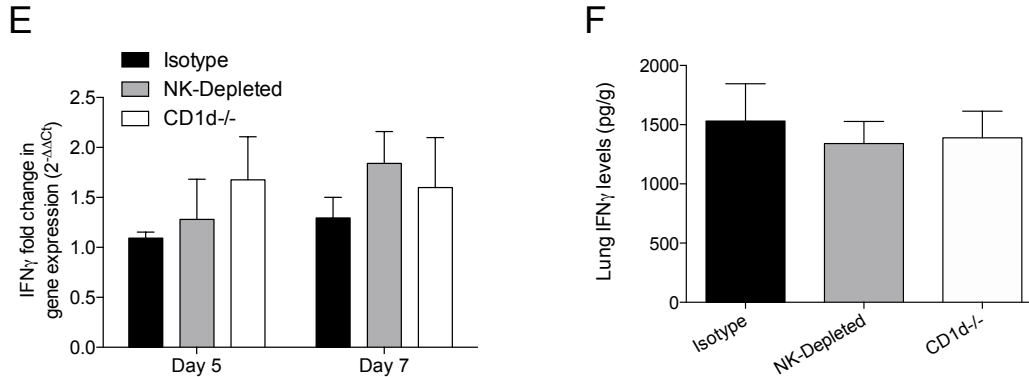


Figure 2-4: Lung cytokine production is maintained despite the absence of NK cells. Mice were infected with HMPV and lung RNA was extracted for quantification of cytokine gene expression using RT-PCR. **(A-E)** Cytokine levels were normalized to the housekeeping gene Hprt, and the relative gene expression was expressed as the fold change compared to the average value in isotype control animals. **(F)** ELISA was also performed for IFN γ protein production and expressed as pg per gram of lung tissue. Data are combined from two to four independent experiments with three to five individual mice per group for each time point and experiment \pm SEM. * $p < 0.05$, two-tailed Student's t test.

Epitope-specific CD8 $^{+}$ T cells remain constant despite NK cell depletion.

Since my previous results indicated that NK cells did not affect host innate immune response during HMPV infection, I wondered whether NK cell depletion would affect the adaptive immune response. T $_{CD8}$ are important for HMPV clearance during primary infection (91); furthermore, the absence of NK cells alters the number of epitope-specific T $_{CD8}$ in a lymphocytic choriomeningitis virus (LCMV) model of infection (181). To study the anti-HMPV T $_{CD8}$ response to HMPV, I used an HLA B7.2 transgenic (B7tg) mouse model. These mice are B6-K $^{bD^b}$ double-knockouts, and can only recognize T $_{CD8}$ epitopes restricted by human HLA-B*0702 (182). Several HMPV epitopes have been previously identified in these mice. M $_{195-203}$ (M195) peptide stimulates a large fraction of HMPV-immune splenocytes to produce IFN γ post-infection (93), thus facilitating my analysis of HMPV epitope-specific T $_{CD8}$.

I quantified lung T_{CD8} by tetramer staining on day 10 post-infection, the peak of the T_{CD8} response to HMPV (93). I found no significant differences in the total number of lymphocytes, T_{CD8}, or HMPV M195-specific T_{CD8} (Figures 2-5 A-C). There was a higher frequency of T_{CD8} in the NK cell-depleted group as compared to the isotype control group (Figure 2-5D); however, the absolute numbers of T_{CD8} were not different between the two groups.

PD-1, an inhibitory receptor, is upregulated on impaired T cells during chronic infections and cancer (183, 184). Since PD-1 signaling also mediates T_{CD8} impairment during acute respiratory infections with HMPV and other viruses (93), and NK cell depletion reduced the expression of T cell exhaustion markers during LCMV infection (181), I next examined whether NK cell depletion had an effect on T_{CD8} PD-1 expression during HMPV infection. I found no significant differences in the percentage or number of total T_{CD8} expressing PD-1, or in the M195-specific T_{CD8} expressing PD-1 (Figures 2-5 E-G). Thus, HMPV epitope-specific T_{CD8} accumulate to the same degree during HMPV infection regardless of the presence or absence of NK cells, and T_{CD8} PD-1 expression remains unchanged.

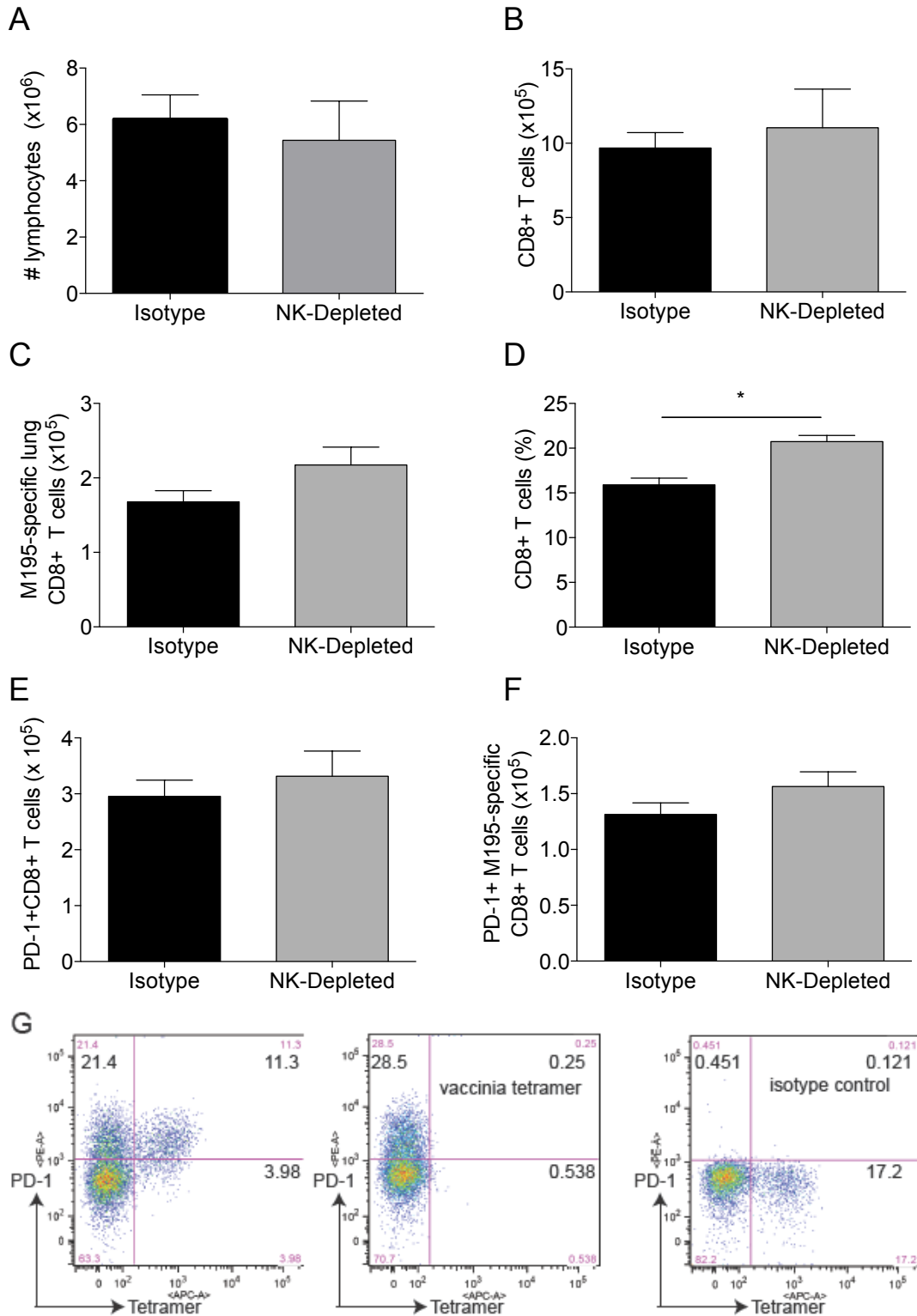


Figure 2-5: Epitope-specific CD8+ T cells remain constant despite NK cell depletion. B7tg mice were infected with HMPV and lung lymphocytes were isolated on day 10 post-infection. Lymphocytes were counted with a hemocytometer and analyzed

by flow cytometry; the total number of **(A)** lymphocytes and **(B)** T_{CD8} are shown. **(C)** M195 tetramer-specific T_{CD8} quantify the T_{CD8} response to the M195 epitope. **(D)** shows the lung T_{CD8} population as a % of total lymphocytes in isotype and NK cell-depleted animals. **(E)** The total number of PD-1+ T_{CD8} and **(F)** the number of PD-1+ M195 epitope-specific T_{CD8} are also shown. **(G)** Representative flow cytometry plots after staining with PD-1 and M195 tetramer (left), PD-1 and irrelevant vaccinia tetramer (middle), and isotype control antibody for PD-1 and M195 tetramer (right). Numbers in each quadrant represent the subset population as a percentage of T_{CD8} . Data in **A-F** are combined from three independent experiments with four mice per group per experiment \pm SEM. * $p < 0.05$, two-tailed Student's *t* test.

CD8+ T cell functionality is unaltered in the absence of NK cells.

To quantify T_{CD8} functionality in the setting of NK cell depletion, I next measured $IFN\gamma$ and CD107a using lung lymphocytes from the same mice analyzed by tetramer staining (Figure 2-6A). As previously reported by our group (93), only a fraction of the HMPV-specific T_{CD8} produced $IFN\gamma$ and degranulated as a result of T_{CD8} impairment (Figure 2-6B). However, comparing the NK cell-depleted and isotype groups revealed no significant differences in the percent or number of T_{CD8} degranulating or producing $IFN\gamma$, or in the $IFN\gamma$ mean fluorescence intensity (MFI) (Figures 2-6B and C, and data not shown). Lung lymphocytes from isotype and NK cell-depleted mice were stained for NK cells to confirm NK cell depletion by flow cytometry (Figure 2-6D). Together, these data suggest that in the context of HMPV infection, NK cell depletion does not have a significant effect on T_{CD8} functionality during the peak of the T_{CD8} response.

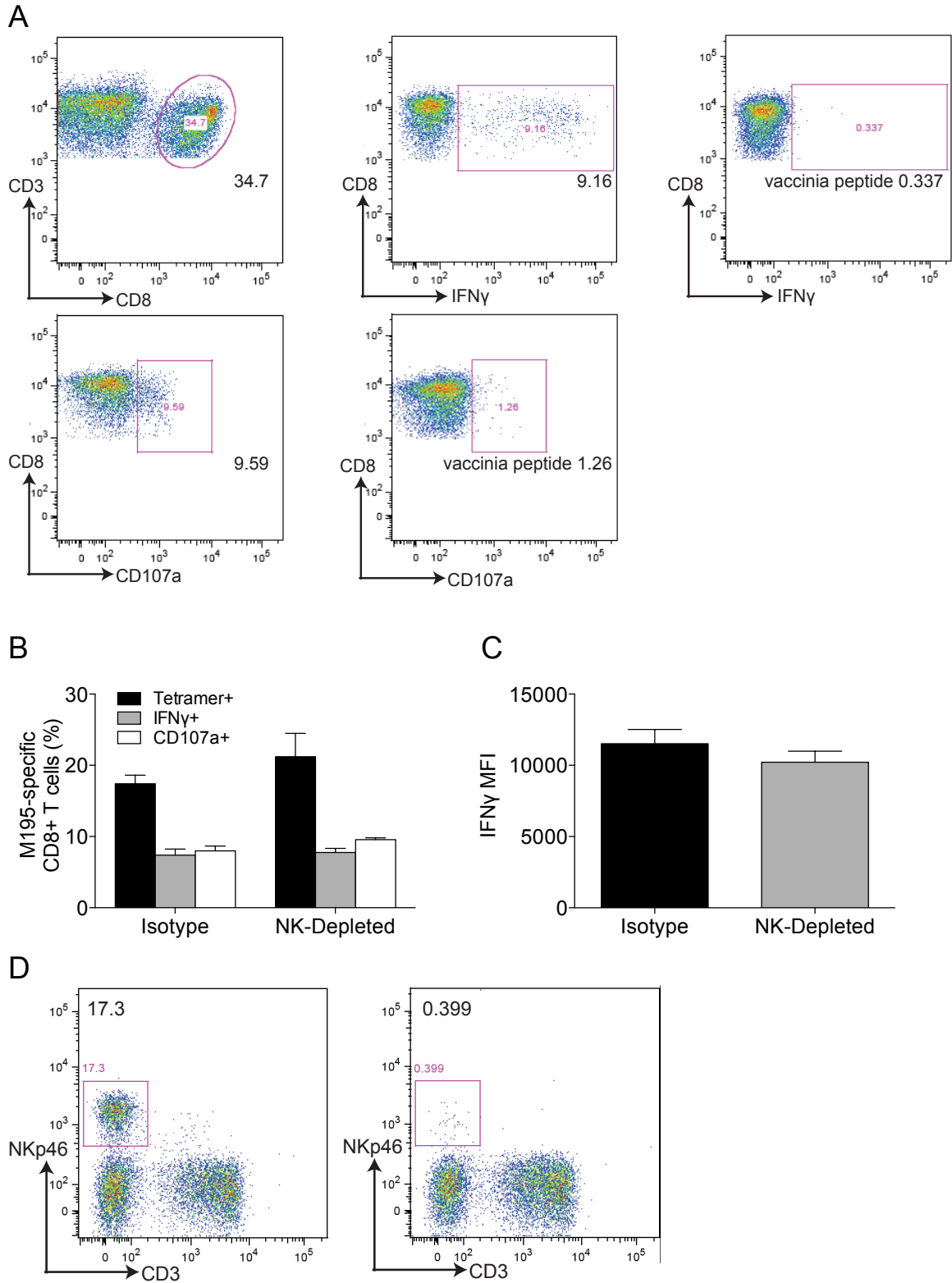


Figure 2-6: CD8⁺ T cell functionality is unaltered in the absence of NK cells. B7tg mice were infected with HMPV and lung lymphocytes were isolated on day 10 post-

infection. **(A)** In parallel with tetramer staining, lymphocytes were stimulated with M195 peptide, irrelevant (vaccinia) peptide as a negative control, or PMA/ionomycin as a positive control and stained for CD3, CD8, IFN γ , and CD107a. **(B)** quantifies the T_{CD8} response to the M195 epitope, and **(C)** quantifies the mean fluorescence intensity (MFI) of IFN γ in M195-stimulated T_{CD8}. 8,000 total T_{CD8} per sample were collected by flow cytometry. **(D)** NK cell depletion was confirmed in lung lymphocytes of isotype-treated (left) and anti-NK1.1 antibody treated (right) animals. Data in **B** and **C** are combined from three independent experiments with four mice per group per experiment \pm SEM.

CD4+ T cell numbers and functionality are preserved in the absence of NK cells.

To determine whether NK cell depletion affected the T_{CD4} population, I stained for CD4 and quantified IFN γ by intracellular cytokine staining on lymphocytes from the same mice as those used in the T_{CD8} analyses. As MHC class II epitopes have not yet been reported for HMPV, and T cells extracted from infected mice do not produce cytokines *ex vivo* without stimulation (data not shown), I stimulated the cells with PMA and ionomycin before analysis by flow cytometry. As in the T_{CD8} experiments, I found no significant differences between NK cell-depleted and isotype groups in percent or number of total T_{CD4}, IFN γ + T_{CD4}, or IFN γ MFI, indicating that T_{CD4} function is preserved during NK cell depletion (Figures 2-7 A-C).

