

EVASION OF TYPE I IFN SIGNALING BY THE SMALL HYDROPHOBIC PROTEIN OF
HMPV AND THE IMPLICATIONS FOR VIRAL REPLICATION AND PATHOGENESIS

By

Andrew Kenneth Hastings

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Approved:

Louise Rollins-Smith

Sebastian Joyce

Mark Denison

R. Stokes Peebles

John V. Williams

To Katherine, for her unending support and encouragement;

and to my family, who never let me say 'I can't' and
always remind me 'Who I belong to'.

ORIGINAL PUBLICATIONS

1. Erickson JJ, Gilchuk P, **Hastings AK**, Tollefson, SJ, Johnson M, Downing MB, Boyd KL, Johnson JE, Kim AS, Joyce S, Williams JV. Viral acute lower respiratory infections impair CD8+ T cells through PD-1. *J Clin Invest* 2012 Aug 1;122(8):2967-82. PMCID: [3408742](#)
2. Cox RG, Erickson JJ, **Hastings AK**, Becker JC, Craven RE, Tollefson SJ, Boyd KL, Williams JV. Human Metapneumovirus Virus-like Particles Induce Protective B and T cell Responses in a Mouse Model. *J Virol* 2014 Jun;88(11):6368-79. PMID: [24672031](#)
3. Amato KR, Wang S, **Hastings AK**, Youngblood VM, Santapuram PR, Chen H, Cates JM, Colvin DC, Ye F, Brantley-Sieders DM, Cook RS, Tan L, Gray NS, Chen J. Genetic and pharmacologic inhibition of EPHA2 promotes apoptosis in NSCLC. *J Clin Invest.* 2014 May 1;124(5):2037-49. PMCID: [24713656](#)
4. Schuster JE, Cox RG, **Hastings AK**, Boyd KL, Wadia J, Chen Z, Burton DR, Williamson RA, Williams JV. A Broadly Neutralizing Human Monoclonal Antibody Exhibits *In Vivo* Efficacy Against Both Human Metapneumovirus And Respiratory Syncytial Virus. *J Infect Dis* 2015 Jan 15;211(2):216-25. PMID: [24864121](#)
5. Erickson JJ, Rogers MC, **Hastings AK**, Tollefson SJ, Williams JV. PD-1 Impairs Secondary Effector Lung CD8+ T Cells during Respiratory Virus Reinfection. *J Immunol* 2014 Nov 15;193(10):5108-17. PMID: [25339663](#)
6. **Hastings AK**, Erickson JJ, Schuster JE, Boyd KL, Tollefson SJ, Johnson M, Gilchuk P, Joyce J, Williams JV. Role of type I interferon signaling in human metapneumovirus pathogenesis and control of viral replication. *J Virol*, 2015 Feb 4; JVI.03275-14. PMID: [25653440](#)
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The pronoun “we” is used throughout the three data chapters of this thesis (Chapters II, III, IV) to reflect the collaborative nature of the work I present. I am grateful to have had the assistance of fellow graduate students and labmates for many of these experiments, and while I performed the majority of work described, without the assistance of several individuals, this endeavor would not be possible. I would like to acknowledge some of them in the proceeding text, but there is not enough paper in the world to thank all of the people who have given me support and enriched my life through this experience. Ultimately, no good thing that I have done in my life has been under my own power, but only through the will of my God and my Savior, Jesus Christ. When I was young, I would pray for wisdom so that I could understand the wonders of His creation, and I feel blessed and privileged that He has led me into opportunities I could never have dreamed of.

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LIST OF ABBREVIATIONS

2B4	NK cell type I receptor protein (Inhibitory receptor)
4-1BB	TNF-nerve growth factor receptor
Ab	Antibody
Ag	Antigen
AIM2	Absent in melanoma 2
ANOVA	Analysis of variance
APC	Antigen presenting cell
Bcl-#	B-cell lymphoma #
BEAS-2B	Bronchial epithelial cell line
BLIMP-1	B-lymphocyte induced maturation protein-1
BSA	Bovine serum albumin
CARD	Caspase recruit domain
CCR#	C-C chemokine receptor type #
CD-#	Cluster of differentiation-#
CD-#L	Cluster of differentiation-# ligand
CTLA-4	Cytotoxic T-lymphocyte protein 4 (Inhibitory receptor)
CXCL#	Chemokine (C-X-C motif) ligand #
CXCR#	Chemokine (C-X-C motif) receptor #
D ^b	Murine MHC class I molecule H2-D ^b
DC	Dendritic cell
DC-CK-1	Dendritic cell-derived chemokine-1
ELISPOT	Enzyme-linked immunosorbent spot assay
EOMES	Human eomesodermin homolog (TBR2 protein)
F	Fusion protein (HMPV)

FBS	Fetal bovine serum
Fox01	Transcription factor in CD4 ⁺ T cells
FoxP3	Transcription factor important for Treg development
GAS	Gamma-activating sequence
HIV	Human immunodeficiency virus
Hr	Hour
HMPV	Human metapneumovirus
ICOS	Inducible T-cell costimulator
ICS	Intracellular cytokine staining
IFIT	Interferon induced proteins
IFN α	Interferon-alpha
IFNAR	Interferon-alpha receptor
IFNGR	Interferon-gamma receptor
IFN β	Interferon-beta
IFN γ	Interferon-gamma
I κ β	nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor
IL-#	Interleukin -#
IRF#	Interferon response factor #
ISGF3	Interferon stimulated gene factor 3
ISRE	Interferon stimulated response element
ITSM	Immunoreceptor tyrosine-based switch motif
JAK#	Janus Kinase #
K ^b	Murine MHC class I molecule H2-K ^b
LAG-3	Lymphocyte activation gene 3 (Inhibitory receptor)
LCMV	Lymphocytic choriomeningitis virus

LRI	Lower respiratory infection
M	Matrix protein (HMPV)
M2-#	Protein encoded by M2 gene (HMPV)
MAVS	Mitochondrial antiviral signaling protein
MDA5	Melanoma Differentiation-Associated protein 5
MIG	C-X-C motif chemokine (CXCL9)
MIN	Minute
MIP-1 β	Macrophage inflammatory protein-1 beta
MPEC	Memory pre-cursor effector CD8 ⁺ T cell
N	Nucleoprotein (HMPV)
NCR1	Natural cytotoxicity triggering receptor 1
Notch2	Neurogenic locus notch homolog protein 2
NS#	Non-structural # protein (RSV)
OX40	TNF-nerve growth factor receptor
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed Death-1 (Inhibitory receptor)
PD-L1	Programmed Death Ligand-1 (Inhibitory ligand)
PD-L2	Programmed Death Ligand-2 (Inhibitory ligand)
PFU	Plaque forming unit
P.I.	Post-infection
PI3K	Phosphoinositide 3-kinase activates CD8 ⁺ T cells with AKT/mTOR
PIV#	Parainfluenza virus - #
PRR	Pattern recognition receptor
RIG-I	Retinoic acid inducible gene I

RSV	Human respiratory syncytial virus
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
Runx3	Runt-related transcription factor 3
S.D.	Standard deviation
S.E.M.	Standard error of the mean
SH	Small hydrophobic protein (HMPV)
SHP-2	Tyrosine-protein phosphatase non-receptor type 11
STAT#	Signal transducers and activators of transcription #
SLEC	Short-lived effector CD8 ⁺ T cell
TANK	TRAF family member-associated NFκB activator
TBK1	Serine/threonine-protein TANK-binding kinase 1
T _{CD8}	CD8 ⁺ T cell
Tcf-1	T-cell specific transcription factor 1
TCR	T cell receptor
TET	Tetramer (MHC class I)
TGF	Transforming growth factor
TIM-3	T-cell immunoglobulin domain and mucin domain 3 (Inhibitory receptor)
TIR	Toll/interleukin-1 receptor homology domain
TLR#	Toll-like receptor #
TNF	Tumor necrosis factor
TRAF	TNF receptor associated factor
TRADD	TNF receptor type 1-associated DEATH domain protein
TRIF	TIR-domain-containing adapter-inducing interferon-β
Wnt	Wingless integration 1
WT	Wild-type

CHAPTER I

Introduction

Thesis Overview

This thesis uncovers a viral protein responsible for the evasion of type I IFN signaling during human metapneumovirus (HMPV) infection, and examines the repercussions of the lack of type I IFN signaling on the CD8⁺ T cell (T_{CD8}) response *in vivo*. Chapter I gives background into the HMPV genome and life cycle, mechanisms of viral detection and host response, as well as discussing known mechanisms of viral innate immune evasion in HMPV and other related viruses. This chapter also explores the data related to the T_{CD8} effector and memory response, impairment of this effector function during acute viral lower respiratory infection (LRI), and what is known regarding the type I interferon (IFN) contribution to T_{CD8} response. In chapter II, I present evidence that HMPV uses the small hydrophobic (SH) protein to inhibit signal transducers and activators of transcription-1 (STAT1) expression and IFN-induced phosphorylation in infected cells to block downstream anti-viral signaling. Chapter III focuses on the *in vivo* contributions of type I IFN on HMPV replication and pathogenesis and shows that while signaling through the type I IFN receptor (IFNAR) significantly contributes to early viral control, it is dispensable for viral clearance. Additionally, type I IFN is shown to contribute to disease pathogenesis during HMPV infection. I examine the T_{CD8} response in IFNAR-deficient animals and show that the magnitude and function of this subset of cells is impaired in these mice compared to wild-type (WT) mice, despite equal programmed death-1 (PD-1) levels and lower overall programmed death ligand-1 (PD-L1) levels. I also demonstrate that this impairment occurs simultaneously with an upregulation of the inhibitory receptor, T-cell immunoglobulin domain and mucin domain-3

(TIM-3), and a defect in dendritic cell (DC) expansion in the lung. Chapter V is a summary of this thesis project, and I also propose future directions for this work.

Human Metapneumovirus (HMPV) Background

In 2001, investigators in the Netherlands looking for undiscovered viruses in respiratory secretions collected from young children with lower respiratory illness first isolated human metapneumovirus (HMPV) (van den Hoogen et al., 2001a). HMPV particles grown in cell culture were examined by electron microscopy and shown to contain pleiomorphic virus particles with diameters between 150 to 600 nm with spike-like envelope projections of 13 to 17 nm (Figure 1-2A) (Schildgen et al., 2011). Further investigation into the genome using PCR and sequence analysis revealed a single-stranded, negative-sense RNA genome. The closest relative to HMPV, according to sequence homology, is avian metapneumovirus (AMPV), an important respiratory pathogen in chickens and turkeys (Cook, 2000). HMPV was classified as the first human member of the *Metapneumovirus* genus, in the subfamily *Pneumovirinae* of the *Paramyxoviridae* family (Figure 1-1) (van den Hoogen et al., 2002; van den Hoogen et al., 2001a).

A comparison of multiple HMPV gene sequences showed that HMPV diverged from an avian metapneumovirus (AMPV-C) between 200-300 years ago (de Graaf et al., 2008; Yang et al., 2009). Epidemiologic studies showed that this virus has been circulating in human populations without being identified for many years. HMPV-specific antibodies were found in sera from patients in the 1950s (van den Hoogen et al., 2001a), and RT-PCR analysis of lung secretions detected HMPV nucleic acid in specimens from 1976 (Williams et al., 2004).

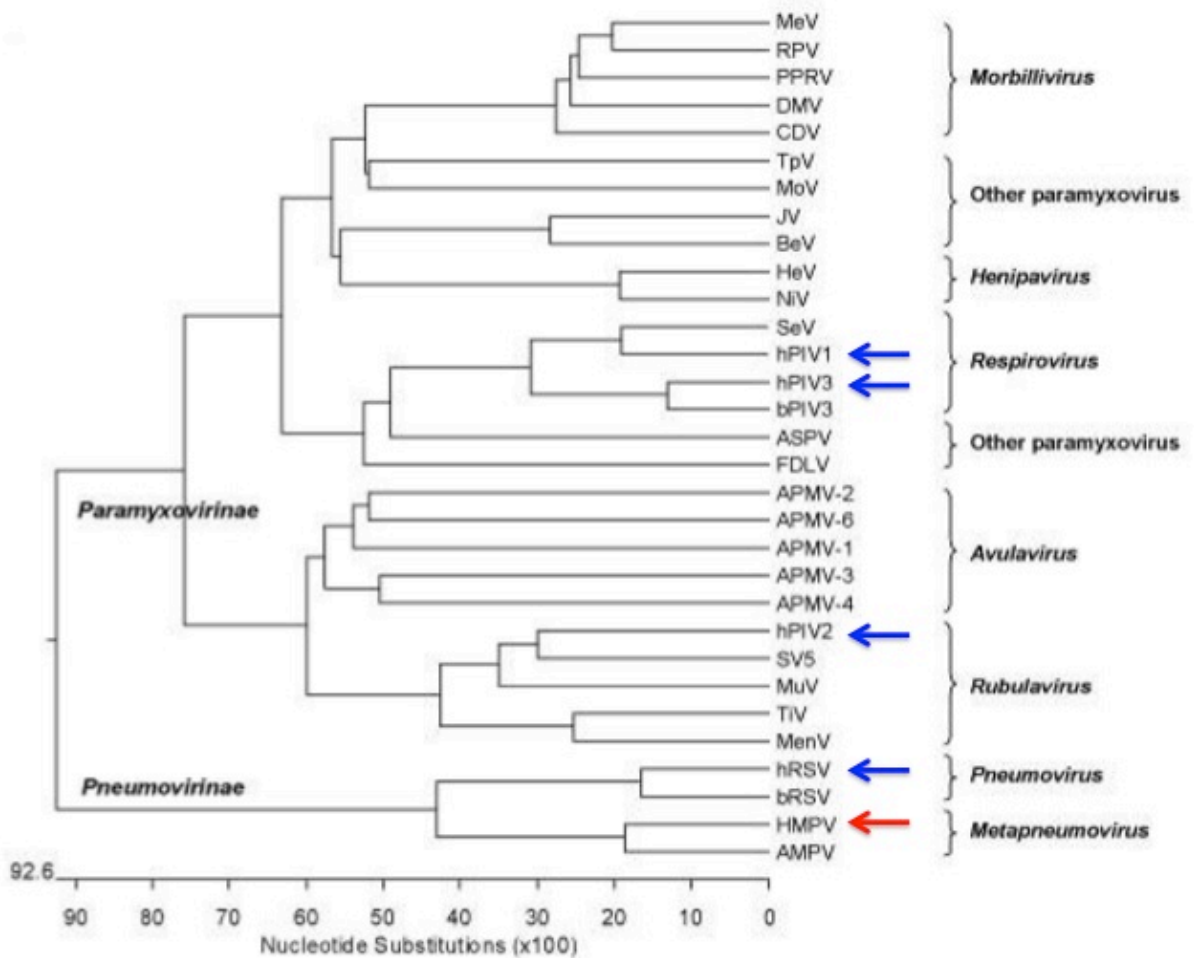


Figure 1-1. Phylogenetic classification of HMPV within *Paramyxoviridae* family. Evolutionary relationships of members classified in the *Paramyxoviridae* family were predicted from multiple nucleotide sequence alignments of whole viral genomes, and a phylogenetic tree was generated using ClustalW program of MegAlign. HMPV (red arrow) can be seen in the *Pneumovirinae* subfamily along with the most closely related human virus, RSV (lowest blue arrow). PIV represents the other paramyxoviruses that are major contributors to acute viral LRI (three top blue arrows). Adapted from (Nayak et al., 2008).

Studies across the globe have shown that HMPV is a leading cause of acute viral lower respiratory infection (LRI) in infants and children in every population (Boivin et al., 2003; Dollner et al., 2004; Ebihara et al., 2004; Esper et al., 2004; Foulongne et al., 2006; Mackay et al., 2006; McAdam et al., 2004; Mullins et al., 2004; Peiris et al., 2003;

van den Hoogen et al., 2003; Williams et al., 2004; Williams et al., 2006a), and immunocompromised hosts or persons with underlying respiratory conditions are at much higher risk for severe disease (Englund et al., 2006a; Larcher et al., 2005; Madhi et al., 2003; Pelletier et al., 2002; Vicente et al., 2004; Williams, 2005; Williams et al., 2005a). Most cases of HMPV in healthy individuals result in a mild upper airway infection, but it is possible to progress to severe lower respiratory tract infections (e.g. bronchiolitis and pneumonia) even in non high-risk populations (Brodzinski and Ruddy, 2009; Williams et al., 2004). Clinical HMPV disease is indistinguishable from closely related pathogens like human respiratory syncytial virus (hRSV) and parainfluenza viruses (PIV), two more leading causes of viral lower respiratory infection in children (Figure 1-1). Infection with HMPV leads to significant inflammation in the respiratory tract, as well as necrosis and subsequent sloughing of the bronchiolar epithelium (Loughlin and Moscona, 2006). Nonhuman primates and small animal models (hamsters, cotton rats, and mice) indicate that replication of HMPV is limited to the upper and lower respiratory tract epithelium, showing that, similar to human infection, HMPV displays a distinct tissue tropism in these models (Hamelin et al., 2006; Kuiken et al., 2004; Williams et al., 2005c).

The genome of HMPV is between 13,280 to 13,378 nucleotides, and contains 8 genes with 9 open reading frames (Figure 1-2C). The virion is similar to other paramyxoviruses, with a viral lipid envelope derived from the plasma membrane of infected cells as they bud from the cell surface. The matrix (M) protein of HMPV lines the inner membrane of the virion, and studded on the outer leaflet are three integral membrane surface glycoproteins, the fusion (F), glycoprotein (G), and short hydrophobic (SH)

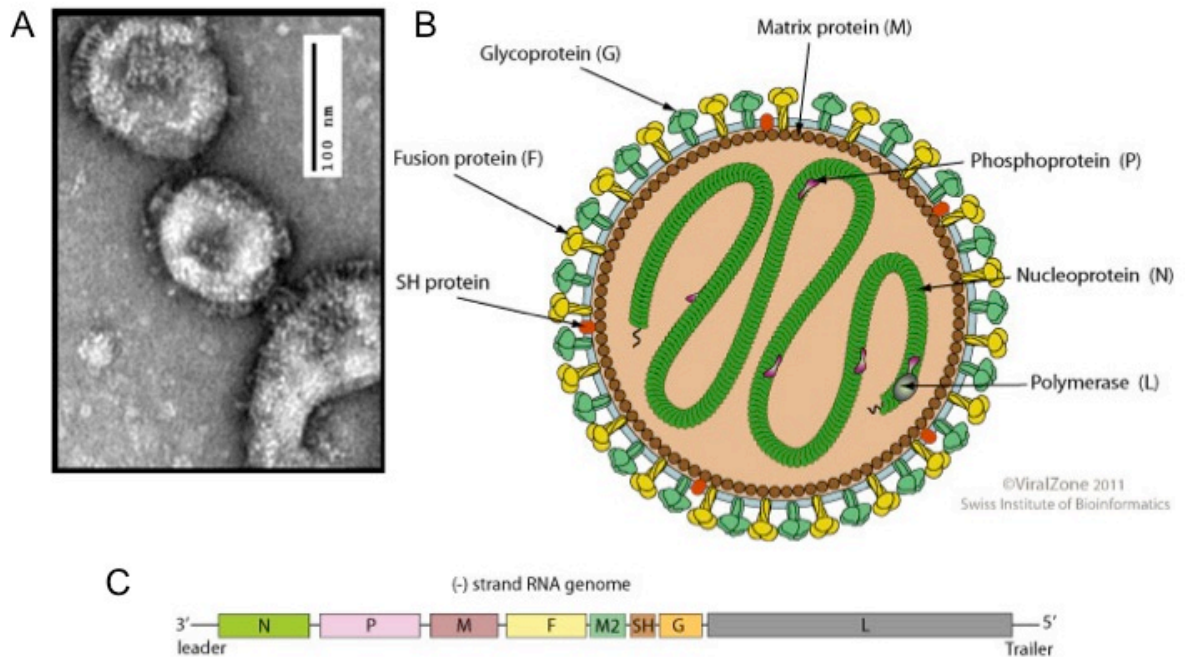


Figure 1-2. HMPV genome structure and virion. (A) Electron micrographs of HMPV were imaged on an FEI Morgagni electron microscope (magnification, $\times 28,000$). Protein spikes can be observed projecting from virion surface. (B) Model of HMPV virion with F, G, and SH proteins studding outer membrane, M protein lining inner membrane, and P, N, and L proteins interacting with (-)-sense single-stranded RNA genome. Not pictured are the non-structural M2-1 and M2-2 proteins. (C) Representation of the ~ 13 kb long (-)-sense single stranded RNA genome of HMPV and its encoded gene products. Adapted from (Cox et al., 2012) and (www.expasy.org/viralzone, Swiss Institute of Bioinformatics)

proteins (Fig. 1-2). Inside the viral envelope, is a helical ribonucleoprotein (RNAP) complex consisting of nucleoprotein (N), phosphoprotein (P), matrix 2 protein (M2), large polymerase protein (L), as well as the single-stranded, negative-sense RNA genome (Figure 1-2B)(van den Hoogen et al., 2002; van den Hoogen et al., 2001b).