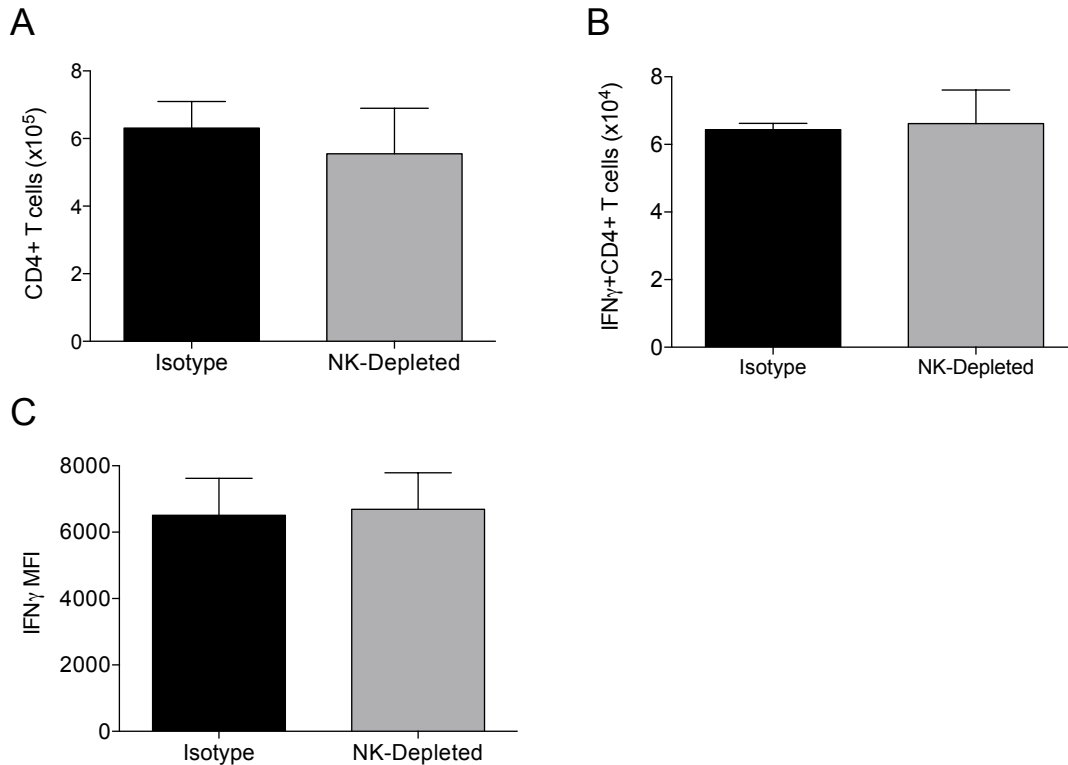


Figure 2-7: CD4⁺ T cell numbers and functionality are preserved in the absence of NK cells. B7tg mice were infected with HMPV and lung lymphocytes were isolated on day 10 post-infection. In parallel with tetramer staining, lymphocytes were stimulated with PMA/ionomycin and stained for CD3, CD4, and IFN_γ. **(A)** quantifies the total number of T_{CD4}, **(B)** quantifies the number of IFN_γ-producing T_{CD4}, and **(C)** quantifies the mean fluorescence intensity (MFI) of IFN_γ in T_{CD4}. 7,000 total T_{CD4} per sample were collected by flow cytometry. Data are combined from three independent experiments with four mice per group per experiment ± SEM.

NK cells are activated during HMPV infection.

One possible reason for the failure of NK cells to contribute to host clearance of HMPV could be that the virus inhibits NK cell function. I thought this unlikely, as I showed in Figure 2-1 that there were higher numbers of NK cells producing IFN_γ and degranulating in HMPV-infected mice than in mice inoculated with cell lysate or UV-inactivated virus (Figures 2-1C and D). To further elucidate the activation of lung NK cells *in vivo* during HMPV infection, I measured surface expression of CD69, an inducible surface marker for activated lymphocytes, including NK cells (185). I found that

on day 1 post-infection, CD69 expression on NK cells significantly increased in infected mice, while it did not increase above background levels in mice from mock and UV-inactivated groups (Figures 2-8A and B). CD69 expression remained significantly higher in HMPV-infected mice on day 3 post-infection (data not shown). These data, in combination with the results shown in Figure 2-1, indicate that HMPV infection results in an activated and functional NK cell response.

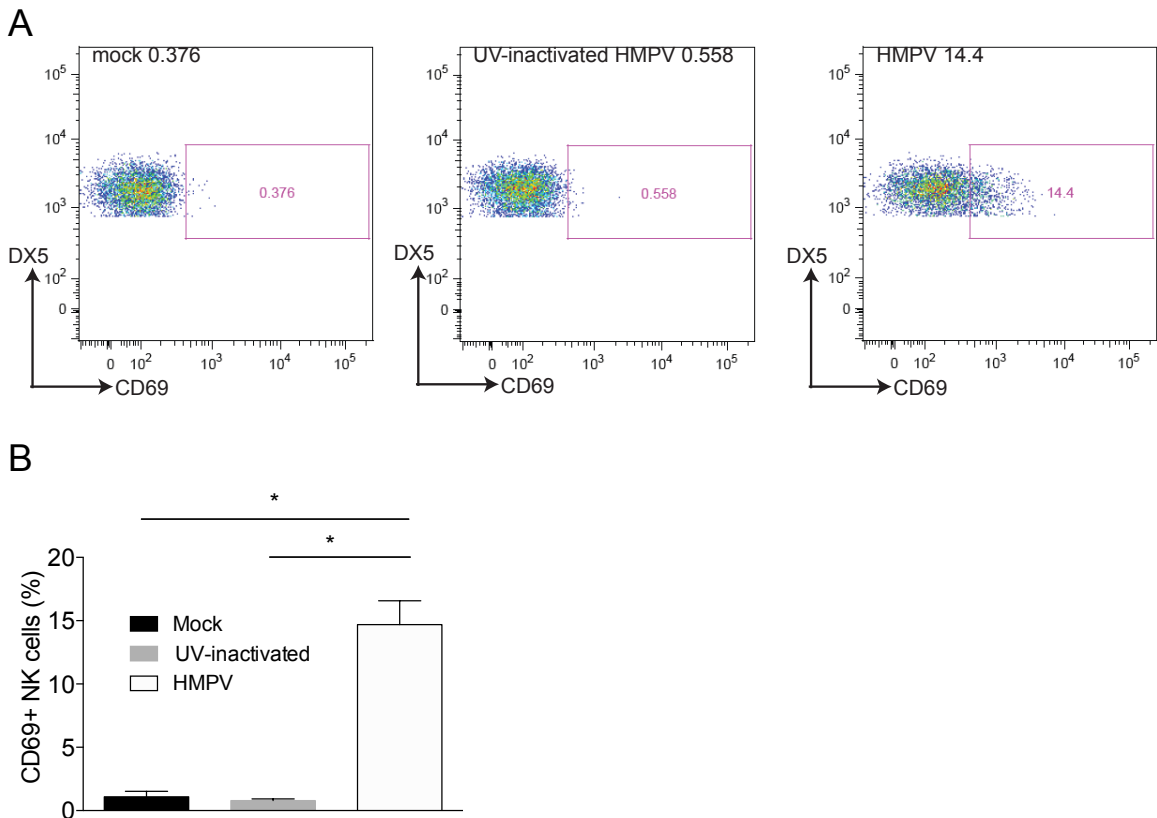


Figure 2-8: NK cells are activated during HMPV infection. B6 mice were inoculated with mock cell lysate, UV-inactivated HMPV, or HMPV and the lung NK cell response was assessed on day 1 post-infection. **(A)** Lung lymphocytes were stained for DX5, CD3, and the activation marker CD69. **(B)** CD69-expressing NK cells were quantified as a % of total NK cells. Data are combined from three independent experiments with three mice per group per experiment \pm SEM. * $p < 0.05$, two-tailed Student's *t* test.

Discussion

In this study, I found that HMPV infection promoted an increase in the number of activated and functional lung NK cells during the early stage of infection. Viral replication was necessary for this increase, as NK cell number and function in mice that received UV-inactivated virus or mock cell lysate did not rise above baseline. In agreement with published reports on influenza virus (70, 186), NK cells had no effect on viral titer, as NK cell-depleted mice had equal HMPV viral load on the day of peak titer (day 5) and cleared infection just as quickly as control mice. These results indicate that in contrast to the critical function of NK cells during infection by members of the herpesvirus family (71, 187), NK cells are nonessential for clearance of HMPV.

A prior report suggested that NK cells had a role in limiting HMPV replication, as NK cell-depleted BALB/c mice had (slightly) higher lung titers (41); however, that model described biphasic viral kinetics and long-term persistence not confirmed by others. The discrepancy with my results could be due to different mouse and virus strains or different depletion antibodies or protocols.

Since NK1.1 is expressed on NK and NKT cells, the anti-NK1.1 monoclonal antibody used for NK cell depletion potentially depleted both types of cells (177). To address this issue, I included CD1d^{-/-} mice in my experiments, as these mice have NK cells but no NKT cells (178), and found that NKT cells are also dispensable for immune control of HMPV. While the depletion of both NK and NKT cells makes anti-NK1.1 antibody an imperfect method of NK cell depletion, this antibody is more specific than anti-asialo-GM1, the other widely-used antibody in the NK cell field, as anti-asialo-GM1 antibody also affects basophils (78) and monocytes (188). Furthermore, the lack of a widely accepted mouse model that is selectively deficient in NK cells makes anti-NK1.1 antibody the current preferred method of studying the consequences of NK cell absence (189-191).

Due to similarities between HMPV and RSV genomes (11), as well as the substantial amount of homology that the two viruses share in their surface glycoproteins (133), some conclusions based on RSV research have been extrapolated to HMPV (11, 130). However, an increasing number of studies have found differences in immune responses to the two viruses (192, 193). Although studies of RSV in mice have found that NK cells contributed to enhanced lung injury with increased inflammatory infiltrates (42, 194), I found no evidence of intensified lung histopathology in HMPV-infected control mice (with intact NK cells) as compared to NK cell-depleted mice, suggesting that immune responses to HMPV cannot always be inferred from RSV research.

Since NK cells have well-established roles in cytokine production, influencing cytokine production by other cell types, and altering the $T_H1:T_H2$ balance (56, 195), I were surprised to find that NK cell-depleted mice did not have lower levels of the various cytokines tested. I speculate that other cell types during the early stages of infection may be producing cytokines to compensate for the lack of NK cells (196). The ability of NK cells to alter the cytokine environment may also depend on the nature of inducing antigens, as NK cell depletion changed the $T_H1:T_H2$ ratio of antigen-driven cytokine synthesis during *Leishmania major* and *Bordetella pertussis* infections (195, 197), but not in an OVA-specific immune response (198).

With regards to the adaptive immune response to viruses, NK cell depletion resulted in increased numbers of virus-specific T_{CD8} , and protected against upregulation of the T cell exhaustion marker PD-1 in an LCMV model of infection (181, 199). In addition, NK cells dampened T cell responses through a mechanism involving perforin-dependent cytotoxicity (200). NK cells also reduced T cell proliferation during human parainfluenza virus infection (201). On the other hand, NK cells were an important source of $IFN\gamma$ in the early response to RSV infection, and subsequently facilitated the emergence of T_{CD8} (202).

Recurrent infections with HMPV occur in humans despite the presence of neutralizing antibodies, indicating that antibodies are not always associated with protection (3, 11). As T cells are crucial in the control of HMPV infection (91), I addressed the possibility that NK cells influence the T cell response to HMPV. While NK cell-depleted mice had a higher percentage of T_{CD8} than control mice, the total numbers of T_{CD8}, HMPV-specific T_{CD8}, PD-1+ T_{CD8}, and functional T_{CD8} were not significantly different between the two groups. Furthermore, NK cell depletion did not alter the magnitude or functionality of immune T_{CD4} to HMPV. Together, these findings demonstrate that in contrast to their role in RSV and other paramyxovirus infections (42, 201, 202), NK cells are dispensable in the host immune response to HMPV in mice.

One potential explanation of the failure of NK cells to influence the course of HMPV infection is the possibility that the virus inhibits NK cell function, as is the case for cytomegalovirus infections (203, 204). However, this explanation is unlikely given that lung NK cells have an activated phenotype, produce IFN γ , and degranulate in response to HMPV infection (Figures 2-1 and 2-8). A limitation of this study is that since mice and other small animals are only semipermissive for HMPV replication (16), the findings may not accurately represent human immune responses to HMPV infection. This is a reasonable concern in research using model organisms, and has been extensively discussed elsewhere (205).

In summary, the present work indicates that in contrast to viruses such as CMV and other herpesviruses that are lethal in the absence of NK cells (71), HMPV can be cleared independently of NK cells. My results are in alignment with previous reports that found certain components of the immune system to be dispensable in some situations, but essential in others (206). Taken together, these findings suggest that aspects of the immune response to HMPV infection are different compared to those of RSV, consistent

with prior findings in humans (207). Further research will contribute to an understanding of the pathophysiology associated with HMPV infection.

CHAPTER III

LUNG CD8+ T CELL IMPAIRMENT OCCURS DURING HUMAN METAPNEUMOVIRUS INFECTION DESPITE VIRUS-LIKE PARTICLE (VLP) INDUCTION OF FUNCTIONAL CD8+ T CELLS

Introduction

Human metapneumovirus is a major cause of acute respiratory morbidity and mortality in infants, older adults, and immunocompromised individuals, although serological studies indicate that almost all humans have been infected by five years of age (11, 208). The HMPV fusion (F) protein, which mediates viral fusion and entry, elicits neutralizing antibodies, whereas antibodies against the other proteins on the virion surface are non-neutralizing (132, 133, 160, 161, 209). Although HMPV subtypes are relatively conserved, reinfections occur throughout life despite the presence of neutralizing antibodies (80, 210). T cell immunity is also important for viral clearance and resolution of illness (20, 87, 88).

Virus-like particles (VLPs) formed from the assembly of viral structural proteins are an attractive vaccine strategy since they are known to elicit both antibody and T cell responses (152). Currently, the functionality of T_{CD8} elicited by VLP vaccination, as compared to infection, is unclear. T_{CD8} are important for viral clearance, and several studies have shown that they contribute to protection from HMPV (18, 141). It is known that several inhibitory receptors, including programmed death-1 (PD-1), mediate T_{CD8} impairment during acute and chronic infections (98). The Williams lab previously reported that HMPV lower respiratory infection led to impairment of T_{CD8} function through PD-1 signaling (93). The memory T_{CD8} response to HMPV was also impaired via PD-1 signaling (141). Blockade or genetic ablation of PD-1 enhanced T_{CD8} function and viral

clearance during both primary and secondary infection, suggesting that a T_{CD8} memory response low in PD-1 expression might be optimal.

In this study, I tested the hypothesis that immunization with HMPV VLPs containing the fusion (F) and matrix (M) proteins could induce functional HMPV-specific T_{CD8} and protection from viral challenge in mice. I found that VLP immunization conferred complete protection in the lungs of mice for at least 20 weeks post-immunization. VLPs elicited F- and M-specific T_{CD8} responses in the lungs of mice, both after vaccination alone and after vaccination followed by HMPV challenge. VLP-vaccination alone elicited T_{CD8} that were functional and expressed lower levels of PD-1 and other inhibitory receptors than those elicited by HMPV infection; however, challenge with live virus after vaccination abrogated these differences. Nonetheless, depletion of T_{CD8} in immunized mice resulted in higher viral titers as compared to immunized non-depleted mice, indicating that VLP-induced T_{CD8} still mediate viral clearance despite higher PD-1 levels after challenge. These results suggest that VLPs containing F and M proteins are a promising HMPV vaccine candidate, and that T_{CD8} contribute to the protection conferred by VLP vaccination.

I acknowledge Pavlo Gilchuk for making the tetramers used in my experiments, Kelli Boyd for lung histopathology analysis, and Jennifer Schuster for contributing B2 HMPV stocks.

Materials and Methods

Virus preparation and cell culture

HMPV (clinical strain TN/94-49, subtype A2, as well as TN/89-515, subtype B2) was propagated in LLC-MK2 cells, as previously described (16). The B2 subtype was sucrose-purified (211). 293-F cells were maintained in Freestyle 293 media (Life Technologies) as recommended by the manufacturer.

Generation of VLPs

HMPV F and M amino acid sequences were derived from a pathogenic clinical strain of HMPV (subtype A2), sequence-optimized for mammalian expression, and cloned into pcDNA3.1 (Life Technologies) as described previously (141). 293-F cells were transfected with plasmids encoding HMPV fusion (F) and matrix (M) proteins using pcDNA3.1-F (20 µg), pcDNA3.1-M (40 µg) and 293fectin transfection reagent (60 µL) as recommended by the manufacturer (Life Technologies). Mock VLPs were generated by transfecting 293-F cells with empty pcDNA3.1 vector. Eighteen hours post-transfection, cell media was changed and 5 µg/mL trypsin added to the flask. Three days post-transfection, cells were pelleted and supernatant was centrifuged at 27,000 rpm for 90 minutes at 4°C using a SW32Ti rotor (Beckman Coulter), and pelleted through 20% sucrose. VLP pellets were resuspended and snap-frozen in a dry ice-alcohol bath for storage at -80°C.

VLP protein content

The protein content of VLPs was determined using the BIO-RAD protein assay as described previously (141). Western blot analysis was conducted to confirm incorporation of F and M proteins. VLPs, mock VLPs, and HMPV samples were lysed

with 1% SDS, heated in NuPAGE LDS Sample Buffer (Life Technologies) containing 5% β -mercaptoethanol (Sigma), run on 10% NuPAGE gels (Life Technologies), and transferred to polyvinylidene fluoride membranes (Life Technologies). Membranes were blocked with 5% milk in PBS/ 0.1% Tween for 30 minutes and then incubated with guinea-pig anti-A2 F or rabbit anti-M serum diluted in 5% milk/PBS/0.1% Tween overnight at 4°C. Membranes were rinsed three times in PBS/0.05% Tween (PBS-T) and then incubated with secondary anti-guinea pig or anti-rabbit antibody for one hour at room temperature. Membranes were rinsed three times in PBS-T and imaged using an Odyssey imaging system (LI-COR). VLP F protein content was also determined by Western blot using recombinant F protein to generate a standard curve. Band intensities were quantified using the Odyssey infrared imaging software. HMPV contained 120 ng/ μ l and VLPs contained 215 ng/ μ l of F protein. Although the amount of M protein was not formally quantified due to lack of a standard, its presence was confirmed by Western blot; HMPV particles had approximately three times the amount of M protein as VLPs (not shown).