Innate Recognition and Type I IFN Signaling

Innate detection of pathogens is facilitated through specialized proteins, termed pattern recognition receptors (PRRs). These molecules are capable of binding to a diverse array of factors specific to microbes, known as pathogen-associated molecular patterns (PAMPs). Viral and bacterial nucleic acid, bacterial carbohydrates,

peptidoglycans and lipoteichoic acids, bacteria-specific amino acids, lipoproteins as well as fungal glucans and chitin all are detected by distinct PRRs (Takeuchi and Akira, 2010). For paramyxoviruses, like HMPV, viral RNA is sensed in endosomal compartments of infected cells by the membrane anchored toll-like receptor 3 (TLR3), and in the cytoplasm by the cytoplasmic helicases retinoic acid inducible gene-1 (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) (Figure 1-3)(Boehme and Compton, 2004; Manuse and Parks, 2010; Matsumoto et al., 2003; Melchjorsen et al., 2005; Rudd et al., 2005). In innate immune cells, like macrophages and dendritic cells, TLR7 and TLR8 are capable of detecting viral nucleic acids in endosomal compartments (Figure 1-3)(Goutagny et al., 2010; Manuse et al., 2010). Detection of very closely related viruses, even within the same sub-family, has been shown to rely on different PRRs (Loo et al., 2008), and, although this phenomenon is poorly understood, evidence suggests that it may be related to the secondary structure of viral nucleic acid or the presence of triphosphates on the 5' end of viral genomes (Hornung et al., 2006; Pichlmair et al., 2006; Saito et al., 2007; Sumpter et al., 2005). HMPV is detected by TLR3 and RIG-I in infected epithelial cells (Banos-Lara Mdel et al., 2013; Bao et al., 2008b; Dou et al., 2010), as well as by MDA5 and TLR7 in cultured DCs (Goutagny et al., 2010).

Upon sensing viral nucleic acids, PRRs transduce signals through adapter molecules. When RIG-I and MDA5 sense PAMPs, the molecule interferon-beta promoter stimulator 1 (IPS-1) interacts with TANK-binding kinase 1 (TBK1) and the inhibitor of kappa B (I κ B) kinase 1 (IKKi) to activate the transcription factors interferon response factor 3 and 7 (IRF3 and IRF7) (Kawai et al., 2005). TLR3 signals through the toll-interleukin receptor (TIR) domain-containing

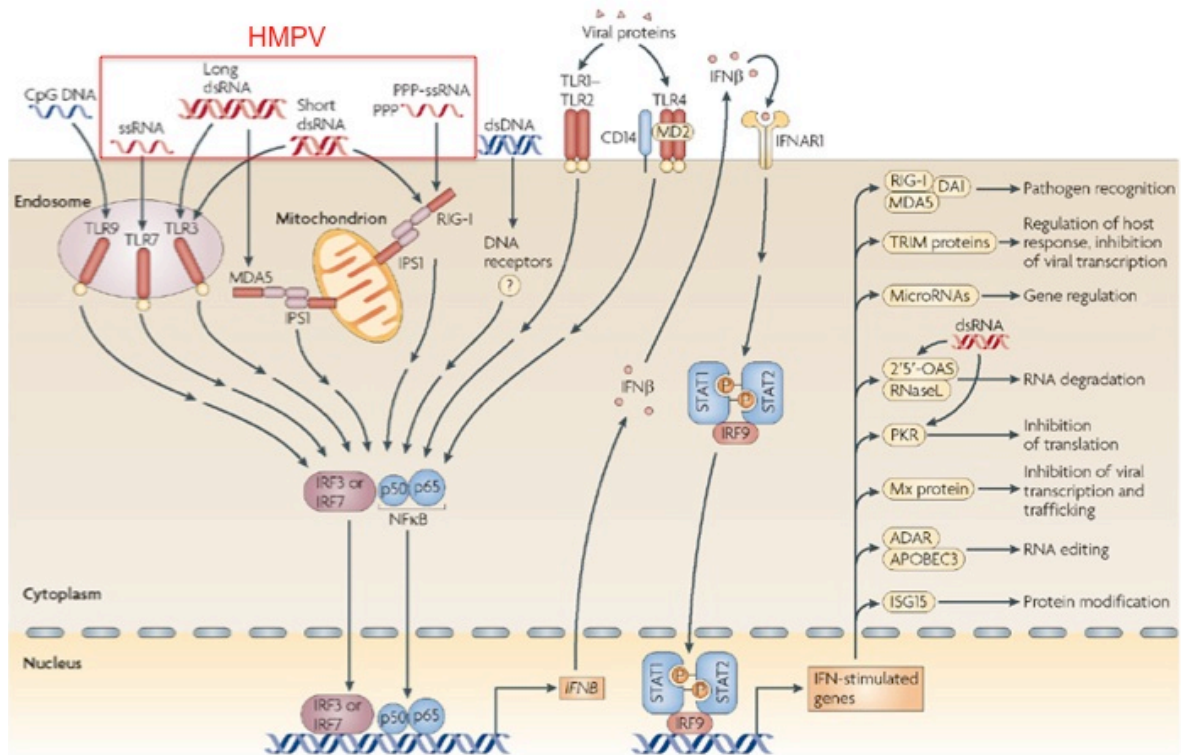


Figure 1-3. Pattern recognition receptors and the type I IFN pathway. PRRs initiate signaling pathways converging at the activation of the transcription factors interferon IRF3, IRF7 and/or NFκB. This leads to the expression of IFNβ, which signals through the type I IFN pathway to produce numerous ISGs. HMPV ssRNA genome and dsRNA replication products are sensed by TLR3/7 in the endosomes of infected or innate immune cells, and RIG-I and MDA5 in the infected cell cytoplasm to trigger IFN production. Adapted from (Bowie and Unterholzner, 2008).

adaptor-inducing IFN-β (TRIF) and tumor necrosis factor (TNF) receptor-associated factor 3 (TRAF3), also utilizing TBK1 and IKKi to activate IRF3 and IRF7 (Kawai and Akira, 2010; Oshiumi et al., 2003; Yamamoto et al., 2003). In TLR7 signaling, the myeloid differentiation primary response protein (MyD88) along with interleukin-1 receptor-associated kinase 4 (IRAK4) and TNF receptor-associated factor 6 (TRAF6) work together to activate IRF7 (Ito et al., 2002; Schoenemeyer et al., 2005).

Both type I and II IFN signaling rely on STAT1 as a crucial component of their signaling pathway. After ligation of IFNα or β to the type I IFN receptor, Janus kinases (Tyk2/JAK1) associated with the receptor cytoplasmic tail to phosphorylate STAT1

(Tyr701) and STAT2 (Tyr689), which together with interferon response factor 9 (IRF9) form a transcription factor, called interferon stimulated gene factor 3 (ISGF3). ISGF3 is capable of binding to promoter elements for cytokines, chemokines and peptides associated with the anti-viral response (Figure 1-3)(Platanias, 2005). For type II IFN signaling, IFN γ binds to the interferon gamma receptor (IFNGR) inducing phosphorylation of STAT1 (Tyr701) by other Janus kinases (JAK1/JAK2) resulting in a homodimer of STAT1 that acts as a transcription factor to bind to the gamma activating sequence (GAS), a promoter for many of the same anti-viral molecules induced by type I signaling (Platanias, 2005). Indeed, type I and II IFN signaling induce an overlapping set of antiviral molecules, and act in combination to produce a cellular antiviral response (Decker et al., 2005; Pestka et al., 2004; Schroder et al., 2004). These signals also prime an efficient adaptive immune response, which will be discussed in detail later (Havenar-Daughton et al., 2006; Kolumam et al., 2005a; Le Bon and Tough, 2008; Luft et al., 1998). The work in this thesis clearly shows that type I IFN signals can contribute to disease pathogenesis (Gautier et al., 2005; Goritzka et al., 2014; Rudd et al., 2007). The further implications of type I IFN signaling in HMPV infection will be explored in chapters III and IV.

Evasion of type I IFN signaling by Paramyxovirus

The type I IFN response exerts a strong evolutionary pressure in the battle between pathogen and host, but viruses are not helpless in this fight. They have evolved varied and effective mechanisms of evading the innate immune response through their viral gene products (Table 1-1). Viruses related to HMPV, such as RSV and PIV, encode one of two classes of proteins responsible for targeting and inhibiting signaling through the innate immune pathways. RSV encodes the nonstructural-1 (NS1) and nonstructural-2 (NS2) proteins and the PIV genome contains an alternative reading frame of the P/C/V

gene that encodes multiple immune evasion proteins. These proteins specifically inhibit aspects of innate immunity in a variety of ways (Andrejeva et al., 2004; Childs et al., 2012; Gainey et al., 2008; Lo et al., 2005). Interestingly, the HMPV genome contains no analogous open reading frames to any of these previously identified as innate immune antagonists (Piyaratna et al., 2011; van den Hoogen et al., 2002). This led us to hypothesize that HMPV is using a viral protein in a unique way to evade the type I IFN signaling pathway.

Paramyxoviruses other than HMPV

Expression of type I IFNs during paramyxovirus infection is dependent on recognition of viral nucleic acids by the cytosolic RNA helicases, RIG-I and MDA5, as well signaling events through adaptor molecules, such as interferon- β promoter stimulator-1 (IPS-1), TNF receptor-associated factor (TRAF) and IRF proteins. Viruses have evolved mechanisms to target virtually every step in this process. Parainfluenza, measles, Sendai and Nipah viruses all encode a V protein, which is capable of binding to MDA5 and blocking the detection of viral nucleic acids (Andrejeva et al., 2004; Childs et al., 2009; He et al., 2002; Poole et al., 2002). Instead, the NS2 protein of RSV has been shown to bind to RIG-I blocking the downstream phosphorylation of IRF3 and the expression of type I IFNs (Ling et al., 2009). An outstanding question in the field is why viruses target one of these RNA helicases over the other, but it is likely related to differences in how the viral nucleic acid of individual viruses are detected by PRRs.

STAT proteins are often targeted by viruses from the *Paramyxoviridae* family to evade innate immune signaling through various mechanisms (Caignard et al., 2007; Takeuchi et al., 2003). The V protein from PIV5 and mumps virus and the NS2 protein from RSV target STAT proteins for proteosomal degradation (Didcock et al., 1999a, b; Kubota et al., 2001; Ramaswamy et al., 2004). Measles virus, by contrast, does not

affect STAT protein expression, but uses the V protein to prevent STAT phosphorylation and therefore block formation of the interferon stimulated gene factor-3 (ISGF3) transcription factor and subsequent translocation to the nucleus (Takeuchi et al., 2003). The Nipah virus V protein binds to STAT proteins to induce high-molecular-weight complexes, which blocks type I IFN signaling (Rodriguez et al., 2004; Rodriguez et al., 2002). The wide variety of mechanisms employed in the inhibition of type I IFN signaling indicates that this pathway presents a substantial evolutionary pressure on the life cycle of *Paramyxovirus*, and thus I hypothesized that a similar evasion mechanism exists for HMPV.

Virus	Viral Protein	Target Protein	Outcome	Reference
PIV-1	C	STAT1	Inhibits nuclear translocation	(Van Cleve et al., 2006)
PIV-2	V	STAT1, STAT2	Degradation of protein	(Andrejeva et al., 2002)
PIV-3	V and D	STAT2	Block formation of ISGF3	(Young et al., 2000)
RSV	NS1 and NS2	STAT1, STAT2	Block nuclear translocation and phosphorylation	(Jie et al., 2011)
	NS2	STAT2	Degradation of protein	(Ramaswamy et al., 2004; Ramaswamy et al., 2006)
HMPV	SH	STAT1	Decreased expression and phosphorylation	(Hastings et al., unpublished)

Table 1-1. Summary of type I IFN evasion by human paramyxoviruses. Multiple mechanisms of type I IFN evasion have been described in the *Paramyxoviridae* family, which target the STAT proteins.

HMPV Innate Immune Evasion

As mentioned previously, HMPV does not encode for the known innate immune antagonists encoded by related viruses, but evidence exists that implicates HMPV gene products in this phenomenon. The phosphoprotein (P), M2-2, the glycoprotein (G) and the SH protein have all been suggested to play a role in the evasion of innate immune responses to HMPV. One group showed a differential ability for the A and B subgroups

of HMPV to induce type I IFN, and suggested that the P protein of the B1 subgroup of HMPV was able to prevent RIG-I activation and the production of IFNs (Goutagny et al., 2010). It is unclear whether this inhibition was due to a direct interaction with host innate immune sensors, or if the P protein's interactions with viral RNA or its effect on the translation of other viral proteins was responsible for this phenotype. The M2-2 protein is not necessary for viral growth (Biacchesi et al., 2005; Buchholz et al., 2005), but data suggest that it might target the important PRR mediator mitochondrial antiviral-signaling protein (MAVS) to prevent innate immune signals by the cytoplasmic helicases RIG-I and MDA5 (Ren et al., 2012). HMPV G protein has been suggested to play a role in the direct inhibition of RIG-I through interactions between the caspase activation and recruitment domain (CARD) of the PRRs N-terminus (Bao et al., 2013), leading to higher levels of inflammatory cytokine production in cells infected with HMPV lacking the G protein. Evidence will be presented in chapter II of this thesis to implicate the SH protein in the evasion of type I IFN through inhibition of STAT1 phosphorylation, and other groups have shown that SH may downregulate TNF α signaling in a cell culture system (Bao et al., 2008a). STAT1 is not a component of canonical TNF signaling, but has been shown to serve as a regulator of activated tumor necrosis factor alpha (TNF-alpha) receptor 1 (TNFR1) recruitment of TNFR1-associated death domain protein (TRADD) and subsequent signaling events (Wang et al., 2000).

CD8⁺ T Cells – Activation and Function during Viral Infection

During viral and intracellular bacterial infections, as well as in the response to cancer, T_{CD8} cells are thought to be primarily responsible for the generation of a protective immune response. These cells are capable of responding to pathogens with clonal expansion and differentiation into a cytotoxic effector cell phenotype. Effector T_{CD8} cells home along a chemokine gradient to infected or tumor tissue and express

molecules capable of clearing infection and eliminating cancerous cells. The activation, differentiation and progression of T_{CD8} to a memory phenotype, capable of responding quickly to future immunological insult, is tightly controlled by a symphony of innate inflammatory responses and antigen presenting cells (APCs) to respond in an appropriate way without damaging host tissues. After initial contact with the pathogen, memory T_{CD8} cells are generated, and can be rapidly reactivated and expand with exposure to a previously encountered pathogen.

During viral infection, the T_{CD8} response begins with rapid clonal expansion, leading to T cell differentiation into an effector phenotype. As the pathogen is cleared, T cells begin to contract via apoptosis of effector T_{CD8} cells, and long-lived memory cells are formed (Figure 1-5A) (Kaech and Wherry, 2007). Specialized APCs scavenge and engulf antigen from the site of infection and subsequently extravasate to secondary lymphoid organs like the spleen and draining lymph nodes where they encounter naïve T_{CD8} cells and prime them for response to the invading pathogen (Zhang and Bevan, 2011). These pathogen-specific T cells begin as around 100-200 naïve precursors that quickly expand 10⁴- to 10⁵-fold (Blattman et al., 2002). Dendritic cells (DCs) are a subset of APC that express high levels of co-stimulatory ligands and are able to efficiently migrate to secondary lymphoid tissues to interact with naïve T_{CD8}. During acute respiratory viral infection, subsets of DCs, specifically those expressing CD103 on the cell surface, have a greater innate ability to prime an anti-viral T_{CD8} response (GeurtsvanKessel and Lambrecht, 2008; Hao et al., 2008; Kim and Braciale, 2009).

More recently, it has been shown that specific DC subsets play specialized roles in the overall development of protective immunity. *Kim et al* showed that during influenza virus infection, CD103⁺ DCs were particularly important for the T_{CD8} effector phenotype, while interaction with CD11b⁺ DCs primed these cells to possess characteristics of

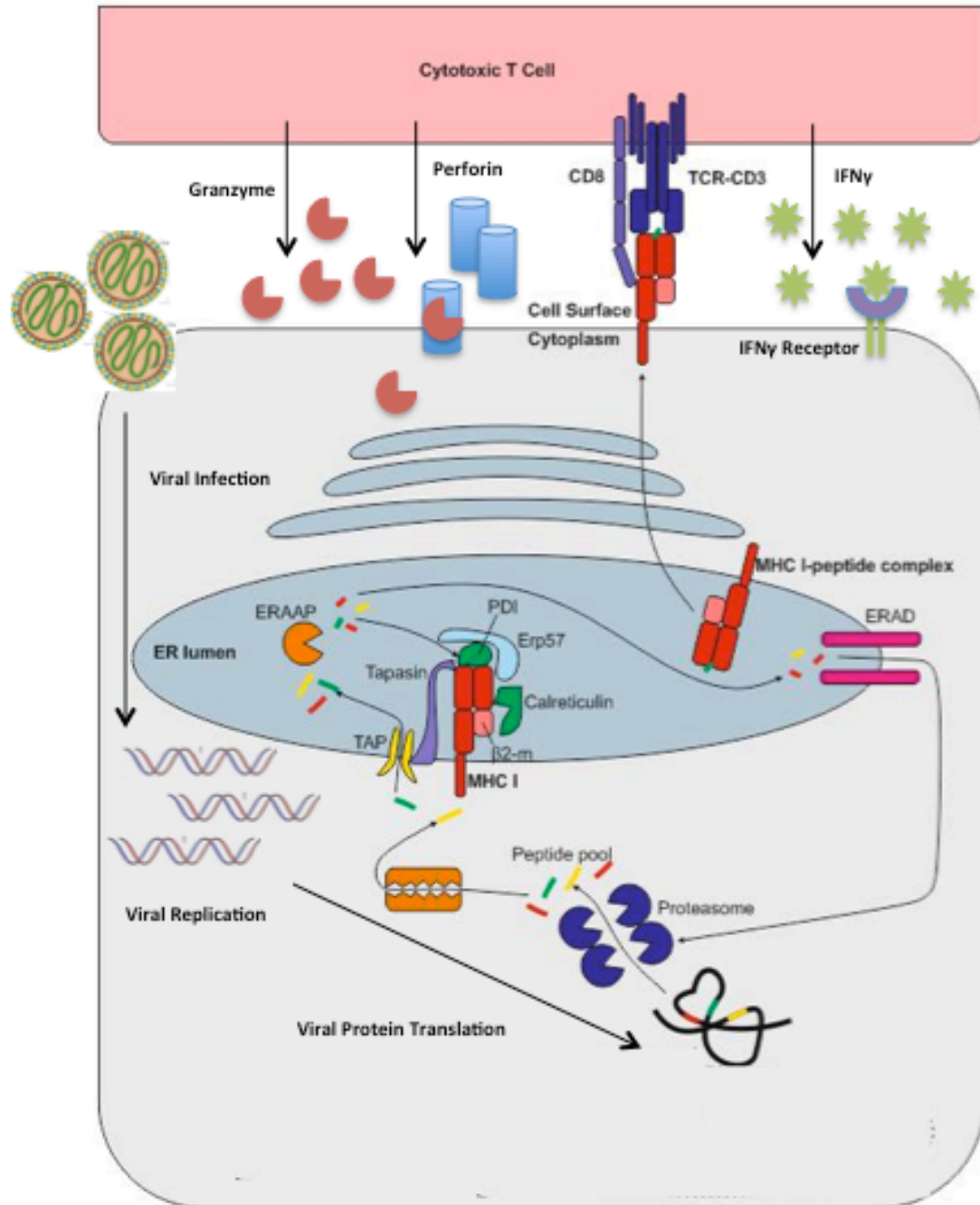


Figure 1-4. T_{CD8} recognition of infected cells and effector mechanisms. Infection of cells triggers the activation of immunoproteasome, which cleaves viral proteins into smaller fragments. These fragments are imported into the ER by the TAP protein, where further processing occurs and viral peptides 8-10 amino acids in length are loaded into MHC class I molecules for presentation at the cell surface. Upon recognition of MHC:peptide complex (Ag), T_{CD8} release the effector molecules perforin and granzyme as well as the cytokine IFN γ leading to the production of anti-viral proteins and apoptosis of infected cells. Adapted from (Nesmiyanov et al.)

central memory CD8⁺ T cells (Kim et al., 2014). Under inflammatory conditions, DCs that have endocytosed viral antigen are capable of cross-presenting foreign peptides of 8-10 amino acids loaded in MHC class I molecules, and express these peptide:MHC complexes (Ag) on their cell surface (Belz et al., 2002; Helft et al., 2012). Once this activated cell reaches the secondary lymphoid organ naïve T_{CD8} can recognize the pathogen-specific Ag via the T cell receptor (TCR) to trigger activation and proliferation.

This initial interaction between T_{CD8} and DC is not sufficient to induce a response, and a second signal is required. CD28 molecules on the T cell surface interact with the costimulatory ligands CD80 and CD86 on APC, inducing signaling in the T_{CD8} through the phosphoinositide 3-kinase (PI3K)-protein kinase B (Akt)-mammalian target of Rapamycin (mTOR) pathway, which supplements TCR-mediated signaling (Salmond et al., 2009). Without this second signal, T cells become unresponsive, a state termed anergy. This phenomenon plays a crucial role in the maintenance of peripheral tolerance and the prevention of autoimmunity (Powell and Delgoffe, 2010). PD-1 and CTLA-4, two inhibitory T cell receptors discussed later, are also implicated in the active induction of peripheral tolerance through a T cell-intrinsic mechanism (Probst et al., 2005). Finally, inflammatory cytokines are also important in the differentiation and expansion of T_{CD8}.

When naïve T_{CD8} become activated, they migrate along a chemokine gradient to sites of infection (Pachynski et al., 1998; Potsch et al., 1999; Stein et al., 2000). Infected epithelial cells can act as APCs by expressing Ag in the context of MHC class I molecules on their surface and making them visible to a pathogen-specific T cell (Figure 1-4) (Rimmelzwaan et al., 2004). Effector T_{CD8} express cytotoxic molecules (perforin and granzymes), and store them in lytic granules that are released upon recognition of Ag to kill the infected cell. Perforin is capable of forming pores in the membranes of infected cells to allow the entrance of other effector molecules (Sad et al., 1996). One important

group of these effectors are granzymes, serine-proteases that activate cytoplasmic caspases to induce apoptosis (Hoves et al., 2010). Interferon gamma ($IFN\gamma$) and tumor necrosis factor alpha ($TNF\alpha$) are also highly expressed and secreted by T_{CD8} to recruit or activate other immune cells, and activate anti-viral pathways in infected cells to inhibit viral replication and induce inflammation. The most efficient protective antiviral immunity is provided by T_{CD8} that are capable of secreting $IFN\gamma$, TNF and $IL-2$ and are capable of rapidly proliferating upon Ag exposure (Betts et al., 2006). These cells are crucial in the clearance of acute respiratory viral infection through the direct lysis of infected cells (Bruder et al., 2006).

During their development in the primary response, T_{CD8} differentiate into either effector or memory phenotype based on signals from APCs and the infectious milieu (Gerlach et al., 2010a). A few models exist for how a response, beginning with a small number of naïve precursor cells, expands into the diverse array of phenotypically distinct T cell subsets observed during infection. The earliest theory was that innate genetic differences are intrinsic within the naïve cell population leading to differentiation during infection, but evidence now exists that shows that a single naïve T_{CD8} has the potential to progress to a myriad of differentiated phenotypes (Gerlach et al., 2010b; Stemberger et al., 2007). The decreasing-potential model, the signal-strength model, and the asymmetric cell fate model are centered around the idea that cell fate is determined by the specific signals received during infection (Kaech and Cui, 2012). The decreasing-potential model posits that repetitive antigen or cytokine stimulation can drive T cells away from a memory state and to an effector phenotype (Ahmed and Gray, 1996). When a DC vaccination approach is used, the inflammatory cytokine response is limited and this has been shown to selectively drive memory differentiation (Badovinac et al., 2005).

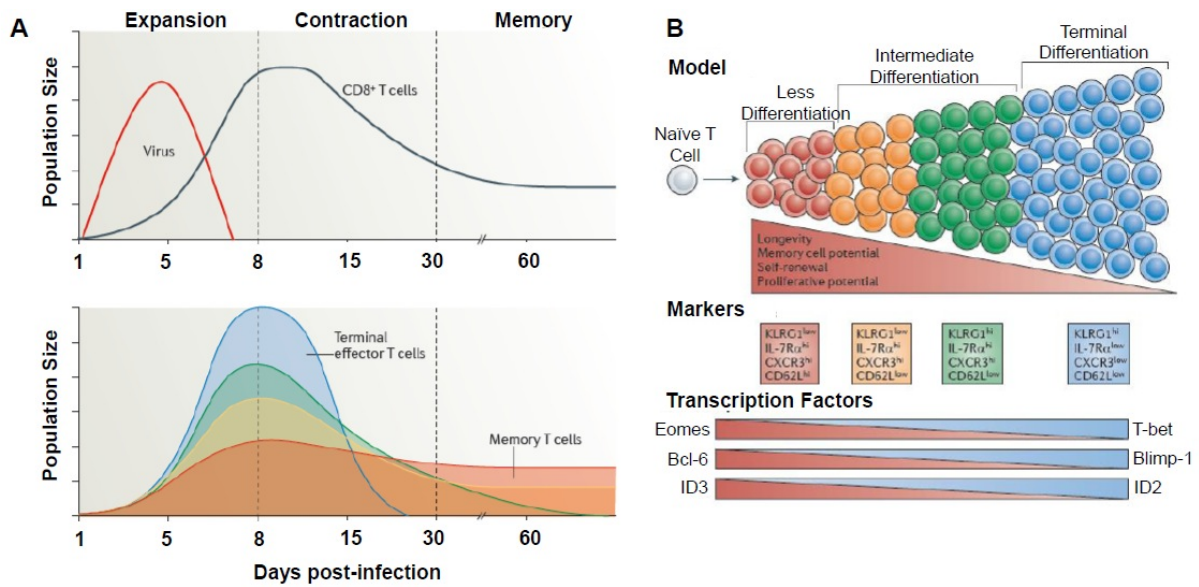


Figure 1-5. T_{CD8} fates during acute viral infection. **(A)** During the expansion phase, Ag-specific T_{CD8} rapidly proliferate (expansion phase) and differentiate into short-lived cytotoxic T lymphocytes that kill infected cells. During the contraction phase, most effector T cells die, but 5–10% survive and mature into memory T_{CD8} capable of rapid reactivation upon reinfection. **(B)** At the peak of the T_{CD8} response multiple subsets of T cells arise. Terminal effector T cells (blue) are KLRG1^{hi}IL-7R^{low}CD27^{low} and memory precursor cells (shown in red) are KLRG1^{low}IL-7R^{hi}CD27^{hi}. The transcription factors important for these distinct subsets are Eomes, Bcl-6 and ID3 for memory development, and T-bet, Blimp-1 and ID2 drive effector differentiation. Adapted from (Kaeck and Cui, 2012).

In studies with varying degrees of inflammation, but a constant level of Ag, a relationship between the balance of effector and memory phenotypes and inflammatory signals became clear (Badovinac et al., 2004; D'Souza and Hedrick, 2006). The signal strength model supports the idea that instead of the frequency of the antigen and cytokine interactions, the intensity of the stimulation controls cell fate; the previously discussed experiments were unable to distinguish between these two models (Lanzavecchia and Sallusto, 2002).

Evidence shows that this differentiation can begin at the first cell division after activation due to the inheritance of the immunological synapse in one daughter cell (Chang et al., 2007) and the unequal distribution of the transcription factor T-bet (Chang et al., 2011). The asymmetric fate model holds that this unbalanced division of signaling

molecules important for effector functions leads to the development of divergent functions. T-bet drives effector T_{CD8} differentiation (Naito et al., 2011), and even small increases favor an effector phenotype (Joshi et al., 2007). The majority of cells will become short-lived effector cells (SLEC), which express high levels of cytotoxic molecules, and undergo apoptosis after clearance of infection, however a small percentage of these cells become memory precursor effector cells (MPEC) which when given the correct signals become long-lived memory cells in the host (Figure 1-2B) (Parish and Kaech, 2009). These final three models all explain experimental evidence well, and likely are not mutually exclusive.

TCR stimulation and inflammatory cytokine signaling (i.e. TNF and IFN) reinforce SLEC development. Signaling strength through the TCR is directly related to the loss of memory cell potential, and the terminal differentiation to an effector phenotype (Ahmed and Gray, 1996). The TCR uses mammalian target of rapamycin (mTOR) to activate the transcription factor IRF4 leading to the expression of effector molecules, shown to be important for the T_{CD8} response to influenza (Yao et al., 2013). The further induction of T-bet is potentiated by IL-12, produced by activated APCs (Joshi et al., 2007), through the Forkhead box protein O1 (Foxo-1)-dependent mTOR pathway (Rao et al., 2010), and IL-2 further drives effector differentiation and acquisition of cytolytic functions through Blimp-1 (Malek and Castro, 2010; Pipkin et al., 2010) Blimp-1 signaling during acute infection leads to the specific upregulation of the effector molecules granzyme B and perforin (Rutishauser et al., 2009), supports T_{CD8} migration to the lung through expression of CCR5 (Kallies et al., 2009), and increases expression of T-bet and ID2, both associated with effector functions. It can also inhibit factors that promote memory development, such as T-cell factor 1 (Tcf-1) and Eomesodermin (Eomes) (Kaech and Cui, 2012). Runt-related transcription factor 3 (Runx3), neurogenic locus notch homolog protein 2 (Notch2) and wingless-related integration (Wnt) are all involved

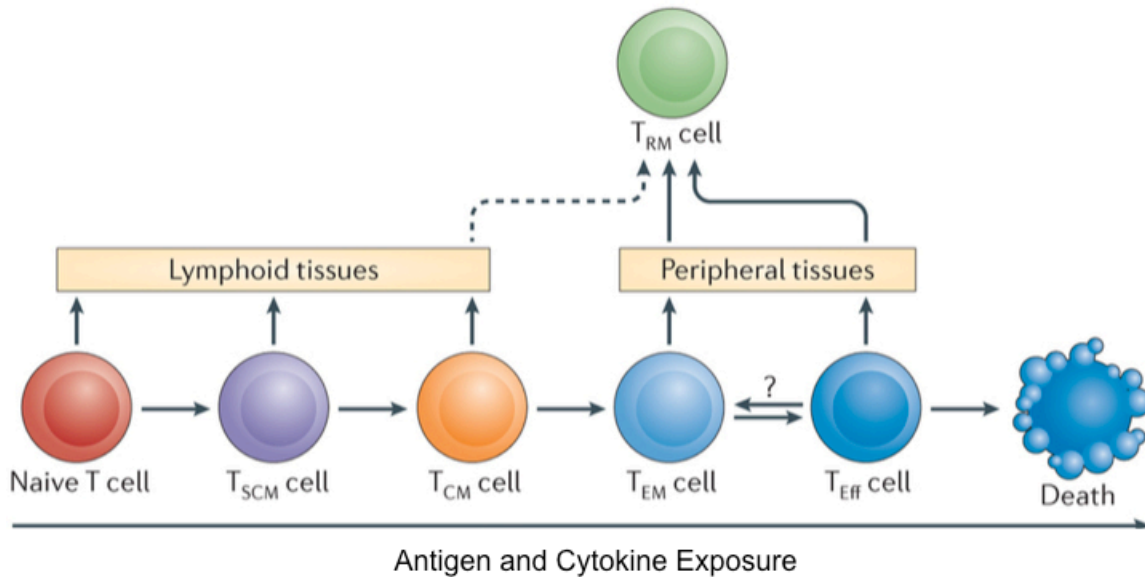


Figure 1-6. T_{CD8} memory differentiation. Naïve T cells progressively differentiate according to their exposure to Ag and specific cytokines. Three major circulating subsets exist — stem cell memory T (T_{SCM}), central memory T (T_{CM}) and effector memory T (T_{EM}) cells —, and two subsets are found mostly in peripheral tissue — effector T (T_{Eff}) and tissue-resident (T_{RM}) cells. Adapted from (Farber et al., 2014).

in the promotion of effector T_{CD8} functions (Cruz-Guilloty et al., 2009; Jeannet et al., 2010; Maekawa et al., 2008; Zhou et al., 2010). Type I IFN also plays an important role in the fate of naïve T cells (Xiao et al., 2009), which will be discussed further in the final section of this introduction.

SLECs are crucially important in the host response to pathogens, and have greater cytolytic function compared to MPECs (Lefrancois and Obar, 2010), but they are short lived and cannot provide future immunity. A subset of MPECs, by contrast, go on to make up long-lasting memory T cells, capable of responding quickly to infection and acquiring effector functions (Jameson and Masopust, 2009). While Blimp-1 expression encourages an SLEC phenotype, the expression of B-cell lymphoma 6 (Bcl-6) leads to memory differentiation (Crotty et al., 2010; Ichii et al., 2007; Ichii et al., 2002; Pepper et al., 2011). Costimulatory receptors cluster of differentiation 28 (CD28), cluster of

differentiation 40 (CD40), 4-1BB, inducible T-cell costimulator (ICOS) and OX40 also impact the balance of the effector and memory T cell populations (Figure 1-8). OX40 promotes MPEC to memory cell transition (Mousavi et al., 2008), 4-1BB has been shown in influenza infection to impact memory response (Bertram et al., 2002), and together these two molecules are crucial for re-expansion during secondary influenza challenge (Hendriks et al., 2005). As mentioned before, when Ag is held constant increasing inflammatory signals, from either IL-12 or IFN can bias away from the memory response through T-bet expression (Cui et al., 2009; Joshi et al., 2007).

In order to persist for extended periods in the host, fully differentiated memory T_{CD8} rely on IL-7 and IL-15 to provide necessary survival signals and do not need TCR signaling. (Hand et al., 2007; Sun et al., 2006; Surh et al., 2006). Memory T_{CD8} have so far been classified into three groups: effector memory (T_{EM}) cells and central memory (T_{CM}) cells are often referred to as circulating memory cells (Sallusto et al., 1999), and another subset that is localized to peripheral sites, called tissue-resident memory (T_{RM}) cells (Figure 1-6). T_{EM} cells recirculate through the host circulatory system, and lack the lymph node homing receptors cluster of differentiation 62 ligand (CD62L) and C-C chemokine receptor type 7 (CCR7), thus remaining outside of the lymphatic system. T_{EM} cells have been shown to progressively lose the ability to home to peripheral tissues due to loss of chemokine receptors (Bedoui and Gebhardt, 2011; Masopust et al., 2010). This was linked to loss of antigen stimulation in these cells (Bedoui and Gebhardt, 2011; Yang et al., 2011), and repeated boosting of memory T_{CD8} pushes more of T_{EM} to the effector-like phenotype (Nolz and Harty, 2011; Wirth et al., 2010). Persisting in the lymph node, T_{CM} cells ($CD62L^{hi}$, $CCR7^{hi}$), can mount a robust secondary responses and establish effector functions upon restimulation (Kaech and Wherry, 2007). The extravasation of these memory T cells from the lymphatics (T_{CM}) or from circulation (T_{EM}) to the site of infection is dependent on the chemokine receptor CXCR3, which increases

in expression as memory cells mature, and also its ligands monokine-induced by γ IFN (MIG) (CXCL9), IFN γ -induced protein 10 (IP-10) (CXCL10), and IFN-inducible T-cell α chemoattractant (I-TAC) (CXCL11) (Groom and Luster, 2011a, b; Hikono et al., 2007). T_{EM} cells can persist in the lung for several months after influenza or Sendai virus infections (Hogan et al., 2002; Hogan et al., 2001), and it appears that the lung microenvironment, independent of Ag, is able to maintain these cells in an activated state (Kohlmeier et al., 2007). T_{CM} expressing CCR5 also protect against influenza challenge (Kohlmeier et al., 2008) and can reacquire effector functions through IFN signaling (Kohlmeier et al., 2010).

T_{RM} cells (CD103^{hi}, CD69^{hi}, CD62L^{lo}, CD27^{lo}) are a newly described subset of memory T cells that remain at the site of initial infection and are capable of quickly responding in those tissues (Kaech and Wherry, 2007; Shin and Iwasaki, 2013). They are thought to arise from a common memory precursor as circulating memory (Mackay et al., 2013), and because of their proximity to sites of pathogen invasion, these cells play an especially important role in the immediate response to virus and the recruitment of circulating memory T cells (Sathaliyawala et al., 2013; Schenkel and Masopust, 2014; Wu et al., 2014). Survival of T_{RM} in the tissue is maintained by interferon-induced transmembrane protein 3 (IFITM3) expression (Wakim et al., 2013), and vaccination approaches targeting these cells in the lung for influenza virus have proven very effective at eliciting protective immunity (Wakim et al., 2015). The expression of CD69L is necessary for the ability for T_{RM} to reside in mucosal tissues (Lee et al., 2011; Mackay et al., 2013). During the response at peripheral sites of infection, circulating memory cells downregulate the transcription factor Krüppel-like Factor 2 (KLF2), which relieves suppression of CD69L and allows for migration (Casey et al., 2012; Skon et al., 2013). Recent work has also shown that the expression of CD103 by T_{RM} is a crucial component in the maintenance of these cells in peripheral tissue, and the T-bet-

dependent expression of this molecule is driven by transforming growth factor beta (TGF β) production from CD4⁺ T cells (T_{CD4}) (Laidlaw et al., 2014). Both circulating and tissue-resident T_{CD8} have shown to be highly effective at host control of previously encountered pathogens (Slutter et al., 2013; Wu et al., 2014), and the T_{CD4} response is also crucial for a heterosubtypic response (McKinstry et al., 2012; Teijaro et al., 2011), defined by cross-protection from serotypes other than the vaccination strain (Nguyen et al., 2007).