Mice and immunizations

C57BL/6 (B6) mice were purchased from the Jackson Laboratory. μ MT mice were a kind gift from Dr. Mark Boothby (Vanderbilt University, Nashville, TN). HLA B7.2 transgenic (B7tg) mice were a gift from Alessandro Sette (La Jolla Institute for Allergy and Immunology, La Jolla, CA) and Francois Lemonnier (Institut Pasteur, Paris, France) (182). Animals were bred and maintained in specific pathogen-free conditions. Six- to ten-week-old mice were used in all experiments. For intraperitoneal (i.p.) vaccinations, mice were injected with 100 μ L of VLPs or mock VLPs mixed with 100 μ L TiterMax Gold (TMG) adjuvant (Sigma). For intranasal (i.n.) vaccinations or infection with HMPV, mice

were anesthetized intraperitoneally with ketamine/xylazine and inoculated intranasally with 6×10^5 pfu HMPV, mock VLPs, or VLPs in a 100 μ L volume. For HMPV challenge, mice were anesthetized and inoculated i.n. with 6×10^5 pfu HMPV unless otherwise indicated. Serum was collected by submandibular bleed and HMPV neutralizing titers were determined by plaque reduction assay as described previously (16).

Viral titration

Lung tissues were collected and pulverized in glass homogenizers before centrifugation at 1200 rpm at 4°C for 10 min. Nasal turbinates (NT) were collected and ground with mortar and pestle prior to centrifugation. Supernatants were collected, aliquoted into cryovials, and snap-frozen in dry ice-ethanol for storage at -80°C until further use. Viral titers were quantified by plaque titration as previously described (16).

Tetramer analysis and staining for inhibitory receptors

Lungs were minced and incubated for 1 hour in 2 ml medium (RPMI-1640 with 10% FBS, 2 mM glutamine, 50 μ g/ml gentamicin, 2.5 μ g/ml amphotericin B, and 50 μ M β -mercaptoethanol) at 37°C with 2 mg/ml collagenase A and 20 μ g/ml DNase (both from Roche). Single-cell suspensions were obtained by pushing digested lung tissue or whole spleens through metal screens, and passing over nylon cell strainers (BD Falcon). Erythrocytes were lysed with red blood cell lysis buffer (Sigma-Aldrich). All antibodies were purchased from BD Biosciences unless otherwise indicated. After staining with live/dead dye (Life Technologies) and blocking Fc receptors with anti-CD16/CD32, lymphocytes were incubated with APC-labeled tetramers (0.1-1 μ g/ml), anti-CD19 (clone 6D5, iCyt), anti-CD8 α (clone 53-6.7), and anti-PD-1 (clone J43) or isotype control antibody (hamster IgG2 κ) in FACS buffer (PBS/1% FBS) containing 50 nM dasatinib (LC

Laboratories) for 1 hour at room temperature. Background staining levels were determined by staining with a tetramer loaded with irrelevant (vaccinia or influenza virus derived) peptide. Tetramers were generated for HMPV epitope HLA-B*0702/M₁₉₅₋₂₀₃ (APYAGLIMI) (M195), H2-D^b/F₅₂₈₋₅₃₆ (SGVTNNGFI) (F528), H2-K^b/M₉₄₋₁₁₂ (VALDEYSKL) (M94), influenza virus H2-D^b/NP₃₆₆₋₃₇₄ (ASNENMETM), and vaccinia epitope HLA-B*0702/A34R₈₂₋₉₀ (LPRPDTRHL) as previously described (212). For additional inhibitory receptors, cells were stained with TIM-3 (clone RMT3-23), LAG-3 (clone C9B7W), and 2B4 (clone m2B4 (B6) 458.1) or isotype control antibodies (Biolegend). The data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Intracellular cytokine staining

Lymphocytes were restimulated *in vitro* with 10 μM of the appropriate (i.e. M195, F528, or M94) peptide for 6 hours at 37°C in the presence of anti-CD107a (clone 1D4B). Brefeldin A and monensin (BD Biosciences) were added for the last 4 hours of stimulation. Restimulation with an irrelevant (vaccinia or influenza) peptide served as a negative control; stimulation with 50 ng/ml PMA and 2 μg/ml ionomycin (Sigma-Aldrich) served as a positive control. Lymphocytes were then stained with anti-CD3 ϵ (clone 145-2C11), anti-CD19 (clone 6D5, iCyt), and anti-CD8 α (clone 53-6.7). After extracellular markers were stained, cells were fixed, permeabilized, and stained for intracellular IFN γ (clone XMG1.2) before flow cytometric analysis.

Lung histopathology

Mice were challenged four weeks post-vaccination. Five days post-challenge, the left lung lobes were inflated with 10% formalin and fixed for 24 hours, embedded in paraffin, sectioned at 5-μm, and stained with hematoxylin and eosin. Immunohistochemistry was

performed on the Leica Bond Max automated stainer for B220 (BD Biosciences, 553086), CD3 (Santa Cruz, SC1127), neutrophils (Santa Cruz, SC71674), or major basic protein (Mayo Clinic, clone MR-14.7). Slides were evaluated by an experienced veterinary pathologist (KLB) blinded to the composition of the groups.

CD8+ T cell depletion

Mice were injected i.p. with 150 µg of CD8-depleting antibody (clone 2.43, BioXCell) five days prior to HMPV challenge, and again with 100 µg of antibody on the day of challenge. Control animals received the same amounts of isotype control antibody. CD8+ T cell depletion was confirmed by flow cytometry.

Statistical Analysis

Comparisons between two groups were performed using an unpaired two-tailed Student's *t* test. Multiple group comparisons were performed using a one-way ANOVA with Tukey's multiple comparisons test. $P < 0.05$ was considered statistically significant. Error bars in each graph represent standard error of the mean (SEM) unless otherwise noted.

Study Approval

All animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were handled according to protocols approved by the Vanderbilt University Institutional Animal Care and Use Committee (IACUC).

Results

VLP vaccination protects C57BL/6 mice from HMPV challenge.

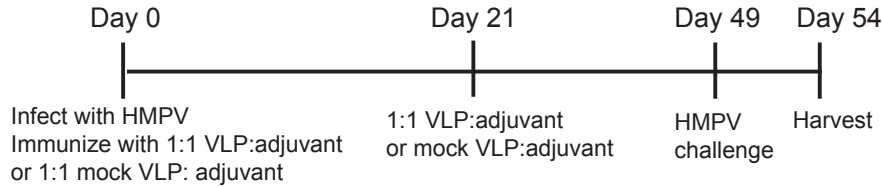
To determine whether VLP vaccination protects mice against HMPV challenge, I injected B6 mice i.p. with two doses of VLPs and TiterMax Gold adjuvant (TMG), given three weeks apart. Control animals were injected with mock VLPs and TMG, or were infected i.n. with HMPV (Figure 3-1A). Mock VLPs were generated by transfecting 293-F cells with empty pcDNA3.1 vector. TMG was chosen as the adjuvant based on its known immunogenicity in rodents (133, 161, 212, 213).

Four weeks post-vaccination, I challenged all mice i.n. with HMPV, and collected tissues five days post-challenge (the day of peak viral titer during primary HMPV infection (93)). VLP-vaccinated mice had significantly reduced nose titers as compared to mock-vaccinated mice, and undetectable lung titers (Figure 3-1B). Previously infected mice had undetectable titers in both noses and lungs. Serum was collected from mice after vaccination and the presence of neutralizing antibodies was determined by a plaque neutralization assay. Both i.n. and i.p. VLP-immunized mice, and HMPV-infected mice had neutralizing antibodies, whereas mock-immunized mice did not (data not shown), as previously reported (141).

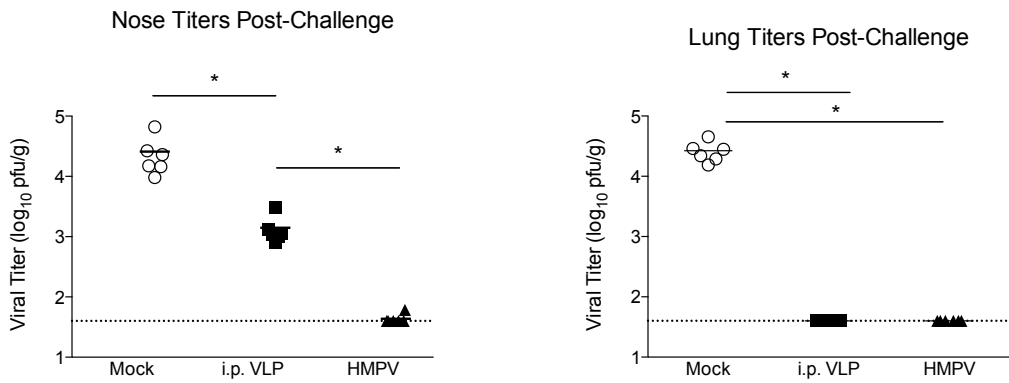
To test the duration of immunity, I waited twenty weeks between the second VLP dose and HMPV challenge. VLP-immunized mice had significantly decreased titers in the noses and undetectable lung titers (Figure 3-1C). Since i.p. vaccination did not fully protect the upper respiratory tract from HMPV challenge, I wondered whether the i.n. route of administration would elicit better protection in the nose. I repeated the experiment using the schedule in Figure 3-1A with i.n. VLPs instead of i.p. VLPs, and found that i.n. vaccination protected lungs completely but did not fully protect the nose (Figure 3-1D). These results indicate that VLP vaccination protects B6 mice completely

in the lower respiratory tract, the site of the most severe HMPV disease in humans, for at least 20 weeks post-vaccination. Furthermore, both i.n. and i.p. vaccination induce partial protection in the upper respiratory tract.

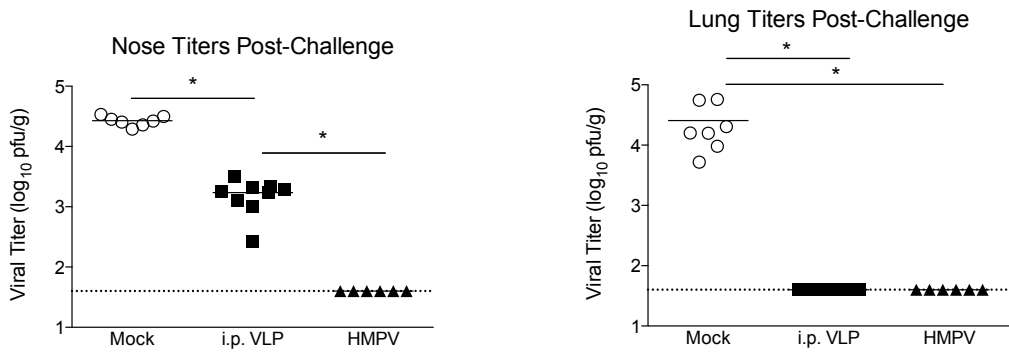
A



B



C



D

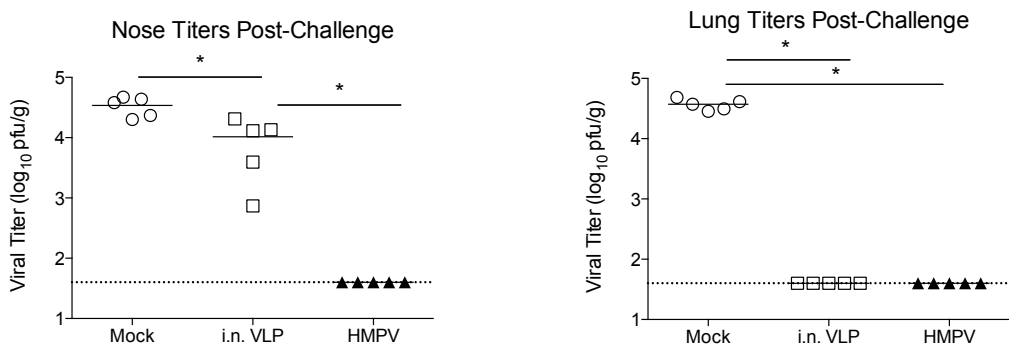


Figure 3-1: VLP vaccination protects C57BL/6 mice from HMPV challenge.

(A) Experimental schematic. B6 mice were vaccinated i.p. with VLPs and TiterMax Gold adjuvant, mock VLPs and adjuvant, or were infected i.n. with HMPV. Three weeks later, vaccinated mice were given a second dose of VLPs. Mice were challenged i.n. with HMPV four weeks after the second VLP dose, and tissues were collected five days post-challenge. **(B)** Nose and lung titers after challenge four weeks after the second VLP dose. **(C)** Nose and lung titers after challenge 20 weeks after the second VLP dose. **(D)** Mice were vaccinated i.n. with VLPs (no adjuvant) and challenged four weeks after the second VLP dose. Dotted lines represent the limit of detection. Data are combined from two independent experiments with two to six mice per group per experiment * $p < 0.05$, one-way ANOVA with Tukey's multiple comparisons test.

VLP vaccination does not exacerbate lung histopathology after HMPV challenge.

Previous reports of a formalin-inactivated HMPV vaccine found enhanced lung pathology when the animals were later challenged with virus (131). To determine whether VLP vaccination enhances lung pathology after HMPV challenge, I vaccinated mice with two VLP doses i.n. or i.p. with adjuvant three weeks apart, or infected them with HMPV, and challenged all groups with HMPV four weeks post-vaccination. Mice were euthanized five days post-challenge, and lung histology was analyzed by an expert pathologist blinded to the different groups. The inflammatory infiltrate post-HMPV challenge was not different between the groups. All groups showed perivascular inflammation consisting of lymphocytes, macrophages, neutrophils, and plasma cells, with no evidence of eosinophilia (Figures 3-2 A-D). VLP-immunized and previously-infected groups showed a similar level of inflammation post-HMPV challenge, whereas the mock-immunized and challenged group had a lower level of inflammation (Figure 3-2E). VLPs given alone without HMPV challenge elicited no inflammation (not shown). Mice that were vaccinated with VLPs or infected with HMPV did not lose more weight post-challenge as compared to mock-vaccinated mice (Figure 3-2F), and none of the mice showed signs of illness. This finding is consistent with prior reports of HMPV infection (192), as mice are semi-permissive hosts. These results indicate that VLP vaccination does not cause enhanced respiratory disease upon viral challenge.

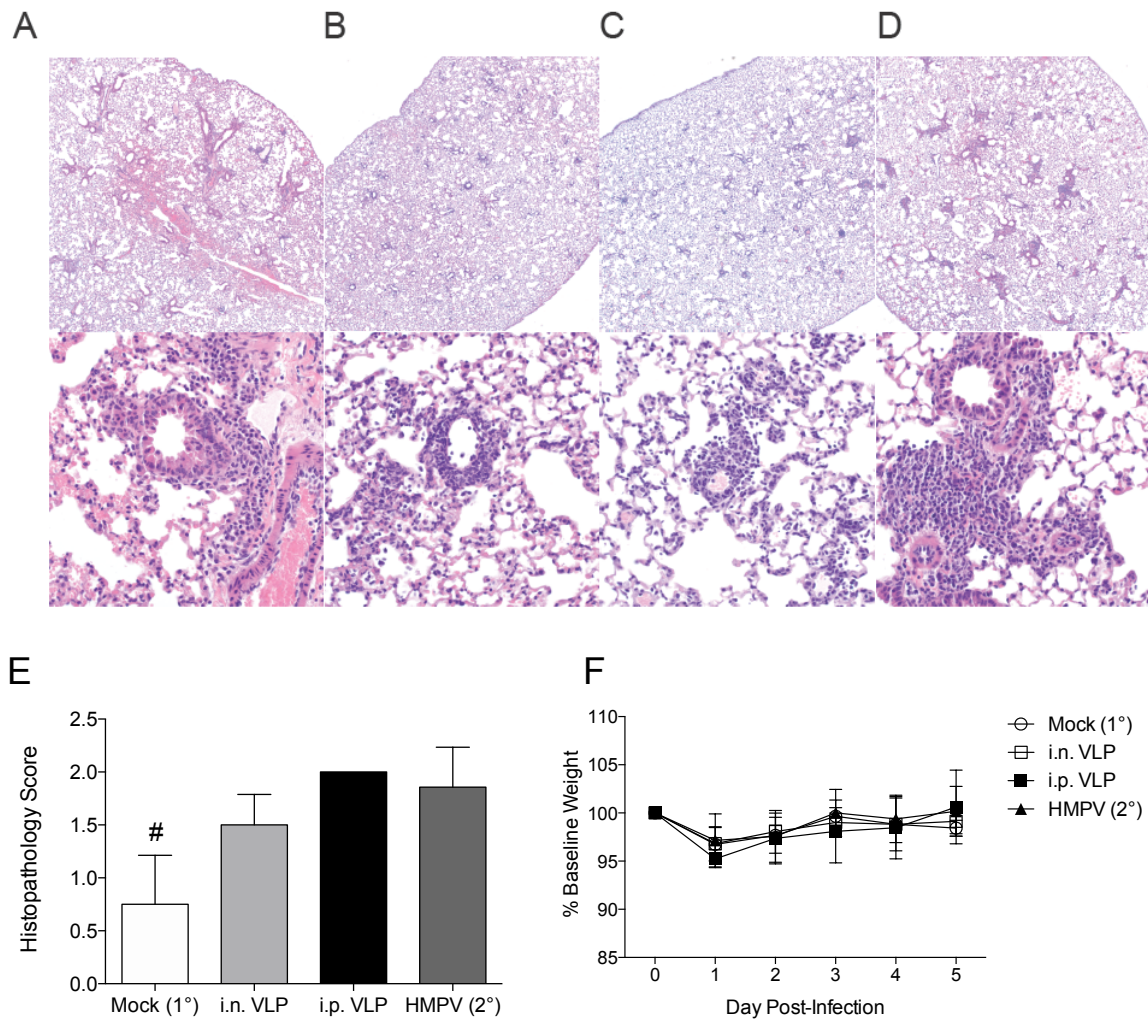


Figure 3-2: VLP vaccination does not exacerbate lung histopathology after HMPV challenge. H&E stain; top row 40x, bottom row 400x magnification. B6 mice were vaccinated with (A) mock VLP, (B) i.n. VLP, (C) i.p. VLP and TiterMax Gold adjuvant, or (D) were infected i.n. with HMPV. Three weeks later, vaccinated mice were given a second dose of VLP. All mice were challenged i.n. with HMPV four weeks after the second VLP dose, and the left lung lobes were collected five days post-challenge for H&E staining. (E) The slides were scored by an experienced pathologist blinded to the composition of the groups.