Both the adaptive and innate immune responses orchestrate an effective immune response to viral infection, and the data presented in this section highlights that depending on the signals received from the pathogen and from the infectious environment, T_{CD8} of diverse effector and memory functions arise. As outlined earlier, viruses have evolved mechanisms to evade innate immunity, and the work presented in this thesis seeks to elucidate the outcomes of this disruption. It is important to keep in mind that since the immune response is so finely balanced, even a slight perturbation of immune response by the pathogen has the potential to have profound consequences. The host also encodes overlapping mechanisms of immune surveillance and response, as well as fine-tuning to protect sensitive tissues, and pathogens are well suited to find and exploit these potential niches in which host pathways can be used for their advantage.

CD8⁺ T Cells – Impairment during Acute Respiratory Virus Infection

While it is clear that the T_{CD8} response to respiratory viruses is crucial for clearance, recent data suggests that lung virus-specific T_{CD8} are impaired in their effector functions. While examining the functional ability of RSV-specific lung T_{CD8} during an infection in a mouse model, researchers showed that only a small proportion of these cells were able to respond to antigenic stimulation by expressing IFN γ (Chang and

Braciale, 2002; Lukens et al., 2006), although spleen and lymph node resident RSV-specific T_{CD8} functioned normally. In subsequent studies, both PIV-5, influenza and vaccinia virus (VACV) resulted in similar T cell impairment during infection (Gray et al., 2005; Vallbracht et al., 2006). The ability for these T cells to make TNF and lyse infected cells was also greatly reduced due to a lessened ability to mobilize lytic granules with lower granzyme B content (Vallbracht et al., 2006). Work by *Erickson et al* showed that the mechanism behind this exhaustion was primarily driven by the inhibitory T cell receptor PD-1 (Erickson et al., 2012), with minor contributions from other inhibitory receptors (Erickson et al., 2014a).

Despite the acute nature of these respiratory viral infections, this impairment of T_{CD8} and the inhibitory markers associated, resembles a unique state of T_{CD8} differentiation that occurs during chronic infections and cancer termed T cell exhaustion. Exhausted T_{CD8} have reduced effector functions, highly express numerous inhibitory receptors and possess a distinct transcriptional profile distinct from other T_{CD8} subsets. The first description of this cell type was in mice chronically infected with LCMV (Gallimore et al., 1998), and this phenomenon is now well studied in the context of chronic viral, bacterial and parasitic infections of mice and humans as well as cancer (Virgin et al., 2009). During chronic LCMV clone-13 infection of mice, T_{CD8} progressively lose effector functions in a manner that is both viral-load- and time-dependent (Wherry et al., 2003) (Blackburn et al., 2009; Mueller and Ahmed, 2009). IL-2 production, proliferative capacity and cytolytic killing are the first functions lost during exhaustion, followed by loss of TNF production, and then IFN γ (Wherry et al., 2003). Finally, terminally exhausted T_{CD8} can undergo apoptosis (Wherry et al., 2007) (Figure 1-7). This apoptosis is related to the downregulation of the IL-7 receptor, which leads to reduced expression of anti-apoptotic molecule B cell leukemia/lymphoma 2 (Bcl-2) (Lang et al., 2005), as well as signaling by PD-1 (Dong et al., 2002; Shi et al., 2011). Viruses can

escape detection by T cells by mutating important amino acids within viral T_{CD8} epitopes, and this results in down-regulation of inhibitory receptors by T_{CD8} no longer recognizing antigen (Ag) (Blattman et al., 2009).

Exhausted T_{CD8} appear to comprise a unique subset of T cells, and their transcriptional profile is distinctly different from that of effector or memory T_{CD8} (Doering et al., 2012; Wherry et al., 2007). Unlike memory cells, which express IL-7R and IL-15R, exhausted T_{CD8} become dependent on TCR signaling for survival, in a phenomenon termed 'Ag addiction' (Shin and Wherry, 2007). Experimental evidence in the LCMV model shows that the loss of virus-specific T_{CD8} upon Ag withdrawal or viral mutation does not restart memory T cell development (Wherry et al., 2004) unless it occurs very early (Blattman et al., 2009), further showing that T cell exhaustion is a uniquely differentiated state (Angelosanto et al., 2012). (Utzschneider et al., 2013). This impaired state appears to be partially controlled by epigenetic modifications of genes since TCR signaling drives demethylation of the *Pdcd1* promoter, which encodes the inhibitory receptor PD-1. This phenomenon primes the exhausted T cell for rapid re-expression of PD-1 under inflammatory conditions (Youngblood et al., 2011). Exhaustion also appears to be wholly unique from the two other well-known states of T cell dysfunction, anergy and senescence. Gene expression patterns in these three states are different between all groups, and regulation of the T cell impairment is specific to each (Jackson et al., 2013; Schwartz, 2003; Teague et al., 2008; Wherry et al., 2007).

Inhibitory Pathways During Acute Infection

An over-exuberant response to pathogens can lead to damage to sensitive tissues, and the immune system has evolved regulatory mechanisms to prevent these unintended consequences. Cell surface inhibitory receptors, soluble factors and

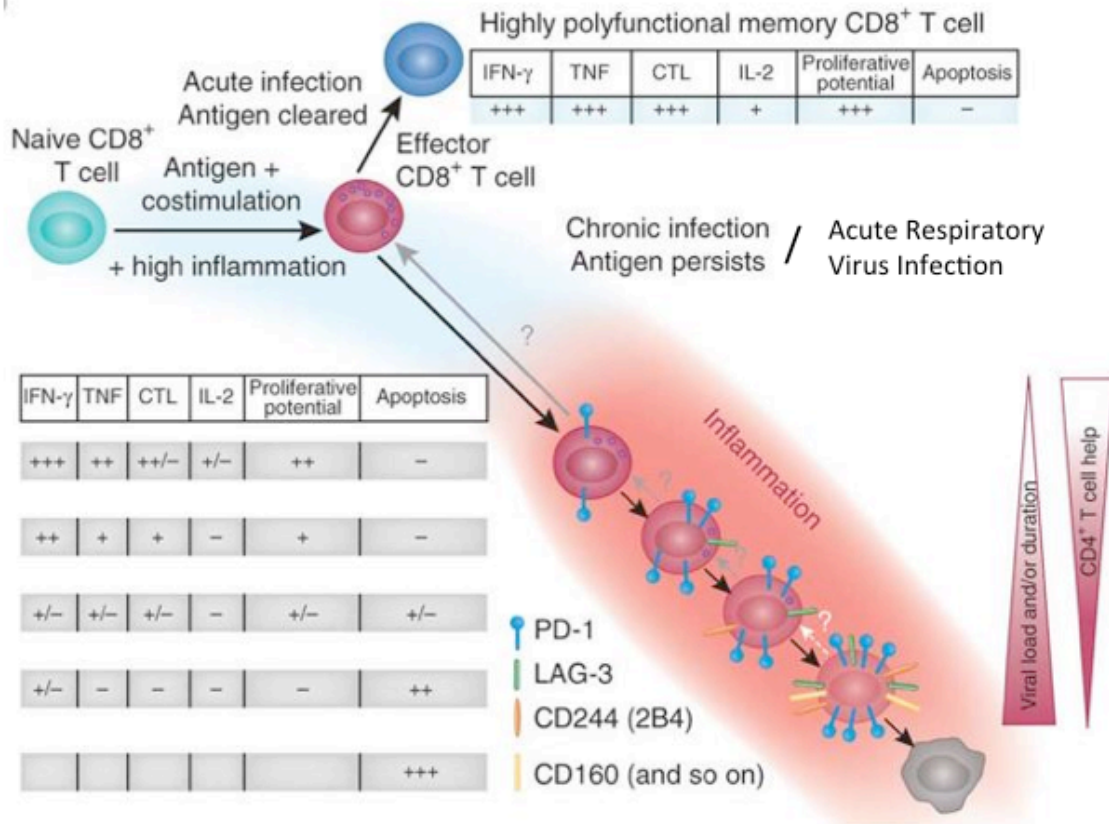


Figure 1-7. Exhaustion of T_{CD8} cells. Functional memory T_{CD8} are generated upon clearance of acute infection, but during both chronic and acute respiratory virus infection, Ag persistence and continued inflammation leads to the progressive loss of effector functions, due to the actions of inhibitory receptors such as PD-1. Adapted from (Wherry, 2011).

specialized immunoregulatory cells (i.e., CD4⁺ regulatory T cells) all work in concert to finely tune host response. Inhibitory receptors, in particular, have been shown to play a key role in inducing self-tolerance and preventing autoimmunity (Kasagi et al., 2011), as well as in the down-regulation of T cell responses during chronic viral infection (Blattman et al., 2003; Trautmann et al., 2006; Wherry et al., 2003; Zhang et al., 2007) and are relatively well studied in that context. Recent data suggests that these receptors also are upregulated during acute viral infection (Barber et al., 2006; Lafon et al., 2008; Phares et al., 2009; Zelinsky et al., 2011), and they serve a particularly important role during

acute viral LRI (Chang et al., 2004; Erickson et al., 2012; Sharma et al., 2011; Workman et al., 2004).

Programmed Cell Death-1 and Its Ligands

PD-1 (CD279) was discovered in studies of cells undergoing apoptosis and is a CD28-family member (Ishida et al., 1992). A 288 amino acid type I transmembrane glycoprotein, containing a single IgV-like domain, PD-1 is expressed on activated T cells, B cells and monocytes (Agata et al., 1996b). This inhibitory receptor exerts inhibitory effects by directly antagonizing TCR stimulation. Ligation of PD-1 results in recruitment of src homology 2 domain-containing tyrosine phosphatase 2 (SHP-2) to a C-terminal tyrosine residue on the cytoplasmic tail, termed an immunoreceptor tyrosine-based switch motif (ITSM). SHP-2 dephosphorylates the TCR-associated proteins cluster of differentiation 3 ζ (CD3 ζ) and zeta-chain associated protein kinase 70kDa (ZAP70), blocking activation of PI3K and inhibiting the PI3K/Akt pathway (Figure 1-4) (Chemnitz et al., 2004a; Okazaki et al., 2001; Pages et al., 1994; Sheppard et al., 2004). PD-1 signaling subsequently inhibits production of the pro-survival factor B-cell lymphoma-extra large (Bcl-xL) (Chemnitz et al., 2004b), and transcription factors, T-bet and Eomes, associated with effector and memory function, respectively (Nurieva et al., 2006).

The expression of PD-1 on T_{CD8} cells is upregulated by Ag exposure. In an LCMV model, increased Ag load led to greater T cell dysfunction (Wherry et al., 2003), and *in vitro* and *in vivo* treatment with an activating anti-CD3 mAb to stimulate the CD3 receptor resulted in PD-1 upregulation (Agata et al., 1996a). Viral T_{CD8} epitopes present a strong evolutionary pressure, and mutations in these epitopes frequently arise facilitating immune escape. When this occurs early in viral infection, the loss of the ability for virus-specific T_{CD8} to detect Ag results in lower PD-1 expression (Blattman et al., 2009), and T_{CD8} present in tissues with higher viral loads show increased PD-1 expression (Blackburn et al., 2009). PD-1 levels in patients with HIV have also been correlated to

viral titers (Trautmann et al., 2006), and T_{CD8} in patients who are able to control the virus without therapy, known as long-term nonprogressors, express relatively low levels of PD-1 (Zhang et al., 2007). The expression of high levels of PD-1 on T_{CD8} is ubiquitous on cells displaying an exhausted phenotype, but analysis of functional effector memory cells in healthy adults showed that PD-1 was present on these cells as well (Duraiswamy et al., 2011). Further investigation in a system in which the expression of PD-1 was tightly genetically controlled demonstrated that levels of PD-1 expression are important. High levels of PD-1 were necessary to inhibit macrophage inflammatory protein-1beta (MIP-1 β) production, while moderate levels blocked cytotoxicity and IFN γ production. Very low levels of PD-1 could impair TNF, IL-2 production, and proliferation (Wei et al., 2013).

Less is known about the function of PD-1 during acute viral LRI, but work by *Erickson et al.* highlights that this inhibitory receptor plays an important part in T cell exhaustion during pulmonary virus infection (Erickson et al., 2012). Before this work, indirect evidence suggested that T_{CD8} impairment occurs during RSV infection of mice, as IL-2 treatment rescues impairment (Chang et al., 2004), similar to what was seen during chronic HIV infection (Blattman et al., 2003). Lung epithelial cells can act as APCs and upregulate expression of MHC class I, CD80 and CD86 during infection (Kim et al., 2005; Papi et al., 2000), and infection with RSV upregulated expression of PD-L1, a ligand for PD-1, on bronchial epithelial cells (Stanciu et al., 2006) leading to the inhibition of T_{CD8} activity (Telcian et al., 2011). These data suggest that the expression of the ligand for PD-1 on infected lung epithelium plays a role in the exhaustion phenotype seen during viral infection.

Blockade of PD-1 has recently been pursued as an attractive option for the reduction of viral titers in both chronic and acute infections, as well as for improvement of T_{CD8} responses in cancer models. During chronic LMCV infection, exhausted T_{CD8} regained functional capabilities through blockade of the PD-1 pathway leading to

enhanced clearance of ongoing infection (Barber et al., 2006). This study demonstrated that PD-1 is a major inhibitory pathway in T cell exhaustion and raised the possibility of a therapeutic intervention that could improve viral control by targeting T cell exhaustion. *In vitro* blockade of PD-1 on T_{CD8} generated by HIV infection also showed efficacy in restoring function (Day et al., 2006; Freeman et al., 2006; Trautmann et al., 2006), and *in vivo* PD-1 blockade during simian immunodeficiency virus (SIV) infection resulted in improved T_{CD8} responses, greater neutralizing antibody levels and enhanced survival (Velu et al., 2009). PD-1 also appears to impair T cells during cancer (Jung et al., 2010), and clinical trials indicate that the anti-PD-1 monoclonal antibody, nivolumab, is safe and effective against refractory hematological malignancies (Ansell et al., 2015; Berger et al., 2008) and solid tumors (Brahmer et al., 2010; Herbst et al., 2014; Powles et al., 2014; Tumeo et al., 2014). These results show that targeting PD-1 is a viable new immunotherapeutic option in the treatment of cancer and chronic infections. Recent work from our lab also shows that lung T_{CD8} rapidly become impaired during acute viral LRI, and demonstrates the potential for blockade of PD-1 in the reduction of viral respiratory titers (Erickson et al., 2014a).

PD-1 has two ligands in the B7-family, PD-L1 (B7-H1, CD274)(Freeman et al., 2000) and PD-L2 (B7-DC, CD273)(Latchman et al., 2001). PD-L1 is thought to be most crucial in suppressing autoimmunity (Greenwald et al., 2005) and inducing T_{CD8} exhaustion (Barber et al., 2006; Blackburn et al., 2009; Butler et al., 2012; Jin et al., 2010b) . PD-L1 is constitutively expressed on the surface of T and B cells, DCs, and macrophages (Yamazaki et al., 2002), and PD-L1 transcript is expressed by a wide range of nonhematopoietic cells (including lung parenchymal cells) (Freeman et al., 2000). Surface expression of PD-L1 in the latter cell type only occurs after an activating signal (Dong et al., 2002). This inhibitory ligand is also expressed on many tumor types, and is thought to inhibit tumor-infiltrating T_{CD8} to promote immune evasion and

malignancy (Curiel et al., 2003; Dong et al., 2002; Grosso and Jure-Kunkel, 2013; Iwai et al., 2002; Soliman et al., 2014). Innate immune responses play a role in the upregulation of PD-L1 as type I IFN signaling as well as double-stranded RNA or lipopolysaccharide (LPS) treatment, in a TLR3- and TLR4-dependent manner, respectively, upregulates PD-L1 (Eppihimer et al., 2002; Groschel et al., 2008; Schreiner et al., 2004; Terawaki et al., 2011). The promoter region of this gene contains two interferon regulatory factor-1 (IRF-1) sites capable of binding both PRR-mediated IRF3/7 and JAK/STAT-mediated ISGF3 (Lee et al., 2006a). IL-27, an innate cytokine associated with a TH17 response, has been shown in a model of autoimmune encephalomyelitis to induce the upregulation of PD-L1 (Hirahara et al., 2012). Type II IFN signaling can upregulate PD-L1 (Eppihimer et al., 2002; Schreiner et al., 2004) by repression of microRNA-513, which prevents PD-L1 protein from being expressed on the cell surface (Gong et al., 2009). Taken together, these results indicate that PD-L1 is upregulated by multiple innate immune mechanisms during infection, while control of T cell PD-1 expression is accomplished through the specific interaction of the TCR with cognate Ag. Chapter III of this thesis will investigate further the dependence of PD-1:PD-L1 interaction on type I IFN signaling, by using a transgenic mouse model lacking the IFN- α receptor (IFNAR).

Other Inhibitory Pathways

Exhausted T_{CD8} also co-express up to seven inhibitory receptors, including TIM-3, LAG-3, 2B4, and CTLA-4 along with PD-1 (Figure 1-5) (Crawford and Wherry, 2009). These inhibitory receptors are capable of working synergistically to affect the severity of dysfunction (Blackburn et al., 2009), but PD-1 appears to act as a master regulator of exhaustion, and blockade of other receptors, without PD-1 blockade, has minimal impact (Barber et al., 2006; Blackburn et al., 2009; Jin et al., 2010b). However, if PD-1 is blocked along with TIM-3 (Jin et al., 2010b), LAG-3 (Blackburn et al., 2009; Butler et al.,

2012) or CTLA-4 (Kaufmann et al., 2007; Nakamoto et al., 2009) a significant enhancement of T_{CD8} function over PD-1 blockade alone can be observed in chronic infections.

CTLA-4 is closely related (Lin et al., 2008) and antagonizes TCR signaling, similar to PD-1, but through a distinct mechanism. During CTLA-4 signaling, this molecule binds to CD80/86 (Masteller et al., 2000) activating phosphatases (Chuang et al., 2000; Chuang et al., 1999; Cilio et al., 1998; Lee et al., 1998; Marengere et al., 1996), which blockade lipid-raft expression (Chikuma et al., 2003; Darlington et al., 2002; Martin et al., 2001; Rudd et al., 2002) and prevent Zap70 microcluster formation (Valk et al., 2006). However, PD-1 signaling also induces the transcription factor Basic leucine zipper transcription factor, ATF-like (BATF), which further inhibits effector functions (Quigley et al., 2010). Signaling mechanisms for TIM-3, LAG-3 and 2B4 are less well understood, and they lack classic motifs associated with T cell signaling (e.g., ITIM or ITAM). Expression of inhibitory ligands represents the other side of the T_{CD8} exhaustion coin. TIM-3 binds Galectin-9, a secreted molecule; LAG-3 binds MHC class II molecules with higher affinity than CD4 (Odorizzi and Wherry, 2012); and 2B4 binds to CD48 present on the surface of all hematopoietic cells (Mooney et al., 2004). The availability of different ligands in host niches could fine-tune the functionality of these T_{CD8} to protect sensitive organs, like the lung.