- 1 – rare perivascular cuffs of lymphocytes and plasma cells 2-5 cells thick
- 2 – lymphoplasmacytic perivascular cuffing of small, medium, and large vessels 5-20 cells thick and sometimes coalescing
- 3 – prominent coalescing perivascular cuffing with interstitial inflammation
- 4 – marked perivascular cuffing with interstitial inflammation and necrosis

(F) The mice were weighed on each day post-infection, and their weights are shown as a % of baseline (day 0).

p<0.05, this group is significantly different from all other groups, one-way ANOVA with Tukey's multiple comparisons test. Data in (E) are combined from two independent

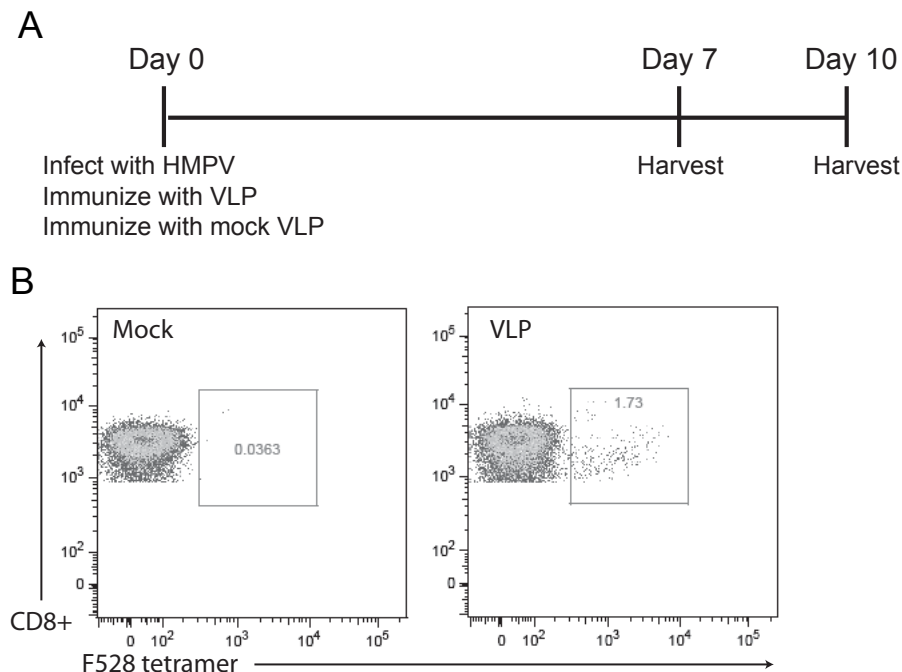
experiments with two to five mice per group per experiment. Data in **(F)** are combined from three independent experiments with two to five mice per group per experiment.

VLP vaccination elicits functional HMPV F-specific T_{CD8}.

To determine whether VLPs are able to elicit a T_{CD8} response after a single dose, I vaccinated mice with i.p. VLPs/TMG or mock VLPs/TMG, i.n. VLPs, or infected them i.n. with HMPV (Figure 3-3A). Seven and ten days later, lungs and spleens were processed to quantify the HMPV-specific T_{CD8} response via flow cytometry (Figure 3-3B). Previously, our group had identified several T_{CD8} epitopes in different HMPV proteins (93). In B6 mice, F₅₂₈₋₅₃₆ (F528) is an immunodominant epitope and M₉₄₋₁₁₂ (M94) is a subdominant epitope. VLP vaccination (i.p. and i.n.), and HMPV infection elicited F528-specific T_{CD8} in both lung and spleen detectable by tetramer assay, but mice that received mock VLPs i.p. did not show a F528-specific T_{CD8} response (Figure 3-3C and data not shown). Mock VLPs given i.n. did not elicit F528-specific T_{CD8} (data not shown). Surface staining for CD107a, a marker of degranulation (214) and intracellular cytokine staining for IFN γ showed that >50% of the F528-specific T_{CD8} were functional whether induced by VLPs or virus (Figure 3-3D). Whereas there was no significant difference between the groups in terms of the percentage of functional cells, the IFN γ mean fluorescence intensity (MFI, a measure of the amount of cytokine produced) was higher in the VLP groups than the HMPV group on day 10 (Figure 3-3E). For the M94 epitope, only the HMPV-infected group had detectable levels of tetramer+ T_{CD8} (data not shown). This finding may be because HMPV contains more M protein than the VLP preparations, or because HMPV M protein is more efficiently processed and presented on MHC class I during viral replication.

A lower percentage of F528-specific T_{CD8} in lungs of i.p. VLP-immunized mice expressed surface PD-1 compared to i.n. VLP-immunized and HMPV-infected animals on days 7 and 10, whereas i.n. VLP-immunized animals had a lower percentage of PD-1

positive T_{CD8} than infected animals only on day 10 (Figure 3-3F). The difference between i.n. and i.p. vaccination was likely due to the higher lung antigen load after i.n. vaccination (since the VLPs were given directly into the respiratory tract), as previous studies showed that higher antigen load correlated with greater T_{CD8} impairment (215, 216). PD-1 levels in the spleens of all three groups were low (<10%) and not significantly different (data not shown), consistent with previous results (93). With regards to the other inhibitory receptors important for T_{CD8} functional impairment (108, 110, 217), infected mice had the highest levels of lung T_{CD8} TIM-3 on both days 7 and 10 (Figure 3-3G). The percentage of PD-1 positive T_{CD8} in VLP-vaccinated mice correlates with the higher IFN γ MFI seen in Figure 3-3E, but the differences between i.n. and i.p. VLP vaccination suggest that the route of vaccine administration plays a role in these different results. Together, these results indicate that VLP-vaccination elicits functional T_{CD8} specific for the dominant F528 epitope. T_{CD8} in VLP-vaccinated mice had lower expression of the inhibitory receptors PD-1 and TIM-3 than those of infected mice, especially on day 10, which corresponds to a higher IFN γ MFI.



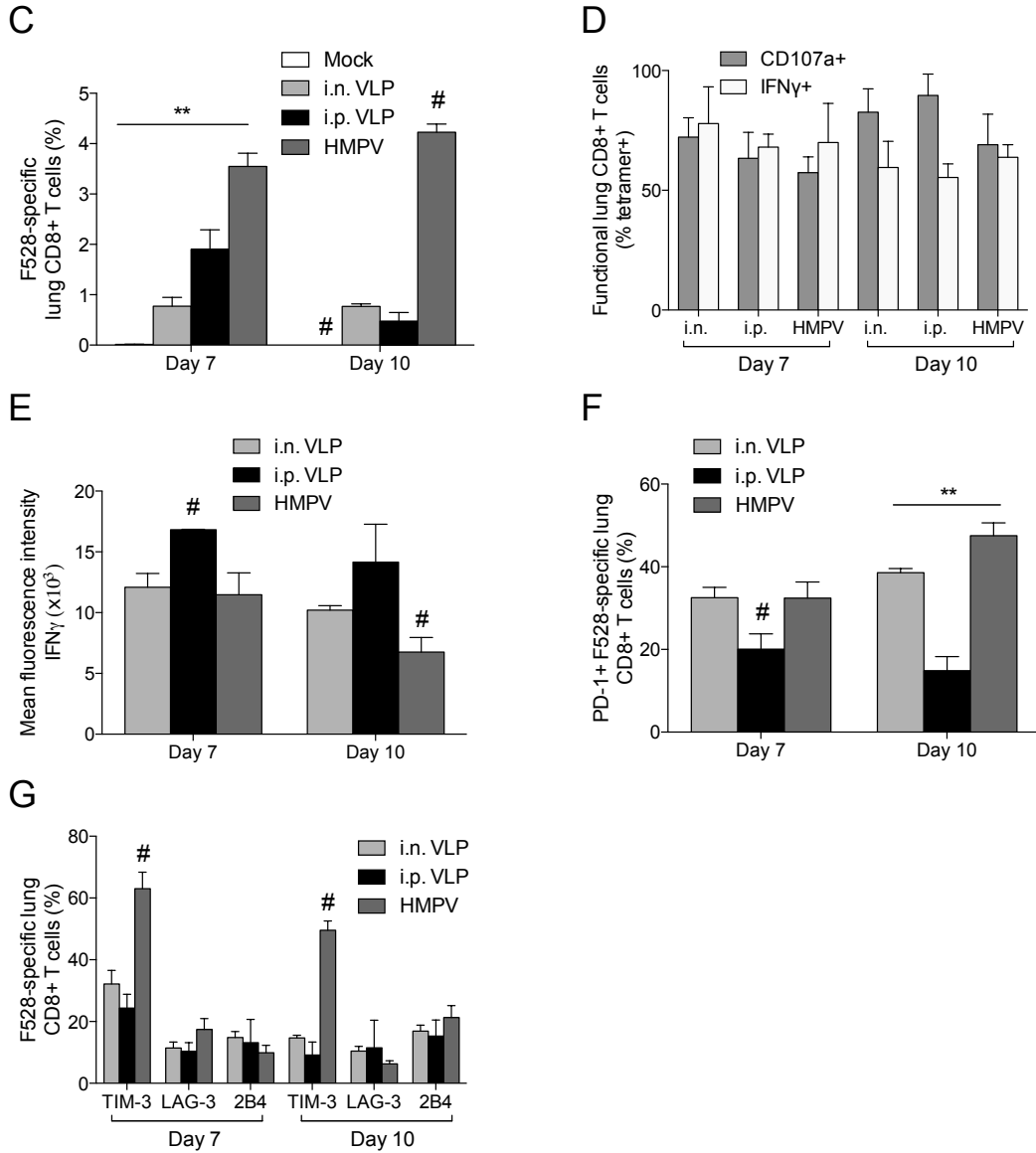


Figure 3-3: VLP vaccination generates functional HMPV F-specific T_{CD8}.

(A) B6 mice were given one dose of mock VLPs, i.n. VLPs, i.p. VLPs + TMG adjuvant or HMPV (i.n.). Lungs were harvested 7 and 10 days later (B) for flow cytometric analysis.

(C) % of total T_{CD8} that recognize the F528 epitope is shown, (D) as well as the % of degranulating (CD107a+) and IFNγ+ T_{CD8} as a % of total tetramer+ cells. (E) Mean fluorescence intensity of IFNγ+. (F) Expression of PD-1 and (G) other inhibitory receptors on lung F528-specific T_{CD8}.

**p<0.05, all groups are significantly different from each other, one-way ANOVA with Tukey's multiple comparisons test.

p<0.05, this group is significantly different from all other groups, one-way ANOVA with Tukey's multiple comparisons test. Data are combined from three independent experiments with three mice per group per experiment. Error bars show standard error of the mean (SEM).

VLP vaccination elicits functional HMPV M-specific T_{CD8}.

We questioned whether the lack of a detectable T_{CD8} response to the subdominant M94 epitope in VLP-vaccinated B6 mice was because VLPs did not elicit M-specific T_{CD8}, or if my assay was not sensitive enough to detect a subdominant response after one dose of VLPs. To distinguish between these two possibilities, I repeated the experiment described in Figure 3-3 using B6-Kb⁰Db⁰-B7.2 transgenic (B7tg) mice, which have T_{CD8} restricted by human HLA-B*0702 (182) and recognize M₁₉₅₋₂₀₃ (M195) as an immunodominant HMPV epitope (93).

One dose of VLPs was sufficient to elicit M195-specific T_{CD8} in mice, with the i.n. VLP group producing a higher response than the i.p. VLP group (Figure 3-4A). The HMPV-infected group had the highest percentage of M195-specific T_{CD8}. Functionally, the i.n. VLP group had the highest proportion of degranulating and IFN γ -producing T_{CD8} (Figure 3-4B). Both VLP groups had a higher IFN γ MFI in M195-specific T_{CD8} than the HMPV group (Figure 3-4C). The percentage of PD-1-expressing M195-specific T_{CD8} was lower in both VLP groups than the HMPV-infected group on day 10, whereas only that of the i.p. VLP group was lower on day 7 (Figure 3-4E). With regards to other inhibitory receptors, the HMPV-infected group had the highest levels of TIM-3 (Figure 3-4F). This higher level of inhibitory receptor expression in HMPV-infected mice correlated with the lower IFN γ MFI shown in Figure 3-4C. These data indicate that VLP-vaccination elicits functional T_{CD8} specific for the M195 epitope with lower inhibitory receptor expression than those of infected mice.

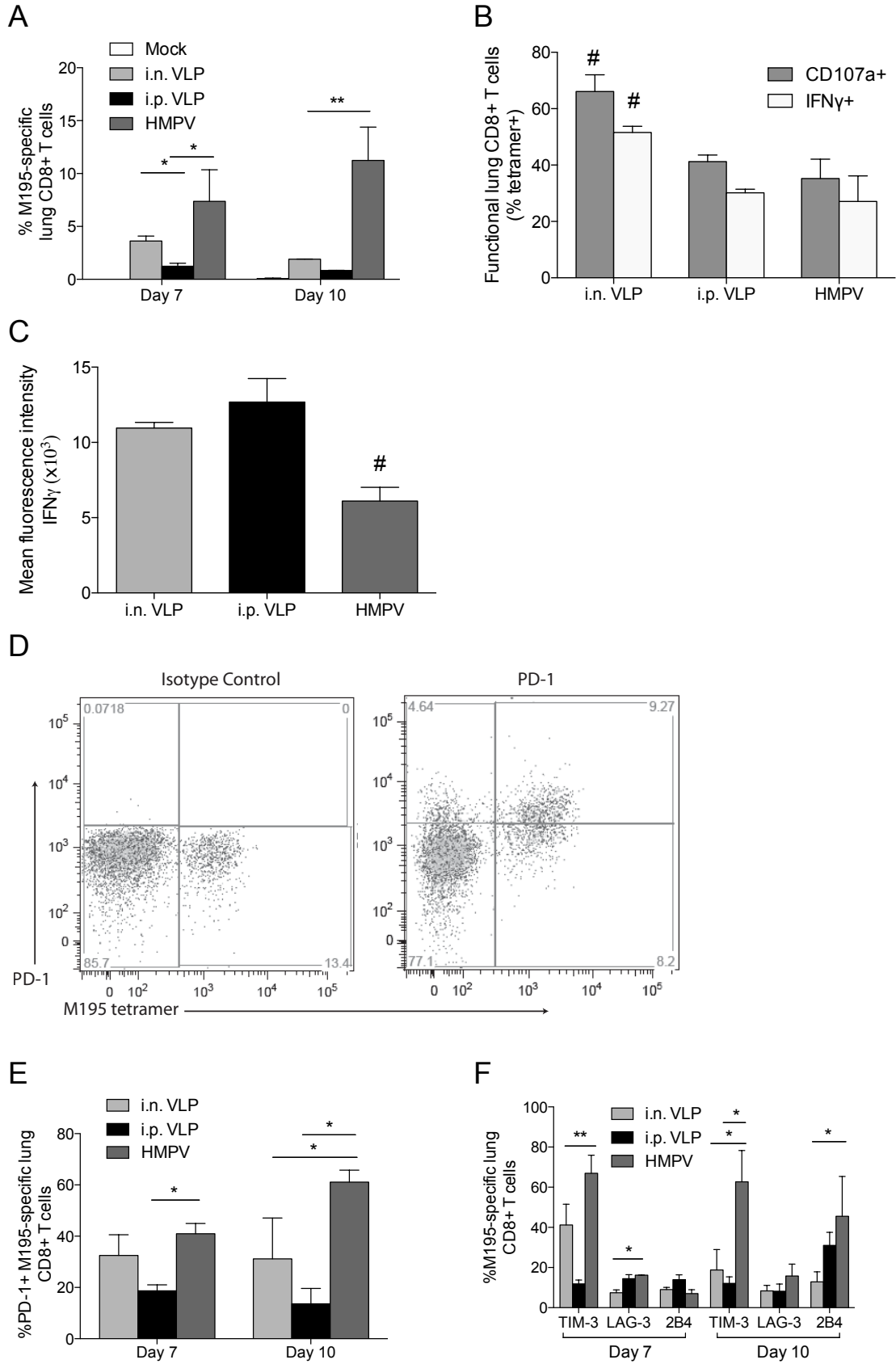


Figure 3-4: VLP vaccination elicits functional HMPV M-specific T_{CD8}.

(A) B7tg mice were given one dose of mock VLPs, i.n. VLPs, i.p. VLPs + TMG adjuvant, or HMPV (i.n.) and lungs were harvested 7 and 10 days later for flow cytometric analysis. % of total T_{CD8} that recognize the M195 epitope is shown, as well as (B) the % of degranulating (CD107a+) and IFN γ + T_{CD8} on day 7. (C) Mean fluorescence intensity of IFN γ + on day 7. (D) Flow cytometric analysis for PD-1 expression on lung M195-specific T_{CD8}. (E) Expression of PD-1 and (F) other inhibitory receptors on lung M195-specific T_{CD8}.

*p<0.05, one-way ANOVA with Tukey's multiple comparisons test.

**p<0.05, all groups are significantly different from each other, one-way ANOVA with Tukey's multiple comparisons test.

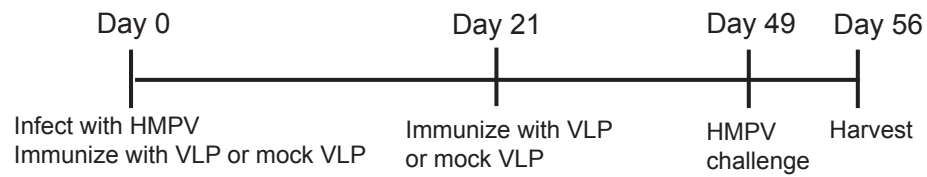
#p<0.05, this group is significantly different from all other groups, one-way ANOVA with Tukey's multiple comparisons test. Data are combined from three independent experiments with three mice per group per experiment. Error bars show standard error of the mean (SEM).

VLP vaccination elicits F- and M-specific T_{CD8} that are similar in function and inhibitory receptor expression to previously infected mice after HMPV challenge.

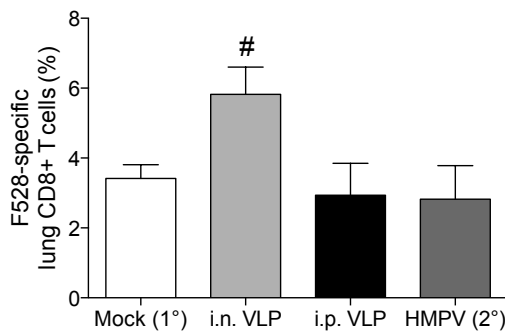
Since one dose of VLPs elicited a lower percentage of PD-1 positive HMPV-specific T_{CD8} than infection, I wondered about the magnitude of the T_{CD8} response after HMPV challenge in VLP-vaccinated mice. B6 mice were given two doses of VLPs (i.n. or i.p.) or mock VLPs, or were infected with HMPV, and challenged four weeks post-vaccination (Figure 3-5A). Seven days post-challenge, all groups had F528 and M94-specific lung T_{CD8} (Figures 3-5B and C). The i.n. VLP group had the highest percentage of F528-specific T_{CD8}, whereas the HMPV group had the highest proportion of M94-specific T_{CD8}. Both VLP groups had a higher absolute number of F528+ T_{CD8} than the other two groups, whereas the HMPV group had the highest number of M94+ T_{CD8} (Figures 3-5D and E). Mock-immunized mice had the highest percentage of functional T_{CD8} for both F528 and M94 epitopes (Figures 3-5F and G). There were no significant differences between the VLP groups and the HMPV group for T_{CD8} IFN γ MFI (data not shown). The percentage of PD-1 expressing F528-specific T_{CD8} was lowest in the mock group, whereas there was no significant difference between the two VLP groups and the HMPV group (Figure 3-5H). There was a similar trend for M94-specific T_{CD8} (Figure 3-

5I). Taken together, these results indicate that while VLP vaccination and prior HMPV infection both enhance the number of memory T_{CD8} responding to HMPV challenge as compared to primary infection (mock group), there is a greater proportion of impaired T_{CD8} in mice that had previously encountered HMPV antigen, either in the context of VLPs or previous infection. Furthermore, while VLP vaccination alone (without viral challenge) elicits epitope-specific T_{CD8} that appear to be more functional and less likely to express PD-1 or TIM-3, HMPV challenge overcomes this increased functionality.

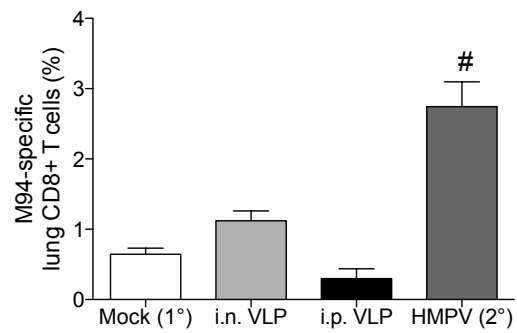
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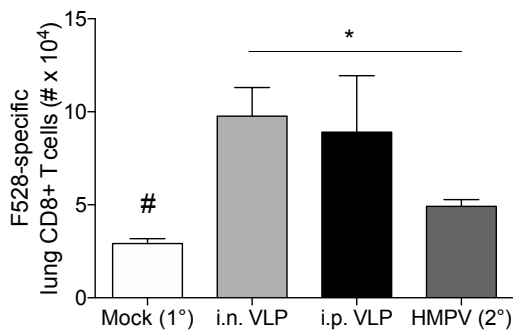
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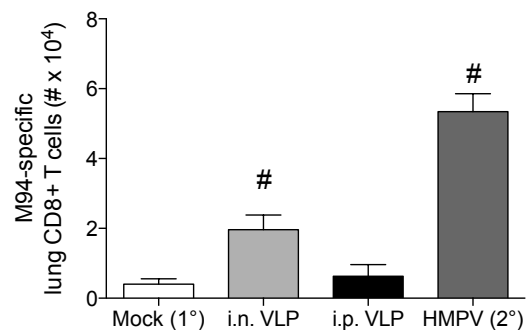
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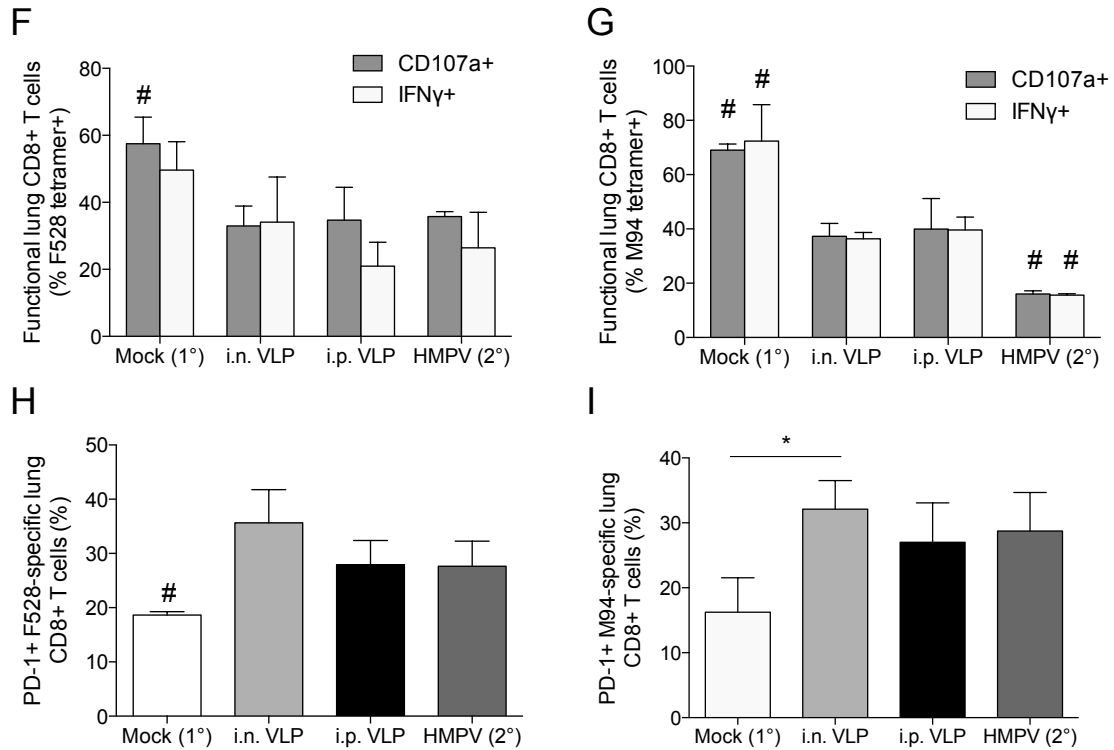


Figure 3-5: VLP vaccination elicits F- and M-specific T_{CD8} that are similar in function and inhibitory receptor expression to previously infected mice after HMPV challenge. (A) B6 mice were vaccinated with i.n. VLPs, i.p. VLPs and TiterMax Gold adjuvant, or i.p. mock VLPs and adjuvant, or were infected i.n. with HMPV. Three weeks later, vaccinated mice were given a second dose of VLPs. All mice (including the mock-vaccinated group) were challenged i.n. with HMPV four weeks after the second VLP dose, and lungs were collected post-challenge for flow cytometric analysis. (B) F528 and (C) M94 tetramer+ T_{CD8} as a % of total T_{CD8} . (D) Number of F528 tetramer+ T_{CD8} , and (E) number of M94 tetramer+ T_{CD8} . (F) % of degranulating (CD107a+) and IFN γ + T_{CD8} as a % of total F528+ T_{CD8} , and (G) % of degranulating and IFN γ + T_{CD8} as a % of total M94+ T_{CD8} . (H) PD-1+F528+ T_{CD8} as a % of total F528+ T_{CD8} , and (I) % PD-1+M94+ T_{CD8} .

* $p < 0.05$, one-way ANOVA with Tukey's multiple comparisons test.

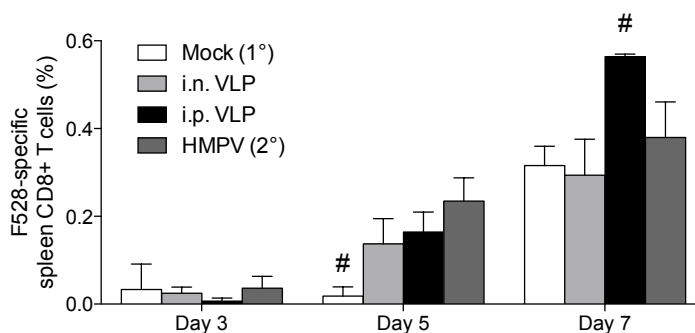
$p < 0.05$, this group is significantly different from all other groups, one-way ANOVA with Tukey's multiple comparisons test. Data are combined from three independent experiments with three mice per group per experiment. Error bars show standard error of the mean (SEM).

Epitope-specific T_{CD8} are impaired in lungs but remain fully functional in spleens post-challenge.

Previously, our group found that after i.n. HMPV infection, T_{CD8} in lungs became impaired starting on day 7 p.i., when only a fraction of lung T_{CD8} responded to antigen by degranulating or producing IFN γ (93, 218). In contrast, splenic T_{CD8} remained fully functional p.i. To determine the T_{CD8} kinetics after vaccination and challenge, I vaccinated B6 mice as shown in Figure 3-5A, then analyzed T_{CD8} tetramer and functional responses on days 3, 5, and 7 post-challenge. F528-specific T_{CD8} increased in both spleens and lungs from day 3 to day 7 post-challenge (Figures 3-6A and B).

In the spleens, I observed a high concordance between tetramer staining and CD107a mobilization or IFN γ production in T_{CD8} on both days 5 and 7 post-challenge (Figure 3-6C). However, in the lungs, T_{CD8} were beginning to show impairment on day 5 post-challenge, and the impairment was more pronounced on day 7, with only 40% or less of tetramer+ T_{CD8} degranulating or producing IFN γ in antigen-experienced mice (Figures 3-5F and 3-6D). Due to the small % of F528-specific T_{CD8} on day 3, I was unable to perform functional assays for that time point. These results, in combination with those shown in Figure 3-5, indicate that a large percentage of pulmonary T_{CD8} in antigen-experienced mice are impaired and fail to respond to antigen. On the other hand, splenic T_{CD8} remain fully functional.

A



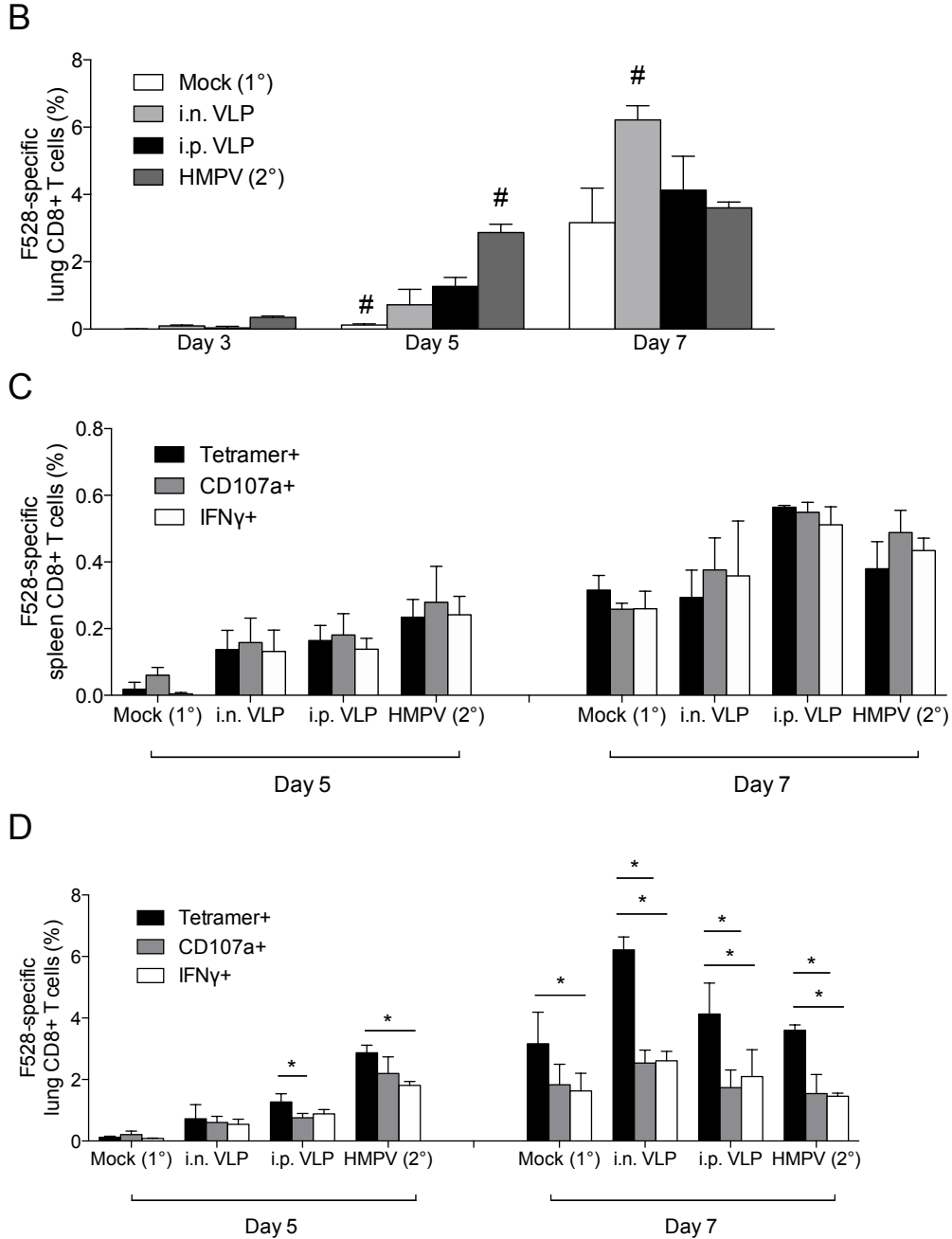


Figure 3-6: Epitope-specific T_{CD8} are impaired in lungs but remain fully functional in spleens post-challenge. B6 mice were vaccinated with VLPs or infected with HMPV, and later challenged with virus, as described in Figure 3-5. Spleens and lungs were collected on days 3, 5, and 7 post-challenge for flow cytometric analysis. F528 tetramer+ T_{CD8} as a % of total T_{CD8} in (A) spleens and (B) lungs. F528 tetramer+, degranulating (CD107a+) and IFN γ + T_{CD8} as a % of total T_{CD8} in (C) spleens and (D) lungs. #p<0.05, this group is significantly different from all other groups, one-way ANOVA with Tukey's multiple comparisons test. *p<0.05, 2-tailed paired *t* test. Data are representative of one experiment with three mice per group. Error bars show standard deviation.