Inhibitory receptors other than PD-1 are also involved in acute viral LRI, although like PD-1, much less is known than in the context of chronic infection. During Sendai virus infection, LAG-3 is capable of negatively regulating T_{CD8} memory development (Workman et al., 2004), TIM-3 limits T_{CD8} effector functions during acute HSV-1 infection (Sehrawat et al., 2010) and influenza infection (Sharma et al., 2011) while LAG3 and 2B4 inhibits T_{CD8} function in HMPV and influenza (Erickson et al., 2014a). These data

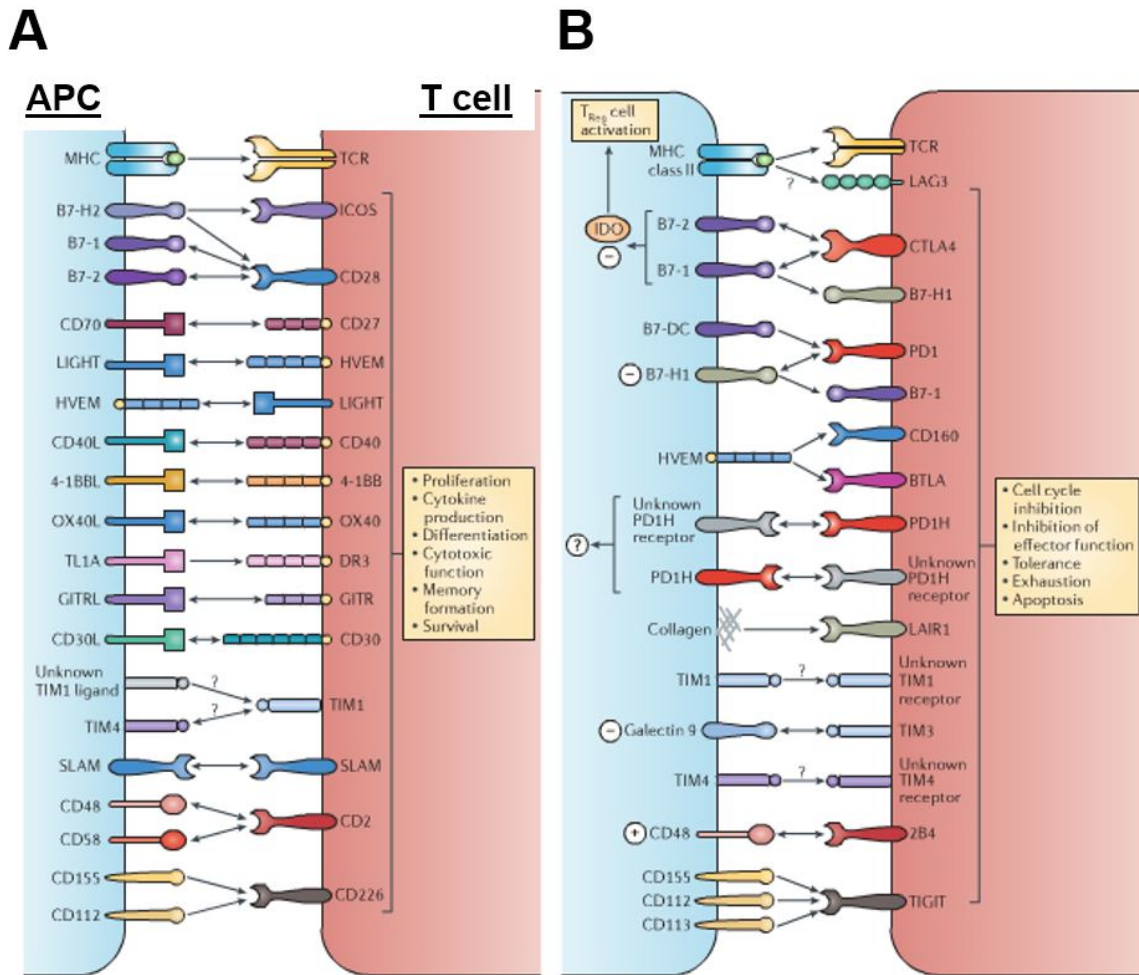


Figure 1-8. T_{CD8} co-stimulatory molecules. **(A)** T cell activation is enhanced through the interactions with multiple cell-cell interactions that supplement TCR signaling. CD28 helps prevent anergy by providing crucial co-stimulation and is required to fully activate T cells. These positive interactions lead to the production of poly-functional effector T response. **(B)** Inhibitory receptors, like PD-1, TIM-3, LAG-3, 2B4 and CTLA-4, antagonize TCR and other positive signals. From (Chen and Flies, 2013).

suggest that synergistic blockade of PD-1 with other inhibitory receptors might be even more effective at restoring functionality to impaired T_{CD8}.

Implications of type I IFN evasion on CD8⁺ T cell response

The link between the innate and adaptive immune response during viral infection has only recently begun to be appreciated, but increasing evidence is mounting that

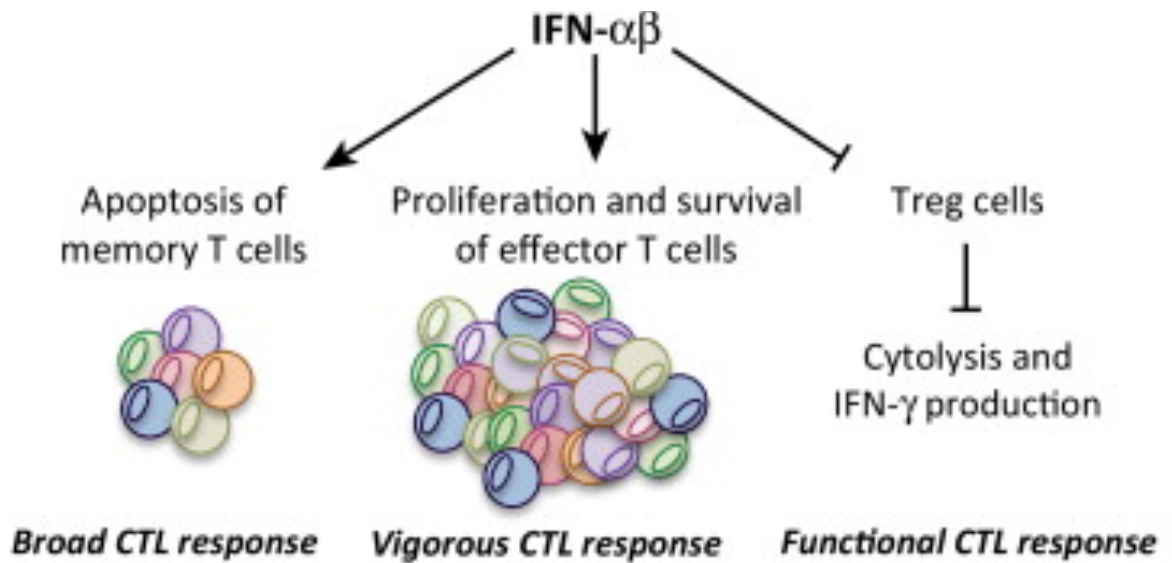
shows that lack of a functional and balanced innate immune response leads to an aberrant adaptive immune response (Figure 1-9). As discussed in a previous section, viruses have evolved many mechanisms with which to evade type I IFN signaling, but not much is known about what this could mean for the adaptive response. There is some data on the contribution of IFNAR signaling to the T_{CD8} response overall, and its importance in generating protective immunity. The timing of type I IFN exposure has been shown to be very important in controlling the pleiotropic effects of this cytokine, as early treatment with type I IFN leads to inhibition of T_{CD8} responses while treatment during or after antigen exposure strongly enhanced immune responses (Gallucci et al., 1999; Le Bon et al., 2003; Le Bon et al., 2001; Proietti et al., 2002).

Type I IFN treatment *in vitro* is capable of directly and potently inhibiting proliferation of T_{CD8} (Figure 1-9)(Lindahl-Magnusson et al., 1972; Petricoin et al., 1997; Zella et al., 2000), but this response appears temporally regulated since 3 hours of IFN pre-treatment leads to enhanced proliferation, while 18 hours had an opposite effect (Feng et al., 2005). Type I IFNs have been shown to act directly on T_{CD8} to induce proliferation *in vivo*, by a mechanism that overlaps with IL-12 signaling (Curtsinger et al., 2005; Kolumam et al., 2005b; Thompson et al., 2006), and this signal is particularly important for the formation of SLECs (Wiesel et al., 2012). This difference in *in vitro* versus *in vivo* effects of type I IFN could also be related to changes in migration due to chemokine receptor expression and the expression of CD69L (Feng et al., 2005; Shioh et al., 2006; Sun et al., 1998). The anti-proliferative properties of type I IFN produces differential responses in naïve compared to activated T cells related to the expression level of STAT1 in these cell types (Stark et al., 1998), providing a potential mechanism for this discordance. Activated T cells express lower levels of STAT1, and this lower expression appears to generate resistance to the negative effect of type I IFNs (Bromberg et al., 1996; Dondi et al., 2003; Gil et al., 2006; Tanabe et al., 2005). Another

mechanism leading to increased survival *in vivo* of IFN-exposed T_{CD8} is related to the expression level of the natural cytotoxicity triggering receptor 1 (NCR1) ligand, which is repressed on the surface of T_{CD8} by type I IFN signaling. Without expression of this molecule, T_{CD8} are sensitive to perforin-mediated killing by natural killer (NK) cells (Crouse et al., 2014). Type I IFNs also directly work on a myriad of other cell types, including lung epithelium and APCs, so the overall affect on T_{CD8} becomes even more complex (Hervas-Stubbs et al., 2011; Le Bon et al., 2001; Le Bon et al., 2006; Tough, 2004).

APCs are an indispensable component of the T_{CD8} response, and the different subsets have been suggested to differentially prime an effector or memory response (Hao et al., 2008; Kim and Braciale, 2009; Kim et al., 2014). Type I IFNs also play a key role in the proliferation and maturation of APCs from immature monocytes (Haicheur et al., 2000; Paquette et al., 1998; Parlato et al., 2001; Santini et al., 2000). This signaling has been shown to be IRF3 mediated, and APCs are able to essentially help themselves mature through autocrine IFNAR signaling (Shalek et al., 2013). GM-CSF and IL-4 are the most commonly known stimulators of APC development from CD14⁺ monocytes, but type I IFNs can take the place of IL-4 in this phenomenon (Paquette et al., 2002). Type I IFNs are also important for the transformation of APCs from an immature to a mature developmental stage and can synergize with TNF α and CD40L to enhance maturation (Luft et al., 2002; Radvanyi et al., 1999). This developmental shift is associated with the upregulation of CD40, CD80, CD86 and MHC class I and II (Ito et al., 2001; Luft et al., 1998; Padovan et al., 2002; Wang et al., 1999).

IFN-matured APCs are capable of potently stimulating T cell proliferation both *in vitro* (Mattei et al., 2001; Montoya et al., 2002; Padovan et al., 2002) and *in vivo* (Le Bon et al., 2001). These cells also secrete chemokines that are important for the trafficking of



T_{CD8} to sites of infection and cytokines that can regulate immune responses. APCs matured in the presence of type I IFN upregulate the expression of CXCL9 (MIG),

Figure 1-9. Direct affect of type I IFN on T cells promotes overall T_{CD8} response. Type I IFN is responsible for pleiotropic affects on the T cell response. It induces the apoptosis of memory T cells, promoting a broad CTL response, and stimulates the proliferation and survival of activated T cells, permitting a vigorous CTL response. Type I IFN also restricts the maintenance of Treg cells, which suppress T cell effector functions allowing for a functional CTL response.

CXCL11 (I-TAC), CXCL10 (IP-10), CXCL19 (MIP-3 β), and/or DC-CK-1 depending on secondary maturation signals. These chemokines bind to activated T cells, except for CXCL19 and DC-CK-1 that act on naïve T cells (Adema et al., 1997; Ngo et al., 1998), and allow for the efficient migration of T_{CD8} to sites of infection (Padovan et al., 2002; Parlato et al., 2001). The secretion of IL-15 by IFN-matured APCs provides a stimulatory signal to maturing T cells as well as to the secreting cell promoting an activating loop for this interaction (Mattei et al., 2001; Santini et al., 2000).

Type I IFN also contributes to the polarization of the CD4+ T cell response towards a Th1-biased response, and away from a Th2 or Th17 response (Eriksen et al., 2004; Huber et al., 2010; Moschen et al., 2008; Schandene et al., 1996; Shibuya and Hirohata, 2005), which generates a favorable environment for T_{CD8} effector functions.

Finally, regulatory CD4⁺ T cells (Treg) suppress effector T_{CD8} functions and proliferation (Almeida et al., 2010), and a recent LCMV study shows that type I IFN signaling in Treg cells suppresses CD28-dependent ICOS expression, ultimately leading to suppression of proliferation of these cells (Figure 1-9)(Srivastava et al., 2014b). My work contributes new information to this question. Contrary to the *Srivastava et al.* study, my data presented in chapter IV of this thesis I showed that during HMPV infection of IFNAR KO mice lower levels of CD25⁺ FOXP3⁺ CD4⁺ T regulatory can be found in the lungs, perhaps indicating a trafficking defect related to chemokine expression.

Taken together, these data show that type I IFN is important for a potent T_{CD8} response, and that the RSV interferon antagonist NS1 is capable of skewing the T cell response *in vitro*. In that study, virus without interferon antagonist ability demonstrated three clear differences from WT virus. First, without NS1, T_{CD8} upregulated CD103, shown earlier to be important for tissue homing during infection. Second, activation and proliferation of Th17 cells was increased, and third, IL-4 producing CD4⁺ T cells, a major contributor to disease pathogenesis, were reduced (Munir et al., 2011). This is strong evidence that innate immune evasion, well described for many viruses, can play a role in modulation of the adaptive response. Work in this thesis will use HMPV to explore the contributions of type I IFN signaling to viral control and pathogenesis, functional and robust T_{CD8} response and the newly described phenomenon of T_{CD8} impairment during acute viral LRI.

CHAPTER II

HMPV evades type I IFN signaling through targeting STAT1 with the small hydrophobic (SH) protein

Introduction

Human metapneumovirus (HMPV) is a negative sense, single-stranded RNA virus in the Paramyxoviridae family (van den Hoogen et al., 2001b). Infection with HMPV is a leading cause of acute lower respiratory infections (LRI), and no treatment or vaccine is currently available to combat this pathogen (Edwards et al., 2013; Englund et al., 2006b; Papenburg et al., 2012; Shahda et al., 2011; Walsh et al., 2008; Widmer et al., 2012; Williams et al., 2004; Williams et al., 2005b; Williams et al., 2006b). By the age of five, nearly all individuals will have been infected with HMPV (van den Hoogen et al., 2001b) and contain neutralizing antibodies toward this virus, but the very young, the elderly, and immunocompromised will be at high risk of developing severe complications from this viral infection (14, 17, 36). HMPV, like other members of the *Paramyxoviridae* family such as respiratory syncytial virus (RSV) and parainfluenza virus (PIV), is capable of blocking or modulating the innate immune response through targeting of molecules involved in signaling through the type I interferon (IFN) signaling pathway (Dinwiddie and Harrod, 2008; Ren et al., 2011). Type I IFN signaling begins with the recognition of viral nucleic acids by pattern recognition receptors (PRRs) in infected cells (Platanias, 2005), as well as in innate immune cells such as macrophages and dendritic cells (Gordon, 2002), which induce the production and release of type I IFNs, IFN α and IFN β . These molecules ligate the interferon α receptor (IFNAR), in both an autocrine and paracrine fashion, and lead to signaling events, involving STAT1, STAT2, and IRF9, which culminate in the expression of anti-viral effector molecules (Barber, 2001; Biron, 2001; Sadler and Williams, 2008). In addition, this pathway is capable of modulating the

adaptive immune response by contributing to both the presentation of antigens as well as the differentiation and maintenance of important adaptive immune cells (Havenar-Daughton et al., 2006; Kolumam et al., 2005a; Le Bon and Tough, 2008; Luft et al., 1998).

Related paramyxoviruses, such as RSV and PIV, encode proteins responsible for targeting and inhibiting signaling through the innate immune pathways. RSV encodes the NS1 and NS2 proteins, and the PIV genome contains an alternative reading frame of the P/C/V gene that encodes the V protein; both types of proteins specifically inhibit aspects of innate immunity (Andrejeva et al., 2004; Childs et al., 2012; Gainey et al., 2008; Lo et al., 2005). Interestingly, the HMPV genome contains no analogous open reading frames to any of these previously identified innate immune antagonists (Piyaratna et al., 2011; van den Hoogen et al., 2002). Specifically, many related viruses have mechanisms to affect STAT1 expression and phosphorylation (Didcock et al., 1999a, b; Kubota et al., 2001; Ramaswamy et al., 2004; Takeuchi et al., 2003), (Rodriguez et al., 2004; Rodriguez et al., 2002), and data in the field indicates that HMPV is no different (Dinwiddie and Harrod, 2008; Ren et al., 2011). This led us to hypothesize that HMPV is using a viral protein in a unique way to evade the type I IFN signaling pathway by targeting STAT1. In this study, we sought to determine the viral protein responsible for inhibition of STAT1 phosphorylation.

Results

HMPV infection increases STAT1 expression and inhibits type I IFN induced STAT1 phosphorylation in BEAS-2B cells

To characterize the effect of HMPV infection on STAT1 expression and phosphorylation, we first used the human bronchial epithelial cell line, BEAS-2B. After 24

hours of HMPV infection, cells were treated with type I IFN for 15 minutes and STAT1 expression and phosphorylation levels were determined using western blotting analysis (Figure 2-1A). Image J software was used to quantify band intensity for this western blot (Figures 2-1B and C). STAT1 expression did not change in cells infected with HMPV (Figure 2-1A and B). However, in infected BEAS-2B cells treated with type I IFN, STAT1 phosphorylation was markedly lower than in IFN-treated uninfected cells (Figure 2-1A and 1C). We next used flow cytometry to measure both expression and IFN-induced phosphorylation of STAT1 in bulk populations of BEAS-2B cells infected with HMPV. Using this assay we observed an increase in total STAT1 expression in BEAS-2B cells over the course of 48 hours of HMPV infection (Figure 2-1D), as well as a significant decrease in the ability of the infected BEAS-2B cells to phosphorylate STAT1 upon type I IFN treatment (Figure 2-1E). This suggests that HMPV is capable of inhibiting STAT1 phosphorylation in these cells.