VLP vaccination facilitates HMPV clearance in μ MT mice in the absence of antibodies.

Wild-type B6 mice cannot be reinfected with HMPV, whereas reinfections in humans are common (85, 210). For this reason, I performed additional experiments in μ MT mice, which lack mature B cells and can be re-infected with HMPV. μ MT mice (on a B6 background) and wild-type B6 mice demonstrate similar HMPV replication kinetics in the lungs and nasal turbinates during primary infection (141). However, while wild-type mice are completely protected against viral replication during HMPV challenge, μ MT mice have similar peak titers as in primary infection.

To determine the effect of VLP vaccination in μ MT mice, I immunized them with two doses of i.n. or i.p. VLPs, mock VLPs, or infected them i.n. with HMPV. Four weeks after the second VLP dose, I challenged all animals i.n. with HMPV (Figure 3-7A). While VLP vaccination did not reduce viral titers in the noses or lungs on day 5 post-challenge, by day 7, vaccinated animals had lower viral titers in both noses and lungs. By day 10 post-challenge, most vaccinated animals had cleared HMPV from the lungs, whereas mice in the mock group still had detectable viral titers (Figures 3-7B and C). These results suggest that in the absence of antibodies, VLP vaccination can still facilitate HMPV clearance in the host.

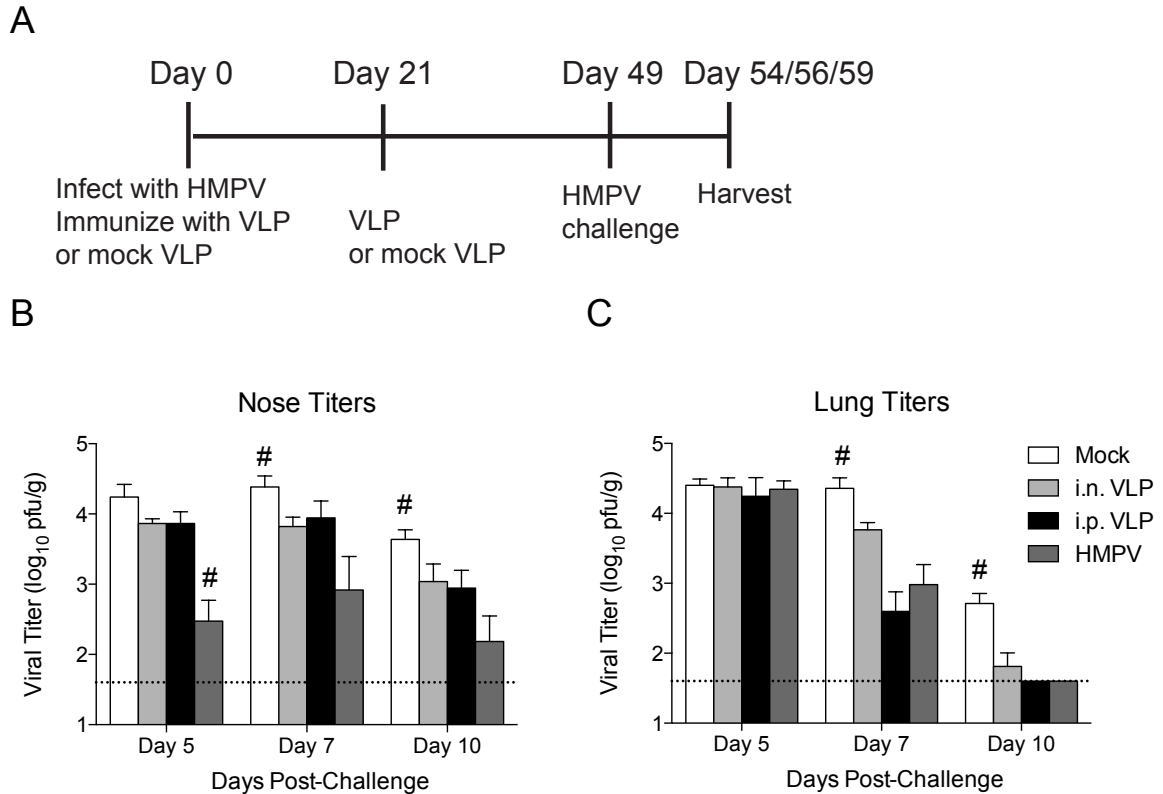


Figure 3-7: VLP vaccination facilitates HMPV clearance in μ MT mice in the absence of antibodies. (A) μ MT mice were vaccinated with i.n. VLPs, i.p. VLPs and TiterMax Gold adjuvant, or mock VLPs, or were infected i.n. with HMPV. Three weeks later, vaccinated mice were given a second dose of VLPs. All mice (including the mock-vaccinated group) were challenged i.n. with HMPV four weeks after the second VLP dose, and tissues were collected post-challenge. (B) Nose and (C) lung titers were calculated.

This group is significantly different from all other groups, with $p < 0.05$ (one-way ANOVA with Tukey's multiple comparisons test). Bars show the means of individual data points combined from two independent experiments with two to four mice per group per experiment. Error bars show standard deviation.

VLP vaccination elicits F- and M-specific T_{CD8} in μ MT mice.

Since T_{CD8} have a known role in clearing viral infections (219, 220), I next sought to determine the magnitude of the HMPV-specific T_{CD8} response. After analyzing lung lymphocytes from μ MT mice on day 7 post-challenge using tetramer and intracellular cytokine staining assays, I found that the VLP groups had the highest percentages and numbers of lung F528-specific T_{CD8} (Figures 3-8A and C), whereas the HMPV group had

the highest percentage and number of M94-specific T_{CD8} (Figures 3-8B and D). Similarly to the results in wild-type B6 mice (Figure 3-5), the mock group had the highest percentage of functional T_{CD8} after challenge for both epitopes (Figures 3-8 E and F).

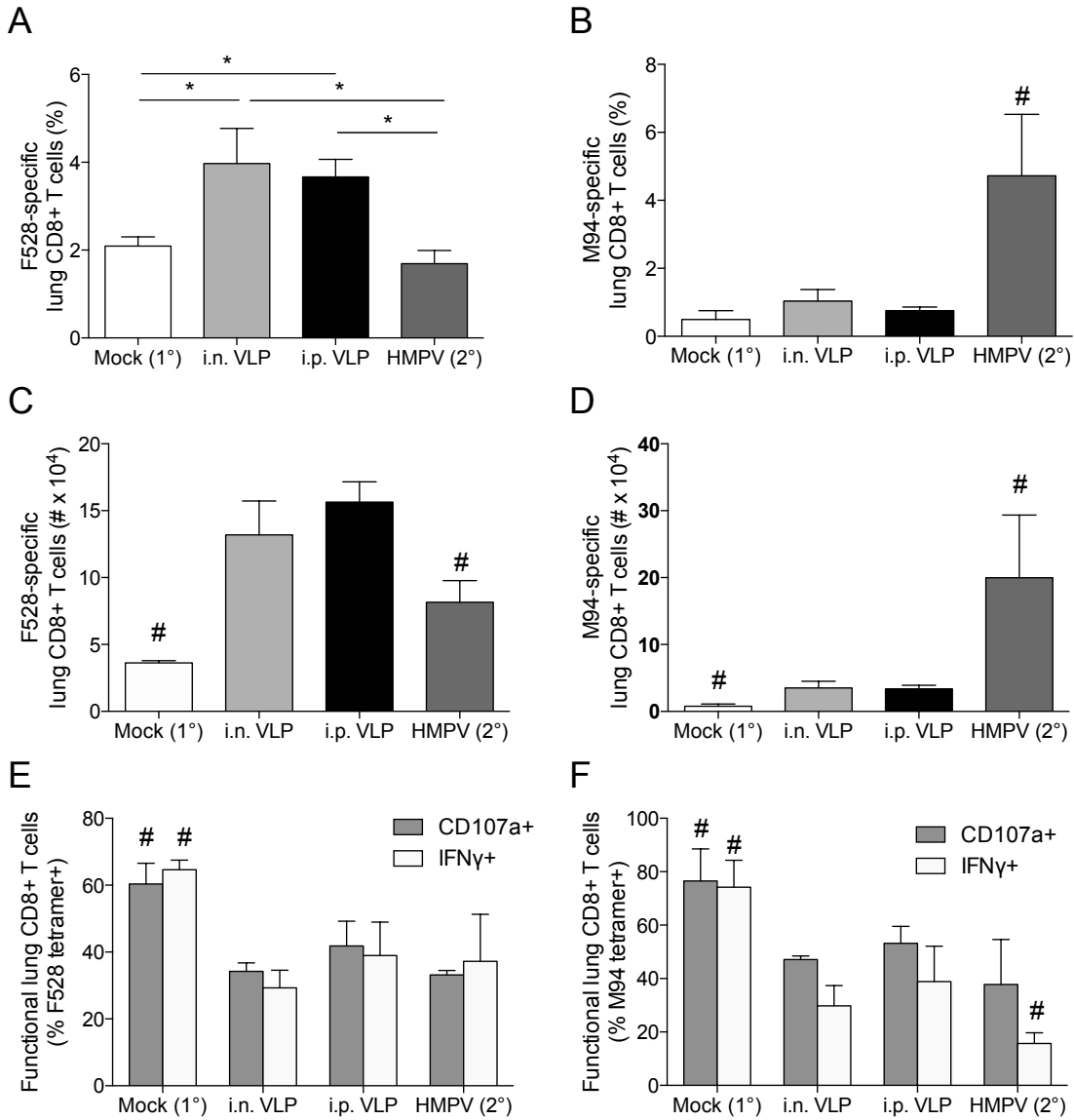


Figure 3-8: VLP vaccination elicits F- and M- specific T_{CD8} in μ MT mice.

μ MT mice were vaccinated with i.n. VLPs, i.p. VLPs and TiterMax Gold adjuvant, or mock VLPs, or were infected i.n. with HMPV. Three weeks later, vaccinated mice were given a second dose of VLPs. All mice (including the mock-vaccinated group) were challenged i.n. with HMPV four weeks after the second VLP dose, and lungs were collected on day 7 post-challenge for flow cytometric analysis. **(A)** F528 tetramer+ T_{CD8} as a % of total T_{CD8}, and **(B)** % of M94 tetramer+ T_{CD8}. **(C)** # of F528 tetramer+ T_{CD8}, and

(D) # of M94 tetramer+ T_{CD8}. **(E)** % of CD107a+ and IFN γ + F528+ T_{CD8}, as well as the **(F)** % of CD107a+ and IFN γ + M94+ T_{CD8}.

*p<0.05, one-way ANOVA with Tukey's multiple comparisons test.

p<0.05, this group is significantly different from all other groups, one-way ANOVA with Tukey's multiple comparisons test. Data are combined from three independent experiments with three mice per group per experiment. Error bars show standard error of the mean (SEM).

T_{CD8} mediate HMPV clearance in μ MT mice.

To determine whether the faster clearance of HMPV in vaccinated animals was due to T_{CD8} or another component of the immune system, I vaccinated μ MT mice with i.p. VLPs+TMG (or mock VLPs+TMG) and injected them with a CD8-depleting antibody or isotype control antibody five days before and on the day of HMPV challenge (Figure 3-9A). T_{CD8} depletion was confirmed by flow cytometry (data not shown). T_{CD8}-depleted mice had higher viral titers than mice treated with isotype control antibody in both noses and lungs (Figures 3-9B and C). In the nasal turbinates, titers in depleted mice were the same as those in mock-immunized mice, suggesting that T_{CD8} were responsible for the decreased viral titer seen in VLP-immunized, non-T_{CD8}-depleted animals. Conversely, while lung titers were lowest in the VLP-vaccinated, isotype antibody-treated group, titers in depleted animals were still lower than those of mock VLP-treated animals. This suggests that in the lungs, T_{CD8} do play an important role in VLP-mediated HMPV clearance, but another component of the immune response (likely T_{CD4}) also contributes.

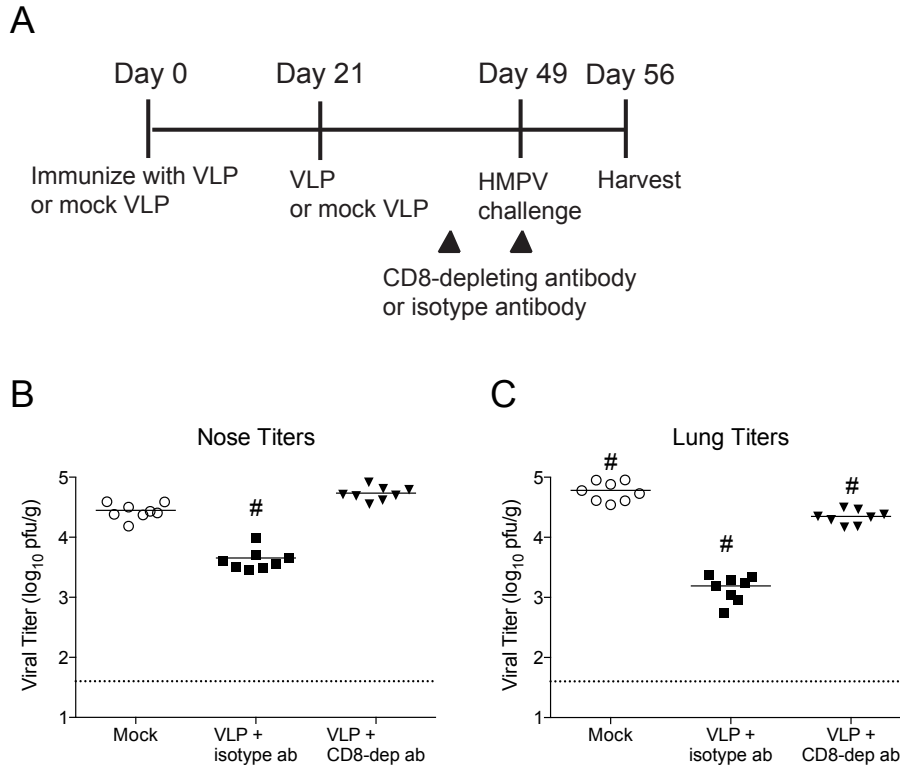


Figure 3-9. T_{CD8} mediate HMPV clearance in μ MT mice.

(A) μ MT mice were vaccinated with i.p. VLPs and TiterMax Gold adjuvant, or mock VLPs and TiterMax Gold. Three weeks later, vaccinated mice were given a second dose of VLPs. i.p. VLP-vaccinated mice were injected with CD8-depleting antibody or isotype control antibody. All mice were challenged i.n. with HMPV four weeks after the second VLP dose, and tissues were collected post-challenge. (B) Nose and (C) lung titers on day 7 post-challenge were calculated.

This group is significantly different from all other groups, with $p < 0.05$ (one-way ANOVA with Tukey's multiple comparisons test). Data are combined from two independent experiments with three to five mice per group per experiment.

VLP vaccination protects DBA/2 mice against heterosubtypic HMPV challenge.

I used the A2 subtype of HMPV to perform all the experiments to this point, as this subtype replicates most efficiently in a mouse model ((93, 211) and unpublished observations). Since clinical HMPV isolates fall into four subtypes, all of which infect humans (221, 222), I decided to determine whether VLP vaccination is protective against challenge with a different HMPV subtype. While the VLPs in my protocol were generated using F and M sequences derived from a clinical isolate of the A2 subtype, there is high amino acid sequence homology to the F and M proteins of different HMPV subtypes.

The Williams lab previously showed that the DBA/2 mouse model is permissive for all four subtypes of HMPV (211). Since the B2 subtype diverges more from the A subtypes than the B1 subtype (223), I vaccinated DBA/2 mice with i.p. VLPs+TMG and challenged them with either the A2 or B2 HMPV subtype using the same protocol as the one shown in Figure 3-1A. Similarly to B6 mice, DBA/2 mice were completely protected from A2 challenge in the lungs and partially protected in the noses (Figures 3-10A and B). In addition, B2 challenged-animals had undetectable lung titers and significantly reduced titers in the noses. My results suggest that VLP vaccination protects mice against different HMPV subtypes.

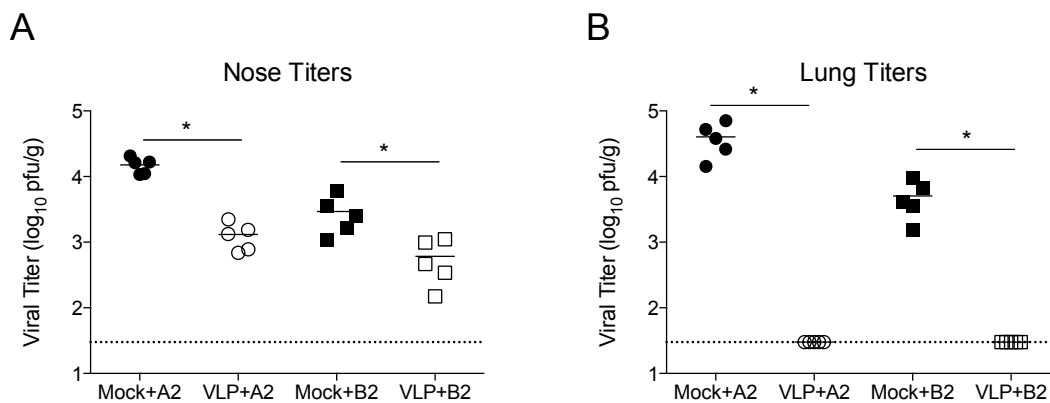


Figure 3-10. VLP vaccination protects DBA/2 mice against heterosubtypic HMPV challenge. DBA/2 mice were vaccinated with i.p. VLPs and TiterMax Gold adjuvant, or mock VLPs and TiterMax Gold. Three weeks later, vaccinated mice were given a second dose of VLP. All mice were challenged i.n. with 10^6 pfu A2 or B2 HMPV four weeks after the second VLP dose, and tissues were collected on day 5 post-challenge. **(A)** Nose and **(B)** lung titers were calculated. Dotted lines represent the limit of detection. * $p < 0.05$, Student's *t* test. Each symbol represents one mouse; small horizontal lines indicate the mean.

Discussion

In this study, I found that VLP vaccination protected mice against HMPV challenge for at least 20 weeks post-vaccination. The protection was complete in the lungs but partial in the noses. This result is probably due to the non-replicating nature of VLP vaccines, as live viruses tend to be more efficient at eliciting mucosal immunity (224). However, since HMPV causes the most severe disease in the lower respiratory tract of humans, and much milder disease in the upper respiratory tract, VLPs are an attractive vaccine candidate for protecting against severe disease.

HMPV VLPs containing F and M proteins can be readily generated (141). While M protein did not elicit neutralizing antibodies, and VLPs containing M protein alone (without F protein) did not protect mice from HMPV challenge, I chose to incorporate M into my VLP vaccine because it enhances VLP yield (RG Cox, unpublished observations). Furthermore, both F and M proteins contain T_{CD8} epitopes (93).

Since HMPV VLPs are fusion competent (225), at least some of the F proteins on VLPs must be in the pre-fusion form. This observation is important since the pre-fusion state of the RSV fusion protein is the target of most neutralizing antibodies in humans (226). Non-neutralizing antibodies have been associated with enhanced respiratory disease by fixing complement and causing tissue damage (124). However, I observed no evidence of enhanced disease in animals vaccinated with HMPV VLPs. Antibodies in vaccinated animals were neutralizing, and lung histopathology revealed no eosinophilia or increased inflammation. In addition, our group previously reported that VLPs elicited a balanced T_{H1}/T_{H2} cytokine response (141).

We found that VLPs elicit T_{CD8} against both F and M proteins after a single dose given either i.n. or i.p. Most vaccines licensed for use in humans are given intramuscularly, including the VLP vaccines for human papillomavirus and hepatitis B virus, but the i.n. route of administration has been used in animal models (227). The

addition of adjuvant increases neutralizing antibody titers when the VLPs are given i.p. (141). Although TiterMax Gold is not licensed for use in humans, other adjuvants have been approved for use in humans, and aluminum adjuvants are a component of both the HPV and hepatitis B vaccines (228, 229).

The mechanism of VLP-induced T_{CD8} responses is unclear. One possibility is that dendritic cells present extracellular antigens on MHC I via cross-presentation (230, 231). Since VLPs are fusion-competent, another possibility is that VLP-cell membrane fusion might deliver HMPV proteins into the cytosol, leading to the presentation of HMPV epitopes onto MHC I molecules. Indeed, studies have shown that VLPs are capable of delivering proteins to the cytosol (232).

In the absence of antibodies, μ MT mice that had been vaccinated or previously infected could be reinfected with HMPV. However, these mice were able to clear virus more rapidly than mice that had not previously encountered HMPV antigens. T_{CD8} depletion resulted in higher viral titers in both nasal turbinates and lungs of mice as compared to their non-depleted counterparts. This result is congruent with previous studies showing that T cell depletion resulted in higher HMPV viral titers (18, 41) and that passive transfer of HMPV-specific T_{CD8} protected mice against HMPV challenge (92). Since μ MT mice, but not wild-type B6 mice, can be reinfected with HMPV, this suggests that while T_{CD8} help to clear virus once an infection had already taken place, antibodies are important in preventing infection, at least in a semi-permissive mouse model.

While VLP-immunized μ MT mice depleted of T_{CD8} had higher lung titers compared to non-depleted mice, their titers were still lower than those of mock-immunized mice. Therefore, it is likely that another component of the immune system plays a role in HMPV clearance in addition to T_{CD8} . I speculate that this contribution is made by T_{CD4} , as both T_{CD4} and T_{CD8} are important in terminating infection by HMPV and

RSV (18, 233). However, HMPV MHC class II epitopes have not yet been reported, limiting the ability to study HMPV-specific T_{CD4}.

While I focused on three HMPV T_{CD8} epitopes, F528, M94, and M195 in the present study, our lab has identified additional HMPV epitopes in B6 mice (unpublished data), and other murine and human epitopes have been reported (91, 92, 162, 234). Both M epitopes analyzed in this study are well-conserved between all HMPV subtypes. F528, which is found in the cytoplasmic tail of the fusion protein, is generally well-conserved but several clinical isolates of other HMPV subtypes show two amino acid sequence changes as compared to the epitope in TN/94-49, the clinical isolate (A2 subtype) used in most of my experiments in this study (235). It remains to be seen whether VLPs containing other HMPV proteins can elicit HMPV-specific T_{CD8} responses.

The F protein expressed on VLPs was derived from an A2 subtype of HMPV; however, VLP vaccination protected mice completely in the lungs from the B2 subtype of HMPV. This result is in concordance with the findings of Levy et al. (160), which showed that vaccinating mice with HMPV F/G VLPs elicited cross-protective antibodies, and of other reports showing that passive antibody transfer protects mice from HMPV infection (83, 236). Furthermore, given that F and M proteins of one HMPV subtype share high amino acid sequence homology with those of different HMPV subtypes, there may also be shared T_{CD8} epitopes between the different subtypes that contribute to heterosubtypic immunity. One study found that T_{CD8} in humans previously infected with HMPV recognized epitopes that were conserved across HMPV subtypes (162).

The lung environment contributes to impaired T_{CD8} function in response to viral infection (95, 237, 238). Previously, our group reported that T_{CD8} responding to HMPV infection are impaired in the lung, but fully functional in the spleen (93). The current study shows that after one dose of VLP vaccine or HMPV infection, a proportion of F528 and M195-specific T_{CD8} were functional, but a substantial percentage of cells in all of the

groups did not degranulate or produce IFN γ . These results suggest that the lung environment regulates T_{CD8} function regardless of whether the mice were exposed to non-replicating VLPs or replicating virus, although the cells responding to HMPV were more impaired as determined by their lower IFN γ MFI.

Since impaired T_{CD8} function in the lung is mediated (at least partially) by PD-1 (93), I determined the inhibitory receptor expression on T_{CD8} responding to vaccination. HMPV-specific T_{CD8} in vaccinated mice expressed lower PD-1 levels than those in infected mice, especially on day 10. This difference could be due to the greater upregulation of PD-1 associated with a longer period of antigen exposure in infected mice due to the replicating nature of HMPV (whereas VLPs are non-replicating). After HMPV challenge; however, there was no longer any difference between VLP-immunized mice and previously-infected mice in the percentage of PD-1-expressing T_{CD8}. T_{CD8} that previously encountered HMPV antigen (whether in the context of VLPs or HMPV) expressed PD-1 in higher percentages than those responding to primary infection. However, despite the presence of impaired T_{CD8} in mice that had previously encountered antigen, there was a higher absolute number of HMPV-specific T_{CD8} in both VLP-vaccinated and previously infected mice as compared to mock-vaccinated mice after viral challenge. Furthermore, mice that had previously encountered antigen were more protected from viral challenge than mock-vaccinated mice.

In summary, my results suggest that VLPs are a promising HMPV vaccine candidate, even though they do not prevent lung T_{CD8} impairment after viral challenge. Vaccination protects mice completely in the lungs and partially in the nasal turbinates against both homosubtypic and heterosubtypic HMPV strains, and elicits neutralizing antibodies and functional HMPV-specific T_{CD8}. Future directions include testing different types of adjuvant and dosing strategies, important considerations for any HMPV vaccine to be used in humans.

CHAPTER IV

SUMMARY AND FUTURE DIRECTIONS

Thesis summary and significance

The focus of my thesis research was the innate and adaptive cellular immune responses to HMPV. In Chapter II, I tested the hypothesis that NK cells contribute to the host immune response by controlling viral replication early post-HMPV infection. While I demonstrated that activated lung NK cell numbers increase post-HMPV infection, and these cells degranulate and produce IFN γ , I found that NK cell depletion did not alter weight loss, peak HMPV titer, or time required for viral clearance. Using CD1d^{-/-} mice revealed that the absence of NKT cells also did not affect viral titers. Thus, my results demonstrate that despite the known value of NK cells during infections by certain viruses, such as MCMV, they are expendable during HMPV infection in mice.

During my initial experiments, the NK cell depleted animals seemed to have more eosinophils upon examination of lung histology, so I reasoned that the absence of NK cells may affect lung inflammation and the T_H1/T_H2 cytokine balance. Upon further investigation; however, this turned out not to be the case. With repeated experiments, there was no difference in eosinophils, perivascular or peribronchiolar inflammation, nor did the absence of NK cells affect the T_H1/T_H2 balance.

In Chapter I, I mentioned that NK cells release cytokines and chemokines that affect other components of the immune system. Since NK cell depletion did not have an effect on viral titers, lung histopathology, or T_H1/T_H2 cytokine balance, I wondered whether there would be a difference in the adaptive immune response. After analyzing HMPV epitope-specific T_{CD8}, total T_{CD8}, and total T_{CD4}, I found no significant differences between NK cell depleted animals and controls. I also measured the number of

CD11c+MHCII+ antigen presenting cells, and found no difference after NK cell depletion as compared to controls.

It is unclear why NK cells are so important for the immune response against certain viruses, but not others. Perhaps the immune response to HMPV is different in mice than in humans, since mice are semi-permissive hosts. If this were the case, I would expect that a mouse-adapted strain of metapneumovirus would have more severe consequences for rodent hosts after infection. In a mouse-adapted model of infection, there might be differences in viral titers between NK cell-depleted and control animals. The HMPV strain I used for most of my experiments in the Williams lab (clinical strain TN/94-49, genotype A2) does not cause signs of clinical disease in C57BL/6 mice even though it replicates to high titer. The other HMPV strains, A1, B1, and B2, do not replicate well in mice, even though they all cause disease in humans.

In my experiments, I did not detect an increased level of eosinophilia in NK cell depleted animals. Most of the literature on RSV infection and NK cell depletion (which I summarized in Chapter I) described experiments performed using BALB/c mice, which are known to be T_H2 -biased. This finding has been attributed to the genetic control of IL-4 production by BALB/c mice, which have T cells that are more likely to exhibit a T_H2 phenotype and lose IL-12 responsiveness than T cells from other strains of mice (239, 240). Performing similar experiments using BALB/c mice might reveal differences in lung eosinophilia and T_H1/T_H2 cytokines. However, BALB/c mice do not express NK1.1, the receptor expressed by NK cells (and a subset of NKT cells) in C57BL/6 mice. Thus, most researchers use anti-asialo GM1 antibody, which also affects basophils and T cells, as I described in an earlier chapter. Therefore, care must be taken when interpreting the results of these studies. If I had used the BALB/c mouse model, it would be difficult to convincingly conclude that the effects I found were solely due to the absence of NK cells.

In Chapter III, I first sought to determine whether VLP vaccination protected mice from viral challenge. I found that both i.n. and i.p. routes of VLP administration protected mice completely in the lungs and partially in the noses. Prior to performing this experiment, I had expected the i.n. VLP-vaccinated animals to have lower titers in the noses. However, that turned out not to be the case. I administered TMG adjuvant with i.p. but not i.n. VLPs, which might explain why i.n. VLPs did not provide better protection. TMG is a viscous, water-in-oil adjuvant that cannot be administered i.n. without killing the mouse. After performing a plaque neutralization assay using serum, I did notice that i.p. VLP + TMG vaccination elicited higher levels of neutralizing antibodies than i.n. VLP vaccination.

Due to the adverse events associated with FI-RSV vaccine, it is important to show that paramyxovirus vaccine candidates do not cause aberrant lung pathology upon challenge. Histological analysis of the lungs of mock VLP-, i.n. VLP-, i.p. VLP + TMG-vaccinated, and HMPV-infected animals (all of which were HMPV-challenged) showed mononuclear perivascular and peribronchiolar inflammation in all groups, with no evidence of eosinophilia. Groups of mice that were previously exposed to HMPV antigen (VLP-vaccinated and HMPV-infected groups) had greater lung inflammation than the mock-vaccinated group, suggesting that there was a memory response in the previously-exposed groups. Additional lung sections for B220, CD3, neutrophils, and major basic protein did not show differences between groups (not shown).

Next, I sought to determine whether VLP vaccination alone could elicit T_{CD8} that recognized specific epitopes in each of the two proteins (F and M) incorporated into the particles. After staining lung lymphocytes of B6 mice that received one VLP dose with fluorophore-labeled tetramers, I detected T_{CD8} specific for the immunodominant F528 epitope, but not for a subdominant M222 epitope. After several trials, I realized that the M222 tetramer was unstable since there was high background staining and the tetramer

stopped detecting any positive cells after a few days. However, even tetramers made for another subdominant epitope, M94, failed to detect epitope-specific T_{CD8}. On the other hand, after performing the same experiment using B7tg mice with the immunodominant M195 epitope, I did detect a tetramer+ response after a single VLP dose. These results show that VLP vaccination induces T_{CD8} responses to both proteins incorporated into the VLPs.

With regards to inhibitory receptor expression after a single dose of VLPs or virus, I found that lung T_{CD8} responding to non-replicating VLPs expressed lower levels of PD-1 and TIM-3 than those responding to replicating virus, especially in the i.p. VLP group. These differences were greater on day 10, when PD-1 expression increased from the level seen on day 7 in HMPV-infected mice but not in VLP-vaccinated mice. In contrast, PD-1 expression on splenic T_{CD8} in all groups remained low as compared to the lungs.

These results suggest that T_{CD8} develop an impaired phenotype in the lung environment, regardless of whether they are induced by non-replicating VLPs or replicating virus. While T_{CD8} in the VLP groups were more functional than those in HMPV-infected animals as measured by their higher IFN γ MFI, there was still a substantial percentage of them that did not degranulate or produce IFN γ , and surface PD-1 expression remained well above that found in the spleen. The i.n. VLP group had higher PD-1 expression than the i.p. VLP group, perhaps due to the higher amount of antigen reaching the respiratory tract, yet even the lung T_{CD8} responding to i.p. VLP expressed higher PD-1 than spleen T_{CD8}.

Although the PD-1/PD-L pathway was initially thought to cause impairment only during chronic infections, it also impairs T cells during acute infections, particularly of the respiratory tract (93, 241). It would be interesting to determine whether this pathway also

functions in other critical organs such as the CNS, heart, or the GI tract to limit acute immune responses. Notably, PD-1/PD-L signaling is important in controlling inflammatory damage to the CNS (242), and there is some evidence that this pathway is associated with survival during acute CNS infection (243).

When I challenged mice with HMPV, T_{CD8} in all antigen-experienced mice (i.e., VLP-vaccinated and previously-infected mice) expressed higher levels of PD-1 and had lower function than T_{CD8} in mice undergoing primary infection. This was true in both wild-type B6 mice and in μ MT mice. Despite this impairment, in both types of mice, VLP-vaccination was just as efficient as previous HMPV infection in limiting lung viral replication upon challenge.

The PD1:PD-L pathway may have evolved to limit immune-mediated damage to critical organs during inflammation. DiNapoli et al. found that infecting mice intranasally or intradermally with the same virus elicited impaired T_{CD8} in the lungs of all mice, even though the intradermal route of infection resulted in no infectious virus in the lungs (95). Splenic T_{CD8} were more functional than lung T_{CD8} in all mice regardless of the route of infection. The decreased functionality of lung T_{CD8} could be due to a combination of factors, including upregulation of inhibitory factors and their ligands (93), suppressive effects of alveolar macrophages (244, 245), or decreased calcium flux (246).

PD-1 functions to promote peripheral tolerance and helps to prevent many autoimmune diseases, such as experimental autoimmune encephalitis. $PD1^{-/-}$ mice eventually develop glomerulonephritis (C57BL/6) or dilated cardiomyopathy (BALB/c) (247, 248). One reason why PD-1 is upregulated further when T_{CD8} re-encounter the same antigen is that signaling through the TCR drives demethylation of the promoter region of *Pdcd1*, the gene encoding PD-1 (249). This epigenetic modification results in antigen-experienced cells rapidly re-expressing PD-1 upon antigen exposure.