Type I IFNs produced by infected BEAS-2B cells induce high STAT1 levels, and cells become refractory to STAT1 phosphorylation

In order to analyze the ability for HMPV to inhibit STAT1 phosphorylation specifically in infected cells, we used fluorescent antibody staining to selectively label cells that were infected with HMPV and expressing viral proteins (Figure 2-2A). Flow cytometry was then used to analyze STAT1 expression and IFN-induced phosphorylation in HMPV-infected and uninfected cells. STAT1 expression significantly increased in both infected and uninfected BEAS-2B cells in the same well compared to cells treated with lysate from uninfected LLC-MKC cells used to grow HMPV (mock) treated cells, but expression in infected cells was lower than the uninfected cells in the same well (Figure 2-2B). When cells infected with virus were treated with type I IFN at time-points during the course of infection, we observed that over 24 hours of HMPV

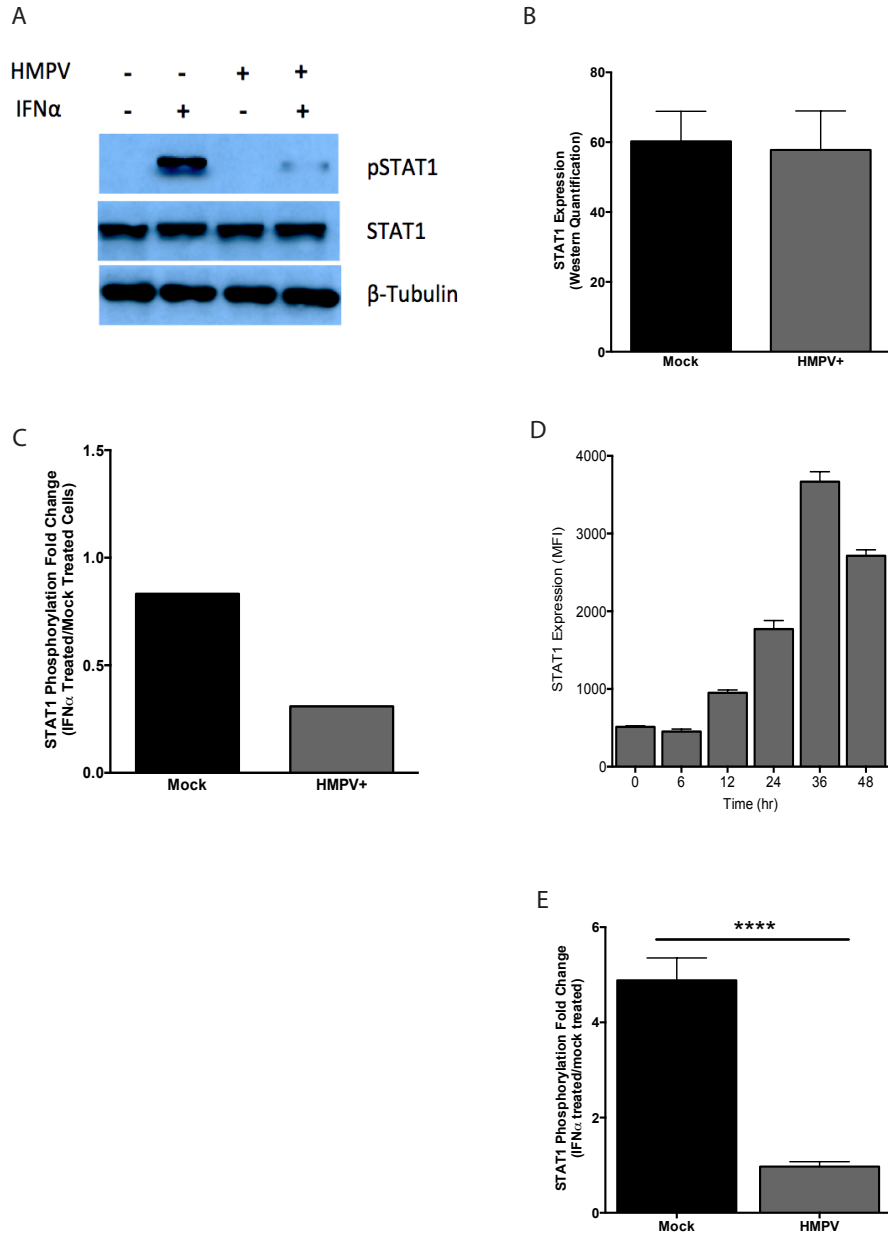


Figure 2-1: Affect of HMPV on BEAS-2B cells. (A) BEAS-2B Cells were infected for 48 hours with HMPV at MOI of 4. Cells were lysed with RIPA buffer, and proteins were separated on 4-15% bis-tris Gel before transfer to nitrocellulose membrane. The membrane was probed with antibodies against STAT1, the phosphorylated isoform of STAT1, and tubulin. (B,C) Quantification of western blot. (D,E) BEAS-2B cells infected for 48 hours with HMPV at MOI of 4. Cells were fixed and permeabilized and fluorescent antibodies and used to probe for STAT1 and the phosphorylated isoform of STAT1 using flow cytometry. Groups were compared using an unpaired Student's t test or one-way ANOVA with Tukey post-hoc test where appropriate. N > 3. Error bars represent SEM. ****=P<0.0001.

infection, both infected cells and uninfected cells in the same well displayed a loss of the ability to phosphorylate STAT1 in response to treatment with type I IFN (Figure 2-2C).

Since uninfected cells cultured with HMPV-infected cells also lost the ability to phosphorylate STAT1, we tested the hypothesis that this inhibition of STAT1 phosphorylation was driven by host factors instead of a direct viral effect. We tested whether prolonged exposure to type I IFN alone could induce this phenotype. We found that after a 24-hour treatment of BEAS-2B cells with exogenous or endogenous type I IFN (the latter induced by poly(I:C) transfection) that these cells significantly upregulated STAT1 expression (Figure 2-2D) and lost the ability to phosphorylate STAT1 when treated with type I IFN (Figure 2-2E). These findings led us to conclude that type I IFN produced by HMPV-infected cells was inducing a non-responsive state in surrounding uninfected cells in the well, thus hampering the ability to detect a specific viral effect within the infected cell population. Vero cells are defective in the production of type I IFNs (Emeny and Morgan, 1979a, b) and provided a tractable alternative. We found no effect of transfection of poly(I:C) on either STAT1 expression (Figure 2-2D) or STAT1 phosphorylation (Figure 2-2E) in the Vero cell line. These data show that the inability of BEAS-2B cells to phosphorylate STAT1 after HMPV infection is partly driven by paracrine type I IFN production during infection, and that use of the Vero cell line can avoid this confounding effect.

HMPV infection of Vero cells leads to lower STAT1 expression and a significant defect in the ability to phosphorylate STAT1 specifically in infected cells

To determine the effect of HMPV infection on STAT1, Vero cells were infected at an MOI of 1, treated with type I IFN 24 hours later, than analyzed for STAT1 expression and phosphorylation by western blot (Figure 2-3A). No difference was detected in total

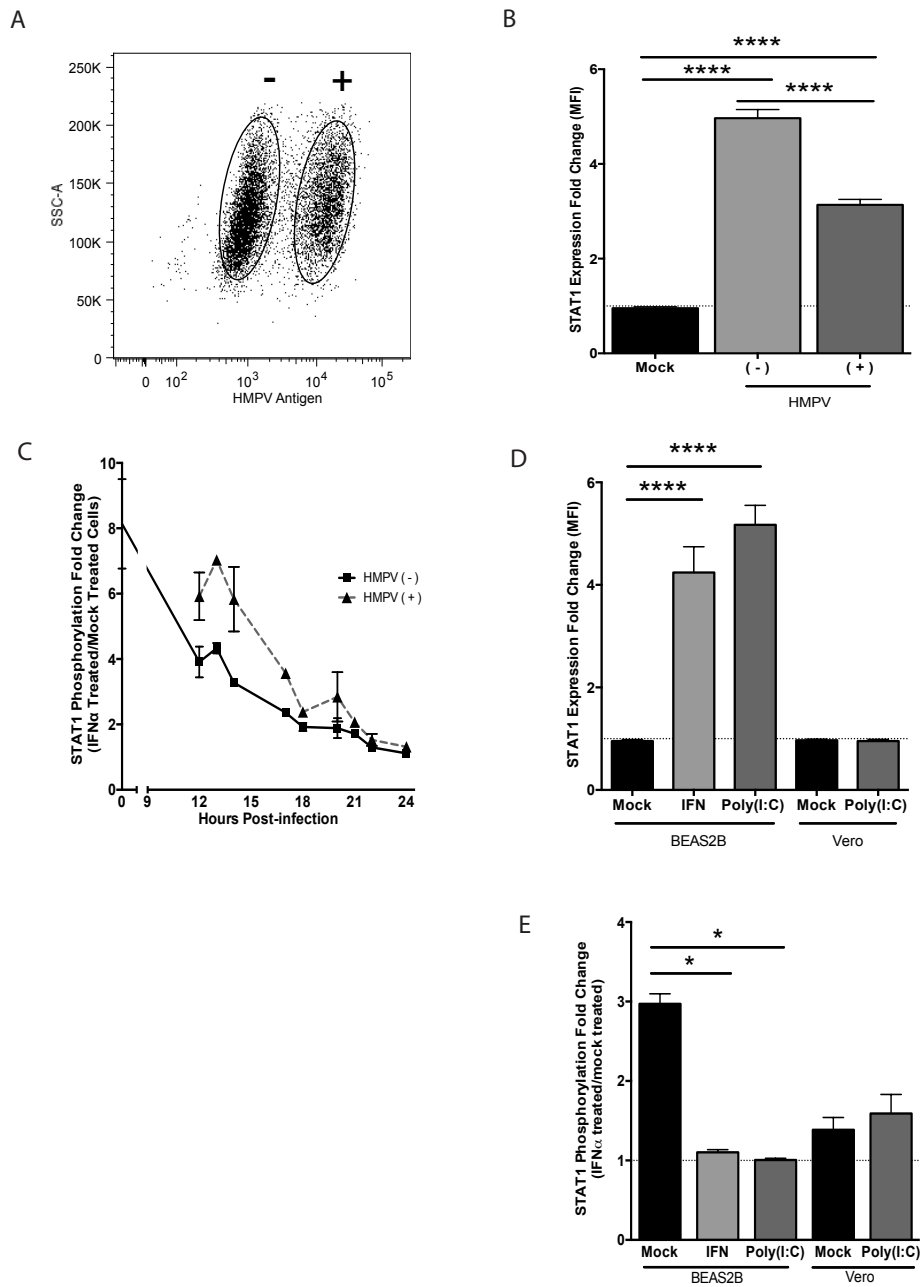


Figure 2-2.: Effect of type I IFN treatment on BEAS-2B cells. (A) Cells infected with HMPV were identified with antibody to HMPV antigen and gated into distinct populations of infected and uninfected cells using flow cytometry. (B) Cells were also probed with fluorescently labeled antibodies against STAT1 (B,D) or phosphorylated STAT1 (C,E), and analyzed via flow cytometry after treatment with HMPV (B,C), type 1 IFNs, or synthetic viral RNA, Poly(I:C), to induce IFN expression (D,E). Groups were compared using unpaired Student's t test or one-way ANOVA with Tukey post-hoc test where appropriate. N = 2-3 duplicate experiments. Error bars represent SEM. *= $P < 0.05$, ****= $P < 0.0001$.

STAT1 expression between infected and mock-treated bulk cell populations (Figure 3A, B), but interestingly lower molecular weight isoforms of STAT1 appear in these cells when they were infected with HMPV (Figure 2-3A). IFN α -driven STAT1 phosphorylation seemed to also slightly decrease STAT1 phosphorylation (Figure 2-3A and C). As observed before, it is important to look specifically at infected cells to determine a viral affect on STAT1. Flow cytometric analysis of Vero cells infected with an MOI of 1 was performed using an anti-HMPV antibody to differentiate between uninfected and infected cells as in Figure 2-2. We found lower STAT1 expression in HMPV-infected cells compared to uninfected cells in the same well (Figure 2-3D). When these cells were treated with IFN α , there was significant defect in the ability of infected cells to phosphorylate STAT1 (Figure 2-3E). These results show that HMPV is capable of decreasing STAT1 expression and inhibiting IFN-induced STAT1 phosphorylation in Vero cells.

Vero cells transiently expressing the SH protein from HMPV display lower STAT1 expression and phosphorylation induced by both type I and II IFN

To identify which HMPV protein was responsible for the inhibition of IFN-induced STAT1 phosphorylation, we generated GFP-tagged viral protein expression plasmids optimized for mammalian cell expression. We used flow cytometry to analyze Vero cells expressing viral proteins compared to non-transfected cells in the same well by gating on GFP (Figure 2-4A). In cells expressing the HMPV SH protein, there was significantly lower STAT1 expression compared to untransfected cells in the same well after 48 hours, but no difference in STAT1 expression was observed upon transfection of other viral proteins or GFP alone (Figure 2-4B). When these cells were treated with IFN α , the cells expressing SH were significantly inhibited in their ability to phosphorylate STAT1

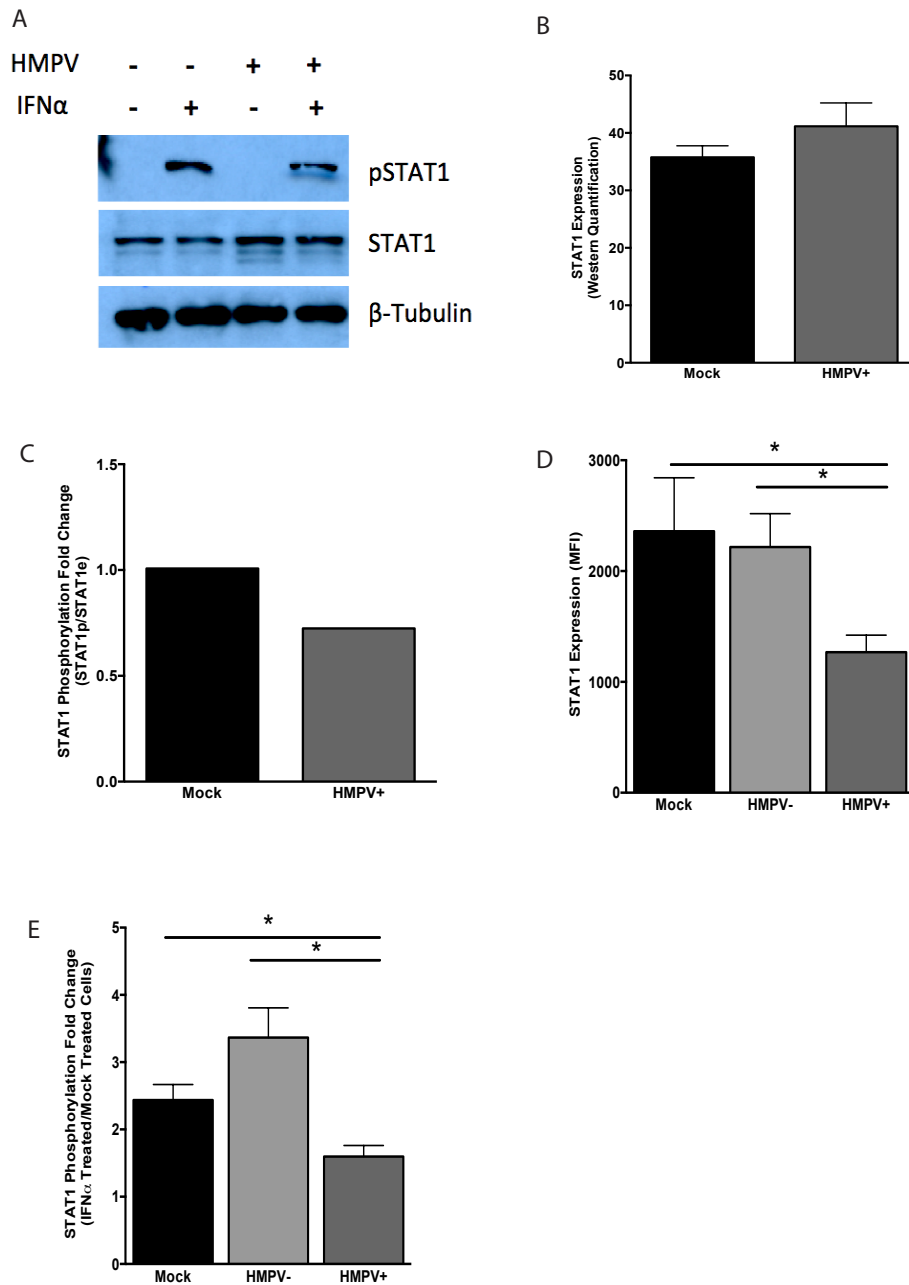


Figure 2-3. Specific viral inhibition of STAT1 by HMPV in Vero cells. (A) Vero Cells infected for 48 hours with HMPV at MOI of 4. Cells were lysed with RIPA buffer, and proteins were separated on 4-15% bis-tris Gel before transfer to nitrocellulose membrane. The membrane was probed with antibodies toward STAT1, the phosphorylated isoform of STAT1, and tubulin. (B,C) Quantification of western blot. (D,E) Vero cells were infected for 48 hours with HMPV at MOI of 1. Cells were fixed and permeabilized and fluorescent antibodies were used to probe for STAT1 and the phosphorylated isoform of STAT1 specifically in cells expressing viral antigen using flow cytometry. Groups were compared using an unpaired Student's t test or one-way ANOVA with post-hoc Tukey test where appropriate. N > 3 duplicate experiments. Error bars represent SEM. *=P<0.05.

(Figure 2-4C). Similar inhibition was observed when treated with IFN γ (Figure 2-4D).

Cells expressing the N protein from HMPV also showed a modest but significant defect in type II IFN-induced STAT1 phosphorylation (Figure 2-4D). Thus, the HMPV SH protein is capable of preventing IFN-induced phosphorylation of STAT1.

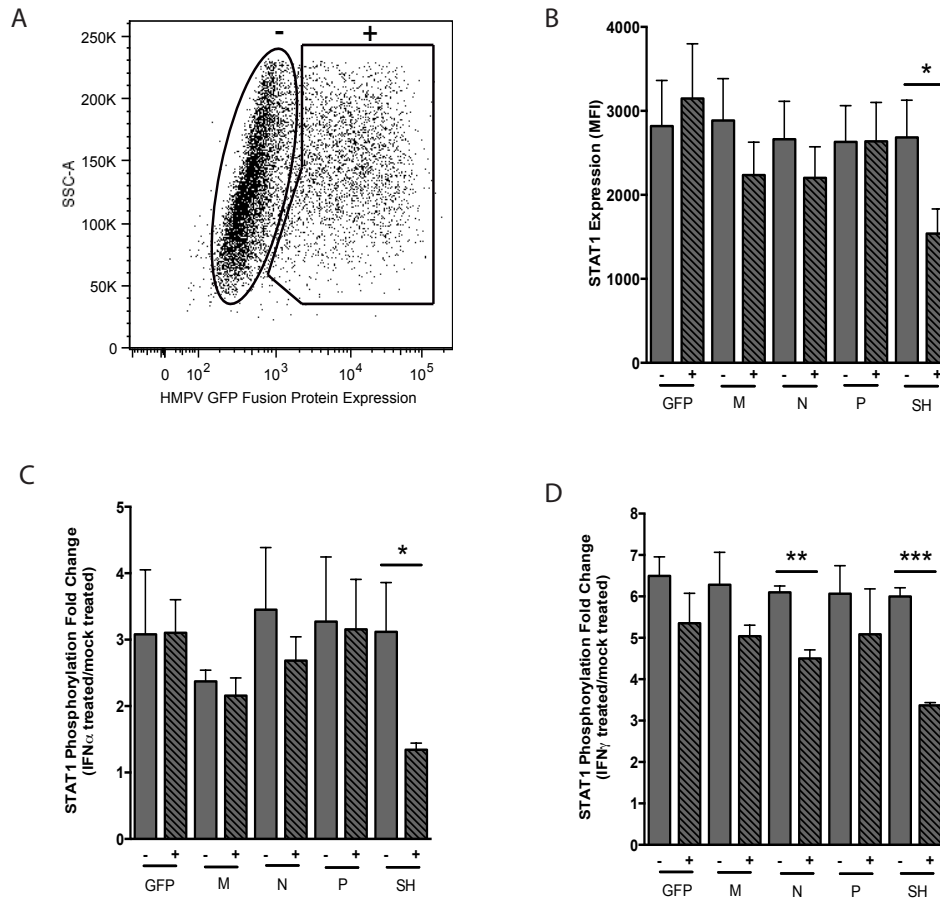


Figure 2-4. Effect of transient HMPV protein expression on STAT1. (A) Vero cells transfected with HMPV GFP-fusion proteins were specifically analyzed using flow cytometry compared to the untransfected cells in the same culture. STAT1 expression (B) and phosphorylation (C,D) was analyzed using fluorescently labeled antibodies in the context of treatment with IFN α (C) and IFN γ (D). Groups were compared using unpaired Student's t test or one-way ANOVA with Tukey post-hoc test where appropriate. N =/>>3 duplicate experiments per group. Error bars represent SEM. *= P <0.05, **= P <0.01, ***= P <0.001.

Stable expression of the HMPV SH protein increases STAT1 expression, but reduces IFN-induced STAT1 phosphorylation and downstream type I IFN signaling

As a complementary approach to transient transfection, cell lines were generated that stably expressed HMPV proteins. There was a significant increase in baseline STAT1 expression in cells constitutively expressing SH protein compared to the parental cells (Figure 2-5A). There was also a modest but significant increase in the level of STAT1 protein in cells expressing HMPV G and P. When treated with IFN α , cells expressing SH protein showed significantly lower levels of STAT1 phosphorylation compared to the parental cells (Figure 2-5B), similar to the defect observed during transient transfection of viral proteins. To confirm that inhibition of STAT1 phosphorylation led to a reduction of downstream signaling through the type I IFN pathway, we utilized a luciferase reporter plasmid containing the promoter region of the interferon stimulated response element (ISRE). Cells stably expressing either the N or SH proteins exhibited a significant defect in the ability to signal through the type I IFN pathway (Figure 2-5C). These data show that the stable expression of the SH protein is capable of inhibiting phosphorylation of STAT1 by type I IFN and that this inhibition decreases signaling through the type I IFN pathway.

Discussion

HMPV is an important human pathogen and is capable of causing significant morbidity and mortality, particularly in vulnerable populations including the very young, elderly and immunocompromised persons (Edwards et al., 2013; Englund et al., 2006b; Papenburg et al., 2012; Shahda et al., 2011; Walsh et al., 2008; Widmer et al., 2012; Williams et al., 2004; Williams et al., 2005b; Williams et al., 2006b) . Similar clinical disease is caused

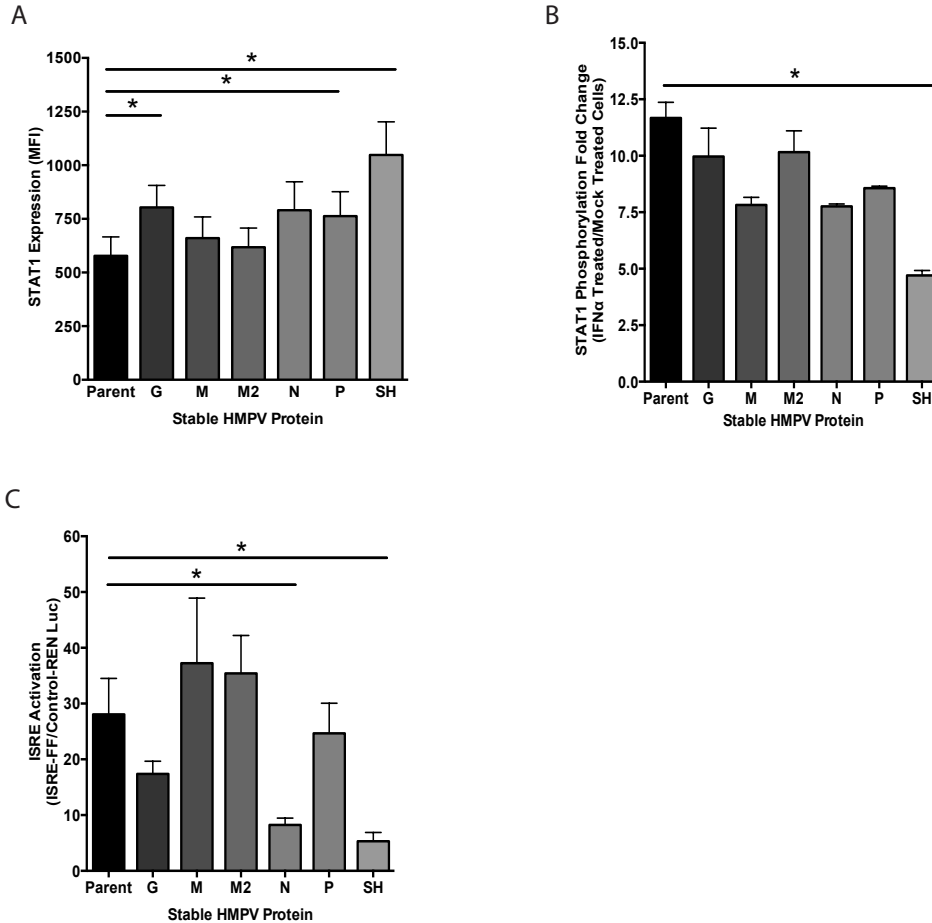


Figure 2-5. Effect of stable expression of HMPV on STAT1 and downstream ISRE signaling. The effect of stable expression of HMPV proteins on STAT1 in 293FLP cells was measured using fluorescently conjugated antibodies for STAT1 expression (A) and phosphorylation (B) induced by type 1 IFN treatment via flow cytometry. Stably transfected cell lines were also used to measure downstream activation of the type 1 IFN pathway by the transfection of an ISRE-luciferase reporter and the subsequent treatment of cells with type 1 IFN (C). N =/≥3 duplicate experiments per group. Groups were compared using an unpaired Student's t test or one-way ANOVA with Tukey post-hoc test where appropriate. N = 3 duplicate experiments. Error bars represent SEM. *= $P < 0.05$.

by HMPV to that seen in other related virus infections, like RSV and PIV (Fox, 2007; Freymuth et al., 1995; Freymuth et al., 2006; Garcia-Garcia et al., 2006), despite the absence of sequence homology in the HMPV genome for previously known innate immune agonists (Piyaratna et al., 2011; van den Hoogen et al., 2002). Both type I and II IFN signaling rely on STAT1 as a crucial component in both pathways. After ligation of

IFN α or β to the type I IFN receptor, Janus kinases (Tyk2/JAK1) associated with the receptor cytoplasmic tail phosphorylate STAT1 (Tyr701) and STAT2 (Tyr689), which together with IRF9 form a transcription factor, interferon stimulated gene factor 3 (ISGF3) capable of binding to promoter elements for genes associated with the anti-viral response (Platanias, 2005). For type II IFN signaling, IFN γ binds to the IFNGR inducing phosphorylation of STAT1 (Tyr701) by other Janus kinases (JAK1/JAK2) resulting in a homodimer of STAT1 that acts as a transcription factor for anti-viral proteins, cytokines, and chemokines (Platanias, 2005). Type I and II IFN signaling induce an overlapping set of anti-viral molecules and act in combination to produce a cellular anti-viral response (Decker et al., 2005; Pestka et al., 2004; Schroder et al., 2004).

In our initial studies, we used the clinically relevant bronchial epithelial cell line, BEAS-2B, to determine the effect of HMPV infection on IFN signaling. Experiments using total cell populations showed that during viral infection, cells upregulated STAT1 expression and showed lower IFN-induced phosphorylation of STAT1. However, by using flow cytometry to discriminate uninfected and infected cells, we found that infection of BEAS-2B cells induced a significant increase in STAT1 expression in both infected and uninfected cells in the same well, along with greatly reducing the ability for IFN α to induce STAT1 phosphorylation in both populations. The effect on uninfected cells in the same well as HMPV-infected BEAS-2B cells led us to speculate that type I IFNs secreted by infected cells were working in a paracrine fashion on uninfected cells, thus confounding the HMPV-specific STAT1 inhibition in infected cells with type I IFN-induced STAT1 downregulation in nearby uninfected cells (Isaacs and Lindenmann, 1957). Treatment of BEAS-2B cells with exogenous IFN α or poly(I:C) confirmed that type I IFN induced a natural downregulation of STAT1 expression and phosphorylation. This phenomenon presented a challenge for analyzing virus-specific effects on STAT1 within the subset of infected cells within an entire cell population. Notably, many studies of

related viruses use cell lines competent for type I IFN production, suggesting that paracrine effects of type I IFN may confound similar experiments (Ren et al.).

Vero cells are defective for the production of type I IFN, but are capable of responding to exogenous type I IFN (Emeny and Morgan, 1979a; Hasler and Wigand, 1978). We confirmed that, unlike BEAS-2B cells, poly(I:C) treatment of these cells neither increased STAT1 expression nor inhibited IFN-induced STAT1 phosphorylation. Analysis of Vero cells via western blotting analysis showed that HMPV infection had no effect on STAT1 expression, but induced a striking inability to phosphorylate STAT1 in response to type I IFNs. Further investigation using flow cytometry to discriminate uninfected from infected cells revealed that HMPV-infected cells displayed significantly lower levels of STAT1 expression and IFN-induced phosphorylation of STAT1 compared to uninfected cells from the same well.

The phenomenon of targeting STAT proteins is a mechanism by which other viruses from the *Paramyxoviridae* family evade innate immune signaling through a variety of inhibitory actions (Caignard et al., 2007; Takeuchi et al., 2003). The V protein from PIV5 and mumps virus in the *Rubulavirus* genus, and the NS1 protein from RSV, in the *Pneumovirinae* subfamily with HMPV, have been shown to target the STAT proteins for proteosomal degradation (Didcock et al., 1999a, b; Kubota et al., 2001; Ramaswamy et al., 2004). The measles virus, by contrast, does not affect STAT protein expression, but uses the V protein to prevent STAT phosphorylation and thereby blocking the association and nuclear translocation of the ISGF3 transcription factor (Takeuchi et al., 2003). The Nipah virus V protein is capable of binding to STAT proteins and inducing the conglomeration of high-molecular-weight complexes, which blocks type I IFN signaling (Rodriguez et al., 2004; Rodriguez et al., 2002).

The HMPV genome contains eight gene segments encoding nine known proteins (van den Hoogen et al., 2002). Some of these proteins have well-defined roles in the

viral life cycle, such as the RNA polymerase (L), the matrix (M) protein which coats the inner layer of the viral membrane, and the fusion (F) protein responsible for binding and fusion of the viral particle to the host cell (Piyaratna et al., 2011; van den Hoogen et al., 2002). Others such as the M2-1, M2-2, and small hydrophobic (SH) proteins play a more enigmatic role. Other groups have shown possible roles for the phosphoprotein (P), M2-2, the glycoprotein (G), and the SH protein in the evasion of innate immune responses to HMPV. One study reported a differential ability for the A and B subgroups of HMPV to induce type I IFN, and suggested that the P protein of the B1 subgroup of HMPV was able to prevent RIG-I activation and the production of IFNs (Goutagny et al.). It is unclear whether this inhibition is a result of direct interactions with host innate immune sensors, if it is a result of the P protein's interactions with viral RNA, or if it is an affect on the translation of other viral proteins. The M2-2 protein is not necessary for viral growth, but published data suggest that it might target the important PRR mediator, mitochondrial antiviral-signaling protein (MAVS), to prevent innate immune signaling and thus block type I IFN production (Ren et al.). The G protein of HMPV has been suggested to play a role in the direct inhibition of RIG-I through interactions between the CARD domain of its N-terminus (Ren et al.), leading to higher levels of inflammatory cytokine production in cells infected with HMPV lacking the G protein.

Transient expression of the SH-GFP protein reduced the level of STAT1 expression and showed a block in IFN γ -induced STAT1 phosphorylation. Surprisingly, cells stably expressing the SH protein expressed significantly more STAT1 compared to the parental cell line, though SH-expressing cells demonstrated a defect in IFN-induced STAT1 phosphorylation and signaling through the ISRE. Since expression of unphosphorylated STAT proteins is important for the constitutive expression of important host molecules (Chatterjee-Kishore et al., 2000) and because signaling through the type I IFN system relies on the accessibility of STAT1 in the cytoplasm (Ramana et al., 2000),

it is possible that these cells are compensating for the SH protein targeting of STAT1. Collectively, these data indicate that the HMPV SH protein is capable of inhibiting IFNAR signaling by targeting the STAT1 protein.

The function of the SH protein of HMPV has not been fully characterized. The SH protein is predicted to be a type II transmembrane protein and is present across the *Pneumovirinae* subfamily, but HMPV encodes the longest SH protein at around 180 aa (van den Hoogen et al., 2002). Sequence homology between HMPV SH and related viruses is very low (Biacchesi et al., 2004a; Yunus et al., 2003) but maintains key characteristics such as similar hydrophilicity and a high percentage of threonine and serine residues (Piyaratna et al., 2011). Recent data indicate that, similar to the RSV SH protein, the HMPV SH protein is capable of forming pores in the membrane of infected cells, termed a “viroporin”, which increases cell permeability (Masante et al., 2014). This is particularly intriguing in the context of our work, because dephosphorylated STAT1 has been shown to interact with the host nucleoporins, Nup153 and Nup214, in order to translocate out of the nucleus after signaling (Marg et al., 2004). The SH proteins of PIV5 and RSV are also capable of inhibiting TNF- α signaling *in vitro* (Fuentes et al., 2007; Lin et al., 2003; Wilson et al., 2006), and a similar role for SH-HMPV has been proposed (Ren et al.) indicating a immunomodulatory role for this protein might not be unexpected.

Recombinant HMPV lacking the SH protein showed no difference in viral kinetics or pathogenesis in a small rodent model of HMPV (Biacchesi et al., 2004b) and gene expression analysis of A549 cells infected with the SH-deficient virus showed few differences from cells infected with WT virus (de Graaf et al., 2013). Despite these data, there are significant limitations in extrapolating animal and cell culture data to human disease, and as our BEAS-2B experiments showed, analysis of gene or protein expression in bulk cell populations may be confounded by paracrine artifacts. Together,

these data show that HMPV SH protein is capable of inhibiting interferon signaling by blocking STAT1 expression and IFN-induced phosphorylation. Further studies are need to elucidate the role of this inhibition in pathogenesis.

Materials and Methods

Cells and Virus. BEAS-2B (ATCC® CRL-9609™) cells were purchased from ATCC. FLP-In 293 cells were purchased from Life Technologies. Vero (ATCC® CCL-81™) cells were kindly provided by Dr. James Crowe. BEAS-2B cells were maintained in Opti-Mem with 2% FBS. Vero cells and 293FLP cells were maintained in DMEM with 10% FBS. HMPV (pathogenic clinical strain TN/94-49, genotype A2) was grown and titered in LLC-MK2 cells as previously described (Williams et al., 2005c). For type I IFN treatment to observe STAT1 phosphorylation, cells were incubated with 1000U/mL recombinant human interferon alpha A (Alpha 2a)(PBL) for 15 minutes. For long-term type I IFN treatment, cells were incubated with 1000U/mL interferon alpha for 24 hours. Poly(I:C) treatment was achieved by transfecting 5µg/well Polyinosinic-Polycytidylic acid sodium salt (Sigma) into a 24-well plate using Lipofectamine 2000 (Life Technologies) and incubating cells for 24 hours. Cell lines stably expressing viral proteins were generated using the FLP-In system (Life Technologies) according to the manufacturer's instructions, and selected using hygromycin.

Plasmids. Mammalian optimized viral protein constructs were designed using the GeneOptimizer® software (GeneArt) by adjusting codon usage and optimizing GC content to be efficiently expressed in mammalian cells. Viral protein sequences were then cloned into a pcDNA3.1 plasmid, and into the pcDNA3.1-GFP plasmid to create

viral protein-GFP fusion constructs. Transfection was performed using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions.

Flow Cytometry. Stable cell lines were detached using 0.1% Trypsin-EDTA, washed, fixed with 3% buffered formalin for 10 minutes at room temperature, and permeabilized with 100% methanol for 10 minutes at -20 °C. After fixation and permeabilizing, cells were washed thoroughly with PBS with 2% FBS. For labeling of HMPV-infected cells, samples were incubated with polyclonal guinea pig anti-HMPV sera and FITC-labeled anti-guinea pig IgG (Southern Biotech). Cells were also probed with a PE-labeled antibody to STAT1 (clone 1/STAT1, BD Phosflow) to determine overall expression levels, and with an AlexaFluor-647 labeled antibody to assess phosphorylation of STAT1 (pY701) (clone 4a, BD Phosflow) after treatment with type I IFN.

Reporter Gene Assay. A firefly luciferase reporter plasmid with 6x copies of the ISRE promoter (Stratagene) and a constitutively active *Renilla* luciferase reporter on a TK promoter (pGL4.74, Promega) were co-transfected into cell lines stably expressing HMPV genes to measure the binding activity of the ISRE transcription factor. Twenty-four hours post-infection, cells were treated with type I IFN for 24 hours, and the Dual-Glo luciferase assay system (Promega) was used to measure the signal for each luciferase type. The constitutively active *Renilla* luciferase signal was used to normalize transfection efficiency across the experiment.

Western Blot. Cells were mock- or HMPV-infected at ~80% confluency and infection was allowed to progress for 48 hours before IFN- α treatment for 30 minutes. Cells were washed on ice with 1x PBS and then lysed using ice-cold RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, and 5 mM EDTA)

supplemented with protease (P8340, Sigma) and phosphatase inhibitors (Roche). Protein concentration was determined using a DC™ protein Assay (BioRad), and samples were diluted in a 5x sample buffer (250mM Tris HCl (pH 6.8), 10% SDS, 0.5% bromophenol blue, 50% glycerol, and 100mM DTT). Clarified lysates were boiled for ten minutes, subjected to SDS/PAGE, and transferred onto nitrocellulose membranes. Membranes were blocked for 1 hour in 5% nonfat dry milk in tris-buffered saline with 0.1% Tween-20 V/V (TBS-T) buffer followed by incubation in p-STAT1 (Clone A-2, Santa Cruz, sc-8394), STAT1 (Clone H-95, Santa Cruz, sc-96783), or β -tubulin (mouse monoclonal, Sigma, #T4026) primary antibodies. Blots were washed in TBS-T and subsequently incubated with HRP-conjugated secondary antibodies (Promega). Signal was detected using the Clarity Western ECL substrate (Bio-Rad). Quantification of western blot analysis in figure 1B and C and figure 3B and C was performed using the Image J software to calculate band intensity. For STAT1 phosphorylation, intensity of phosphorylated band was calculated relative to STAT1 expression for each sample.

Statistical Analyses. Data analysis was performed using Prism v4.0 (GraphPad Software). Groups were compared using an unpaired Student's t-test or one-way ANOVA with Tukey post-hoc test for multiple comparisons. $P < 0.05$ was considered to be significant.