To confirm that the enhanced viral clearance in VLP-vaccinated μ MT mice was due to T_{CD8} and not another type of cell, I challenged VLP-vaccinated, T_{CD8} -depleted mice and found that they had similar nose titers as mock-vaccinated control animals and higher nose titers than vaccinated, non-depleted animals. This suggests that in the nose, T_{CD8} contributes to the decreased titer seen in vaccinated mice. On the other hand, VLP-vaccinated, T_{CD8} -depleted animals had lung titers in between those of mock-vaccinated and VLP-vaccinated, non-depleted mice. This result suggests that while T_{CD8} play an important role in decreasing lung viral titer, other cell type(s) are also involved. I hypothesize that T_{CD4} also function to decrease lung titers in μ MT mice.

T_{CD4} are known to contribute protective immunity following acute and chronic viral infections by recruiting key lymphoid cells to sites of infection or secondary lymphoid tissue, providing help for expansion or function of effector cells, or directly producing cytokines or mediating cytotoxicity. In fact, T_{CD8} require T_{CD4} help for priming, effector function, expansion, and memory (250, 251). B cells require follicular helper T_{CD4} for high-affinity, neutralizing antibody responses (252). Further examination of T_{CD4} during vaccination and HMPV infection will address an important knowledge gap, which I will discuss in further detail under the Future Directions section.

To confirm that vaccination with VLPs made from F and M of an A2 HMPV strain also protects from viral challenge with a different strain, I vaccinated DBA/2 mice and challenged them with B2 HMPV. As with A2 HMPV challenge, the animals were partially protected in the noses and fully protected in the lungs, suggesting that a VLP vaccine candidate would be able to protect against infection by different HMPV strains. To further develop VLPs as a vaccine candidate for use in humans would require testing the vaccine in other animal models along with different dosages and adjuvants (TMG is not approved for use in humans). However, these results suggest that a single VLP vaccine would provide protection against all subtypes of HMPV.

The results of my thesis work have implications for vaccination strategies against respiratory viruses. T_{CD8} in VLP-vaccinated and subsequently HMPV-challenged μ MT mice were able to clear virus more rapidly from the lungs than mock-vaccinated mice despite their higher PD-1 expression; by blocking the PD-1/PD-L pathway, it is likely that the virus would be cleared even faster. Indeed, Lee et al. showed that PD-1 signaling limited the effectiveness of a peptide RSV vaccine (237). On the other hand, Erickson et al. showed that blocking this pathway resulted in greater breath distention than in control animals (93). Care must be taken to ensure the balance between greater T_{CD8} functionality (and more efficient viral clearance) and immunopathology.

Future directions

VLP vaccines and mechanisms of protection: lessons learned from HPV

Human papillomavirus virus-like particles are generated by the self-assembly of the major capsid protein, L1. There are currently two HPV vaccines licensed for use in humans: Cervarix, a bivalent HPV-16/18 L1 VLP vaccine (GlaxoSmithKline), and Gardasil, a quadrivalent HPV-16/18/6/11 L1 VLP vaccine (Merck and Co). These vaccines elicit high titer anti-L1 antibodies that persist at levels >10 times that of natural infections for at least 48 months (253). In contrast, serum neutralizing antibody titers in natural HPV infections are low. This is thought to be due to the intra-epithelial infectious cycle of papillomaviruses and absence of viremia, resulting in low uptake of antigen by APCs. On the other hand, VLP vaccines are delivered intramuscularly (i.m.), which allows access to draining lymphatics and could explain the higher immune response.

Currently, the relative contribution of neutralizing antibodies and cell-mediated immunity to HPV vaccine efficacy is unknown. VLPs are highly immunogenic, and there have been few vaccine failures (i.e., patients who don't sero-convert) that could be used

to study immune correlates of protection. It is assumed that vaccine protection against HPV infection is mediated by serum neutralizing IgG: anti-L1 IgG is associated with protection against challenge, and passive antibody transfer confers protection in animal models (254). Mucosal IgA may not mediate significant protection since only 50% of vaccinees developed mucosal anti-HPV antibodies (255). On the other hand, patients with antibody immunodeficiency are not more susceptible to re-infection with cutaneous HPVs than healthy subjects, suggesting that antibodies may be sufficient but not necessary for protection (253). If an HMPV VLP vaccine were to be tested in humans, I would expect cell-mediated immunity to play an important role in vaccine-mediated protection, since serum antibody in previously-infected individuals does not prevent re-infection. Further research will be needed to determine the immune correlates of protection, and longer-term surveillance will be required to identify waning immunity.

Other vaccine strategies

As mentioned in a previous chapter, live-attenuated vaccines for HMPV tested in animal models were protective against viral challenge without exacerbating immunopathology. However, attenuated pathogens have the rare potential to revert to a pathogenic form and cause disease in vaccinees. Thus, this would cause safety concerns if such a vaccine were to be used in the populations most at risk for severe HMPV infections: premature infants and the elderly.

Another approach known to elicit both humoral and cellular immunity is DNA vaccination. DNA vaccination consists of a plasmid containing the DNA sequence encoding an antigen of interest. In the case of HMPV, a promising antigen for a DNA vaccine would be F protein, since this protein elicits antibody responses and contains several T_{CD8} epitopes.

DNA vaccine candidates have been extensively studied in the cancer and HIV fields; however, there is currently no DNA vaccine licensed for use in humans (256, 257). This approach offers a number of advantages over traditional vaccine approaches, including high vaccine stability (no need for cold-chain storage), the absence of any infectious agent, and the relative ease of large-scale manufacture. The drawbacks associated with this strategy include low immunogenicity and difficulty in increasing cellular uptake of DNA.

So far, clinical trials show that DNA vaccines are well tolerated and have an excellent safety profile (258). While early DNA vaccine designs failed to demonstrate sufficient immunogenicity in humans, several newer strategies include: adding genes encoding adjuvants to the DNA (e.g. cytokines and chemokines), targeting antigens to DCs via ligands for DC receptors (e.g. CTLA-4, PD-1, and DEC-205), and electroporation to enhance cellular uptake of plasmid DNA (256, 259). Since the initial DNA vaccine clinical trials in the 1990's, a great deal of progress has been made to enhance immunogenicity. Several ongoing clinical trials are investigating this strategy for preventative and therapeutic applications; the results of these trials will be pivotal for providing insight into the progress of DNA vaccines and for designing new vaccines in the future.

The effect of dosage

While I did not test the effect of vaccine dosage in my studies, I would expect different VLP dosages to affect the T_{CD8} response as well as inhibitory receptor expression. Since PD-1 expression increases with TCR activation and antigen exposure, I hypothesize that lowering the VLP dose would decrease inhibitory receptor expression and increase the percentage of functional T_{CD8} .

The contribution of other cell types to HMPV immunity and pathogenesis

Innate lymphoid cells (ILCs) are recently identified members of the innate immune system that communicate with hematopoietic and non-hematopoietic cells in immunity, inflammation, and homeostasis. ILCs are characterized by lymphoid cell morphology, but unlike T and B lymphocytes, they do not express rearranged antigen receptors and do not exhibit antigen specificity (260).

Non-cytotoxic ILCs consist of three groups: group 1 ILCs (ILC1s), group 2 ILCs (ILC2s), and group 3 ILCs (ILC3s). ILC1s produce $\text{IFN}\gamma$, $\text{TNF}\alpha$, and have been implicated in immunity to intracellular bacteria and parasites (Figure 4-1). ILC2s produce $\text{T}_\text{H}2$ -associated cytokines and promote anti-helminth immunity, allergic inflammation, and tissue repair. ILC3s are a heterogeneous population of cells that promote antibacterial immunity, chronic inflammation, or tissue repair.

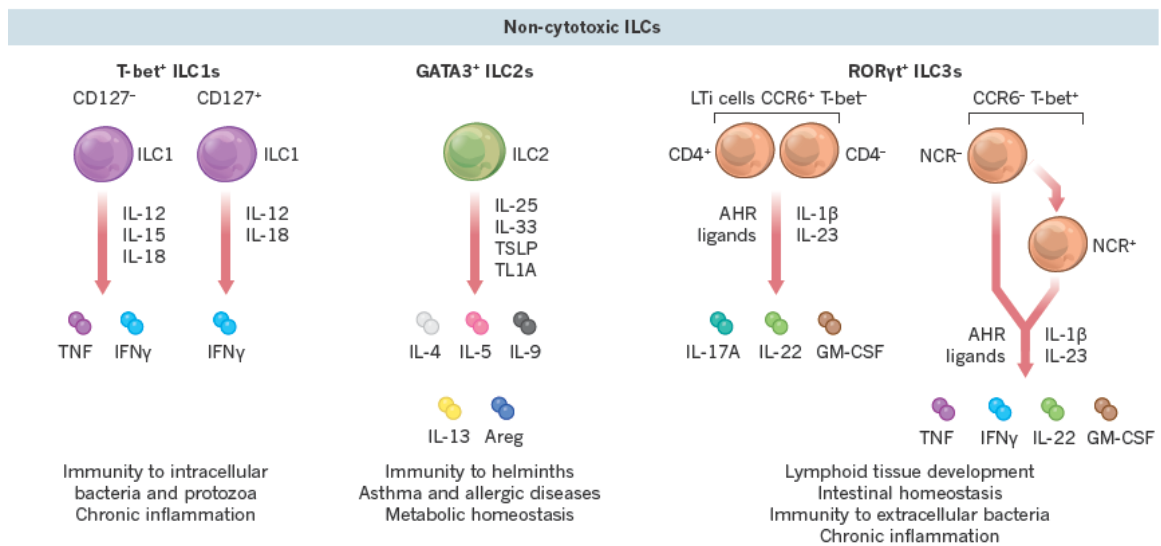


Figure 4-1: Classification of non-cytotoxic innate lymphoid cells. Non-cytotoxic ILCs consist of three groups: group 1 ILCs (ILC1s), group 2 ILCs (ILC2s), and group 3 ILCs (ILC3s). These subsets are defined on the basis of their differential requirements for transcription factors, patterns of cytokine expression, and acquisition of other effector functions. ILCs lack expression of cell-surface molecules that identify other immune cells, and are defined instead as cell lineage marker-negative (Lin^-) cells. Adapted from (261).

ILCs are enriched at barrier surfaces that are common sites of infection, and are poised for rapid activation by cytokines and growth factors. ILC1s and ILC3s promote innate immunity to viruses, intracellular bacteria, and parasites. In addition to mediating innate immune responses to infection, ILCs are also involved in tissue healing and chronic inflammation. Thus, ILC activation can contribute to healing or pathology.

With regards to respiratory viruses, there is a population of lung-resident ILCs that accumulates in the lung after influenza infection (70). ILC depletion leads to loss of airway epithelial integrity, diminished lung function, and impaired airway remodeling. On the other hand, there is some evidence that ILCs contribute to virus-induced asthma exacerbations (262-264). Little is known about ILC function during paramyxovirus infections, although there is some evidence that RSV infection causes an increase in IL-33 expression and ILC2 numbers that may contribute to T_H2 inflammation. It would be interesting to determine the role of ILCs during HMPV infection – do they contribute to respiratory epithelial integrity during infection, or do they exacerbate immunopathology? Further research regarding ILC responses will allow greater knowledge of these immune cells and potentially offer therapeutic strategies in the treatment of a wide range of diseases.

Role of CD4+ T cells in HMPV infection and vaccination

The results of Figure 3-8 show that while VLP-vaccinated μ MT mice depleted of T_{CD8} had higher lung viral titers than non-depleted VLP-vaccinated mice, they still had lower lung viral titers as compared to mock-vaccinated mice after HMPV challenge. This suggests that another component of the immune system contributes to the clearance of HMPV in these antigen-experienced animals. Since μ MT mice are deficient in mature B cells and antibodies, a likely candidate is T_{CD4}.

The contribution of T_{CD4} to HMPV immunity is poorly understood. Depletion of both T_{CD4} and T_{CD8} results in higher HMPV lung viral titers in mice, suggesting that both T cell compartments contribute to viral clearance (18). Furthermore, T_{CD4} produce both T_{H1} and T_{H2} cytokines (IFN γ , TNF α , IL-2, IL-4, IL-5, IL-6, and IL-10) in response to HMPV infection, although HMPV epitope-specific T_{CD4} responses to infection were not analyzed (82).

A key function of T_{CD4} is providing help to T_{CD8}. During chronic infection, T_{CD8} develop more severe exhaustion when T_{CD4} are depleted, suggesting that T_{CD4} can help prevent functional impairment. The role of PD-1 signaling in T_{CD4} during acute respiratory viral infection is unknown. As for other viral infections, PD-1 signaling inhibits T_{CD4} production of T_{H0}, T_{H1}, T_{H2}, and T_{FH} cytokines in the peripheral blood of HIV+ patients, whereas blockade of the PD-1 pathway *in vitro* increases cytokine production (265). Likewise, PD-1 expression on T_{CD4} is correlated with viral load and inversely with absolute T_{CD4} counts, whereas blockade of the pathway augments HIV-specific T_{CD4} count and function (266). During recurrent HPV infection, T_{CD4} isolated from lesions show significantly increased in PD-1 expression, which correlates inversely with cytokine production (267). Blocking PD-1 signaling restores cytokine production.

HMPV MHC II epitopes have not yet been mapped. Challenges of MHC II tetramer staining include the relatively low frequency of T_{CD4} of interest (especially in peripheral blood), and low TCR-MHC avidity (certain antigen-specific T_{CD4} do not stain despite using appropriate MHC class II tetramers) (268, 269). While it would be possible to examine cytokine production and PD-1 expression in the bulk population of T_{CD4}, it would be more revealing to examine HMPV epitope-specific T_{CD4} responses for inhibitory receptor expression and potential functional impairment, as results for T_{CD8} were more significant in epitope-specific cells than in the total population. Since T_{CD4} provide help to B cells, it would also be interesting to examine whether PD-1 signaling blockade affects

antibody generation. Not much is known about the effect of PD-1 signaling on T_{CD4} function (especially during acute infection), so experiments addressing this question would fill an important knowledge gap.

Role of B cells in HMPV infection and vaccination

PD-1 is expressed on T_{CD4} , T_{CD8} , as well as B cells. PD-1 is recruited to the B cell receptor upon activation and functions to inhibit the B cell activation cascade (270). Furthermore, T_{FH} express high levels of PD-1 (271). $PD1^{-/-}$ mice show increased B cell proliferation in response to stimulation, and increased serum levels of IgG2b, IgG3, and IgA (272). Since PD-1 is upregulated during HMPV infection as well as after vaccination, it would be interesting to determine how blocking the PD-1 pathway might alter memory B cell responses and levels of different kinds of antibodies following infection and/or vaccination.

Inhibitory receptor signaling in limiting acute immune responses in other organs

The host immune response to pathogens requires regulation to minimize tissue damage while still achieving defense. During infections, some bystander tissue damage usually happens because of inflammatory reactions or cell destruction. For example, during some infections, immune-mediated tissue damage would be more severe if it were not for host components such as inhibitory receptors, regulatory T cells, and immunosuppressive cytokines such as IL-10. On the other hand, these anti-inflammatory mechanisms could constrain the efficiency of protective immune components.

It was initially thought that PD-1 only functions during chronic antigen stimulation to prevent excess immunopathology. This idea has been revised to include acute infections, especially respiratory tract infections. The lungs are critical organs; excessive inflammation could damage alveolar walls and prevent efficient gas exchange. It is likely

that PD-1 and other inhibitory receptors also function to inhibit excessive inflammation due to acute infection of other critical tissues such as the heart and CNS.

Taking the CNS as an example, the role of the PD-1/PD-L pathway in regulating immune-mediated tissue damage in the CNS during autoimmunity and chronic viral infections is well documented (273-275). On the other hand, the importance of this pathway during acute infection is less clear (276). The work of Phares et al. suggests that T_{CD8} responding to acute CNS infection by neurotropic coronavirus JHM strain upregulate PD-1, with expression increasing over time even after infectious virus had been cleared from the CNS (243). CNS T_{CD8} expressed higher levels of PD-1 than those from spleen or lymph nodes. Blocking PD-L1 with antibodies enhanced $IFN\gamma$ secretion by T_{CD8} ; however, disease symptoms were more severe in infected PD-L1^{-/-} mice. Further research is needed to elucidate the roles of PD-1 and other inhibitory receptors in acute infections.

Conclusions

My thesis work involved studying the NK cell immune response during HMPV infection and the T_{CD8} response following VLP vaccination. I found that NK cells do not play a role in altering viral titer, lung pathology, or cytokine production during acute HMPV infection, suggesting that the immune system has built-in redundancy, at least in a mouse model of HMPV infection. Future directions include elucidating the role of non-cytotoxic ILCs and other innate immune cells during HMPV infection.

My VLP work began to elucidate the contribution of inhibitory receptors to the T_{CD8} immune response following vaccination. VLP vaccination elicited lower lung T_{CD8} inhibitory receptor expression than HMPV infection; however, there was a large percentage of impaired T_{CD8} in both vaccinated and infected mice. Spleen T_{CD8} inhibitory receptor expression was much lower than that of lung T_{CD8} in all groups. Despite these

these impaired T_{CD8} , VLP vaccination completely protected mice from HMPV challenge in the lungs for at least twenty weeks post-vaccination. Further research will elucidate whether there are other critical organs in which acute infection elicits impaired T_{CD8} .

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