CHAPTER III

Type I IFN signaling controls early viral replication, but also contributes significantly to disease pathogenesis

Work presented in this chapter also published in the Journal of Virology (Hastings et al., 2015)

Introduction

HMPV, like other members of the Paramyxoviridae family such as respiratory syncytial virus (RSV) and parainfluenza viruses (PIV), can subvert the innate immune response through modulation of the type I interferon (IFN) signaling pathway (Dinwiddie and Harrod, 2008; Ren et al., 2011). Type I IFN signaling, which is initiated through activation of the interferon α receptor (IFNAR), is thought to be integral to the early immune response through the induction of anti-viral effector molecules (Barber, 2001; Biron, 2001; Sadler and Williams, 2008). Recent data has also revealed a role for type I IFN signaling in the pathogenesis of respiratory diseases. An IFNAR-deficient model of RSV infection shows significantly greater inflammatory cytokine levels and weight loss than infected WT mice (Goritzka et al., 2014).

Type I IFNs have been shown to initiate cyclic signaling cascades responsible for the production of an inflammatory response. In studies of Francisella, researchers have shown that type I IFN is capable of upregulating the absent in melanoma 2 (AIM2) inflammasome (Choubey et al., 2010; Fernandes-Alnemri et al., 2010). AIM2 activation results in caspase1 activation and the production of IL-1 β and IL-18, as well as pyroptosis in infected cells (Fernandes-Alnemri et al., 2009). Type I IFN are also able to induce apoptosis, in conjunction with TLR3 and TLR4, through the suppression of Akt signaling and degradation of the CDK inhibitory protein, p27. RIG-I, and MDA5, shown to be important in innate signaling of HMPV (Banos-Lara Mdel et al., 2013; Goutagny et

al.), are also upregulated by type I IFN signaling (Haller et al., 2006; Jaitin and Schreiber, 2007). These receptors signal through the mitochondrial adaptor IPS-1 to induce the production of proapoptotic BCL-2 homology 3 (BH3)-only proteins Puma and Noxa, and the activation of caspase-9 and Apaf-1-dependent mitochondrial apoptosis (Besch et al., 2009).

This study used an established model of HMPV infection in a mouse model lacking the type I IFN receptor (IFNAR^{-/-} mice) to elucidate the role of type I IFN on the control of early viral replication and the pathogenesis of the virus. We found that IFNAR^{-/-} mice had significantly higher viral titers early and at peak replication (Day 5) but were not impaired in the ability to clear HMPV. Mice challenged with virus 3 weeks after initial virus infection were immune to HMPV, and investigation into the neutralizing antibody (nAb) response showed higher nAb titer in IFNAR^{-/-} animals. Higher levels of inflammatory cytokine transcripts and protein expression were also seen in WT, compared to transgenic animals, and the pulmonary dysfunction, measured by pulse oximetry, indicated significantly higher breathing effort in WT mice compared to IFNAR^{-/-}. Analysis of histological lung sections from both groups showed that, corroborating our breath distension data, WT mice had significantly more lung inflammation during HMPV infection.

Results

Type I IFN signaling limits HMPV replication and spread, but also contributes to disease pathogenesis

To characterize the role of IFNAR signaling in HMPV infection, we investigated the kinetics of HMPV replication in an IFNAR deficient (IFNAR^{-/-}) transgenic mouse model. IFNAR^{-/-} mice exhibited significantly more infectious virus in the lungs at the peak

of HMPV infection (3 and 5 days post infection) compared to wild-type (WT) controls. However, viral clearance in IFNAR^{-/-} mice was similar to WT animals, with decreasing virus titers at day 7 and no detectable virus by day 10 (Figure 3-1). WT and IFNAR^{-/-} mice were challenged 22 days post-infection and neither had detectable viral replication.

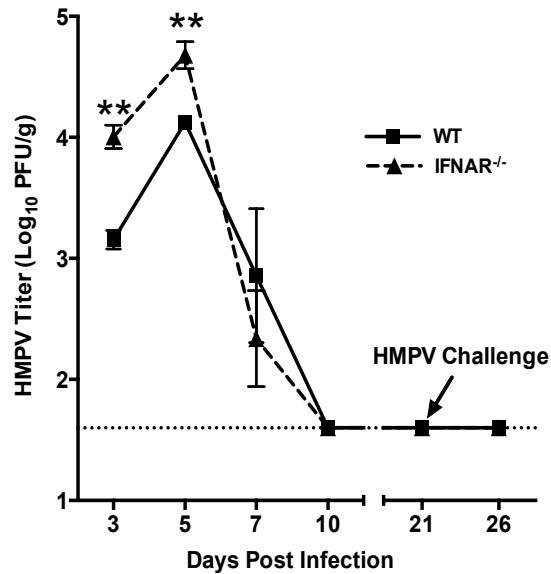


Figure 3-1. Type I IFN signaling limits early HMPV replication, but is not necessary for clearance. Plaque assays to measure infectious HMPV titer in the lungs of WT and IFNAR^{-/-} mice were performed on days 3, 5, 7, and 10 post-infection. A group of mice were challenged 3 weeks after initial infection, and plaque assays were performed 5 days post-infection. N = 9 per group for day 3, 5, and 7, and an N = 5 for each group on days 10 and 26. Groups were compared with two-way ANOVA. * = P < 0.05, ** = P < 0.02.

To investigate the role of IFNAR in disease severity during HMPV infection, we first assessed weight loss and breath distension. IFNAR^{-/-} mice showed significantly less weight loss compared to WT mice early after infection (Figure 3-2A). We utilized a mouse oximeter to quantify airway dysfunction, a key feature of severe LRI in humans (Hartert et al., 1999). Airway dysfunction and subsequent air trapping during HMPV infection leads to pulsus paradoxus, an exaggeration in the pulse volume during respiration as a result of increased breathing effort (Rebuck and Pengelly, 1973) which we quantified as breath distension. IFNAR^{-/-} mice had significantly decreased breath

distension compared to WT animals throughout the course of HMPV infection (Figure 3-2B). Measurements from HMPV-infected IFNAR^{-/-} mice were similar to those of uninfected WT mice (not shown).

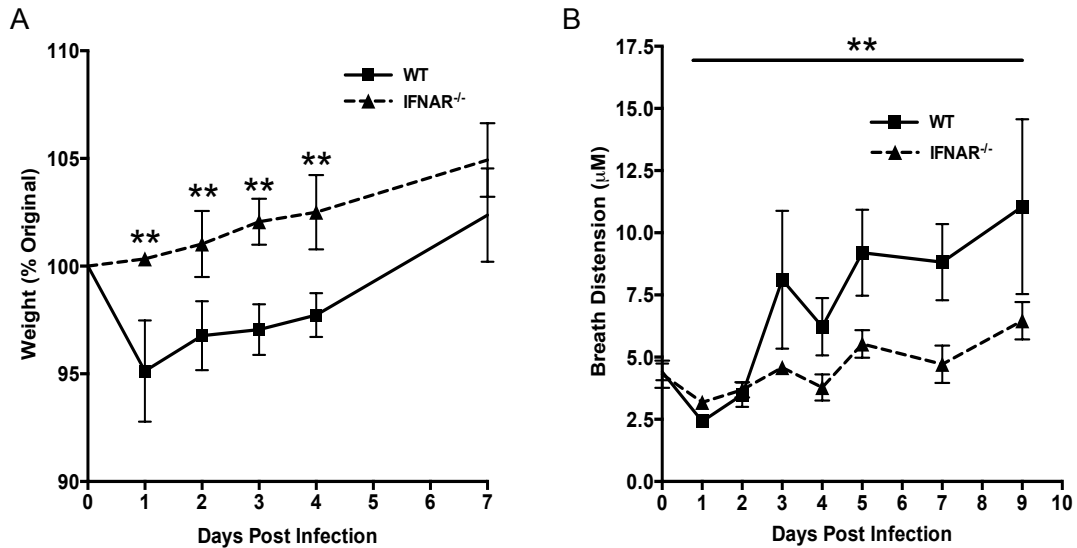


Figure 3-2. Type I IFN contributes to disease pathogenesis during HMPV infection. (A) WT and IFNAR^{-/-} mice were weighed following infection with HMPV. Data are expressed as a % of average original body weight in each group; error bars represent the SEM. N = 4 mice per group. (B) Breath distension of IFNAR^{+/+} mice and IFNAR^{-/-} mice was measured using pulse oximetry following infection HMPV. N = 4-10 mice per day per group with at least two independent experiments. Groups were compared with unpaired t test or two-way ANOVA. * = P < 0.05, ** = P < 0.02.

Type I IFN signaling contributes to histopathological disease during HMPV infection, and IFNAR deficiency leads to lower levels of inflammatory cytokines

To determine whether the marked lack of airway dysfunction in IFNAR^{-/-} mice corresponded with histopathological disease, HMPV infected lungs from WT, IFNAR^{-/-} mice, and uninfected control mice, were stained with H&E (Figure 3-3A) and periodic acid-Schiff (PAS), which preferentially stains polysaccharides in mucus (Figure 3-3B). HMPV-infected WT mice had increased inflammatory infiltrate compared to uninfected animals, while the lung architecture of infected IFNAR^{-/-} mice appeared similar to uninfected controls with less inflammatory infiltrate (Figure 3-3A). Additionally, there

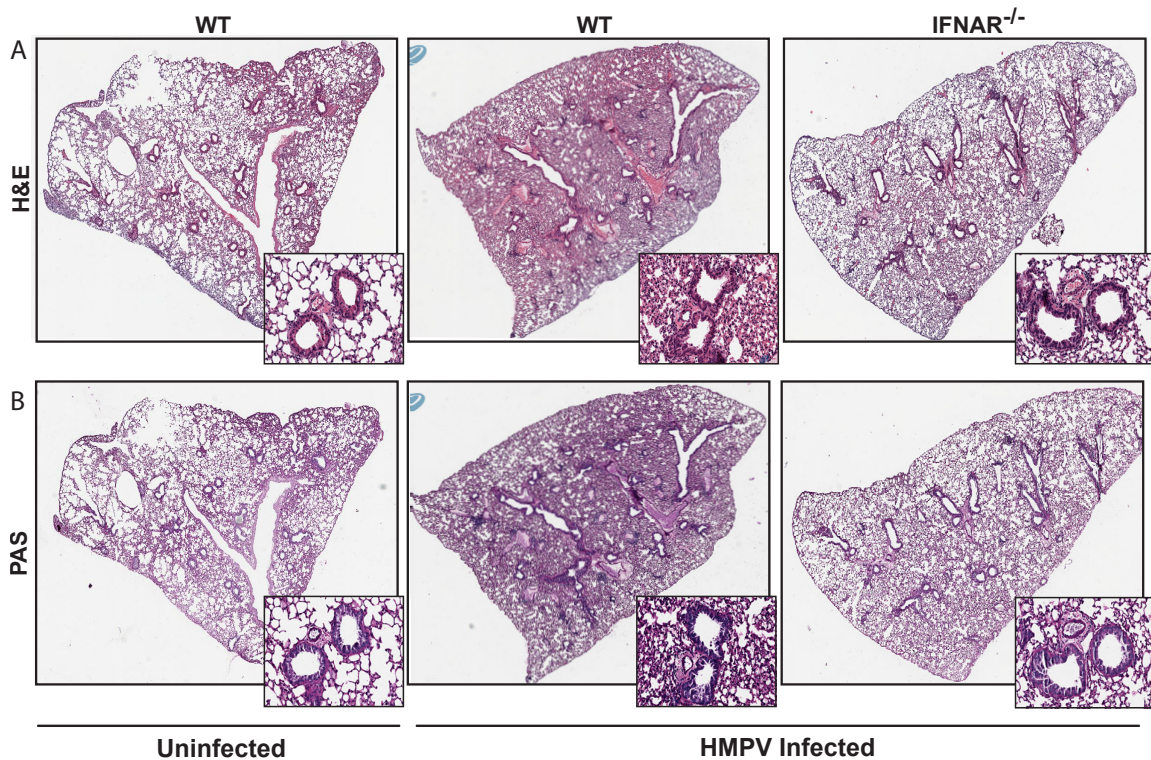


Figure 3-3. Type I IFN signaling contributes to histopathological disease during HMPV infection. Lungs from WT and IFNAR^{-/-} mice 5 days after HMPV infection, or uninfected control animals, were stained with (A) H&E or (B) PAS (polysaccharide specific). Images of whole lungs from WT and knockout animals were taken at 1x using Aperio scope. 20x representative lung images are inset.

appeared to be more mucus production in the lungs of WT as compared to IFNAR^{-/-} mice (Figure 3-3B).

Analysis of the lung histopathology using a formal scoring system identified significantly higher inflammation scores in the WT compared to IFNAR^{-/-} groups (Figure 3-4A). We also measured cytokine levels from whole lung homogenates collected from HMPV infected, as well as uninfected, WT and IFNAR^{-/-} mice using RT-PCR. In uninfected animals, cytokine levels are undetectable by this assay, or present at very low levels (data not shown). Therefore we compared infected WT to infected IFNAR^{-/-} mice. These analyses revealed that TH₁ cytokines, IFN- γ , IL-2 and IL-12, and IL-4 were

decreased in HMPV-infected IFNAR^{-/-} mice as compared to HMPV-infected WT mice (Figure 3-4B). IFN γ protein levels were also measured by ELISA, and found to corroborate transcript data indicating higher inflammatory cytokines in WT mice (Figure 3-4C) Together, these data suggest that IFNAR signaling contributes to disease pathogenesis in the lungs of HMPV infected mice.

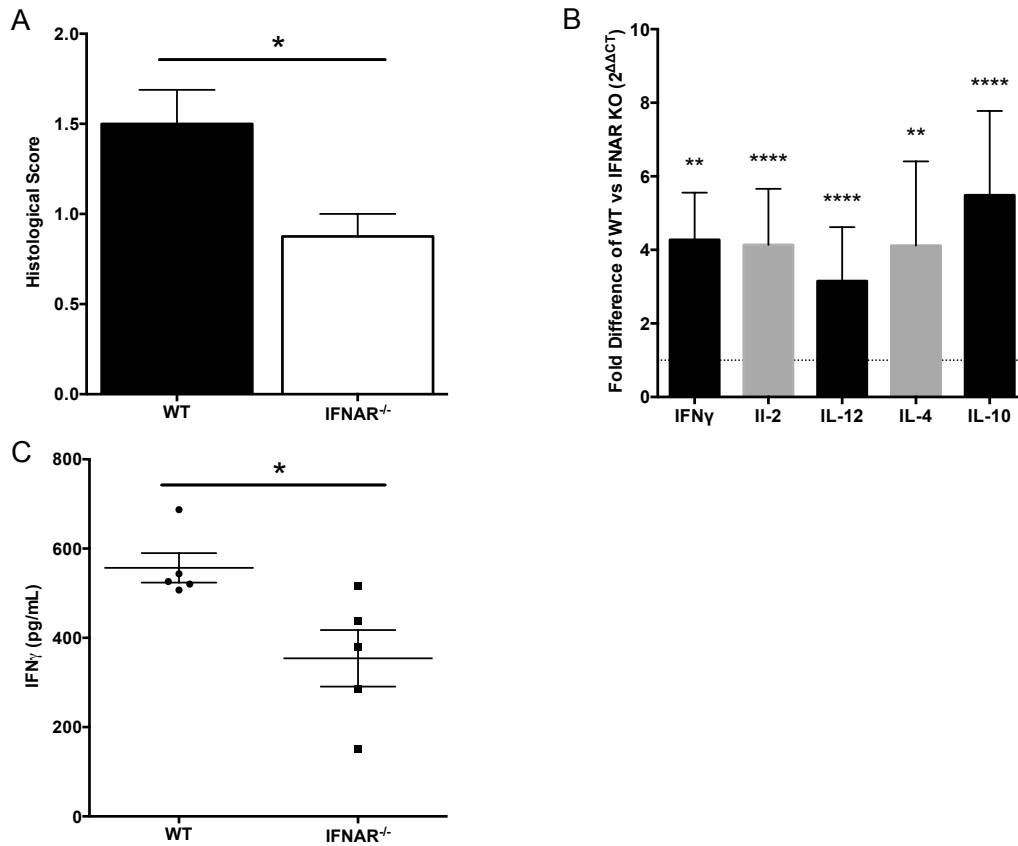


Figure 3-4. IFNAR deficiency leads to lower level of inflammatory cytokines. (A) A board-certified pathologist scored lungs stained with H&E from WT and IFNAR^{-/-} mice 5 days after HMPV infection, or uninfected control animals. N = 8 mice per group. (B) At 7 days post-infection, lungs were harvested and cytokine levels were measured using RT-PCR. Data are expressed as a $\Delta\Delta CT$ analysis between IFNAR and WT groups. N = 8 mice per group. (C) At 7 days post-infection, lungs were harvested and IFN γ levels were determined using ELISA. N = 5 mice per group. Groups were analyzed by one-way ANOVA. Error bars represent SEM. * = P < 0.05, ** = P < 0.02, **** = P < 0.0001.

Discussion

We investigated early and late immune responses in IFNAR-deficient mice to elucidate the effect of IFNAR deficiency on the control and clearance of this virus *in vivo*. These data demonstrate that IFNAR signaling contributes to limiting both replication and spread of HMPV, similar to what has been observed in related viruses (Johansson et al., 2007; Johnson et al., 2005; Schilte et al., 2010; Seo et al., 2011). One group found that for RSV in BALB/c mice, both type I and type II IFNs were important (Durbin et al., 2002; Johnson et al., 2005). IFNAR^{-/-} mice displayed significantly higher levels of infectious virus and more HMPV antigen⁺ lung epithelial cells. By 10 days after HMPV infection, virus was not detectable by either plaque assays or direct staining of lung epithelial cells. We were not able to detect any HMPV⁺ CD11c⁺ lung macrophage/DCs at either 5 or 10 days after HMPV infection. IFNAR^{-/-} mice were immune to HMPV challenge after primary infection and had a higher serum titer of neutralizing antibody than WT mice. This increase in nAb titer could be due to increased viral antigen in IFNAR^{-/-} mice, and potentially highlights an advantage to a more finely tuned modulator of the type I IFN pathway in using host innate immunity to prevent antigen loads from reaching levels necessary to develop sterilizing immunity. Moreover, these data show that in this model, type I IFN is not required to develop a fully protective response.

Recent studies have shown that RSV infection of IFNAR deficient mice leads to lower levels of inflammatory cytokines (Gautier et al., 2005; Goritzka et al., 2014; Rudd et al., 2007), and our data show that, similarly, IFNAR^{-/-} mice infected with HMPV have fewer inflammatory cytokine transcripts. Histological analysis of infected animals showed significantly less lung inflammation in IFNAR^{-/-} compared to WT mice, and IFNAR^{-/-} mice had significantly less lung dysfunction and weight loss during infection, suggesting that IFNAR signaling contributes substantially to the major disease symptoms associated with HMPV infection (Edwards et al., 2013; Kolli et al., 2008; Williams et al., 2004). Thus,

although IFNAR signaling suppresses early viral replication, it is not essential for clearance of HMPV and contributes to disease pathogenesis. A recent paper focused on the response to HMPV in neonatal mice deficient in important adaptors of the innate cytokine response, IPS-1, IRF3 and IRF7, also corroborate our findings regarding the importance of the balance between protection and immune-induced pathogenesis for type I IFN (Spann et al., 2014).

Materials and Methods

Mice and Viruses. C57BL/6 (B6) mice were purchased from The Jackson Laboratory. IFN alpha/beta receptor deficient (IFNAR^{-/-}) B6 mice were kindly provided by Dr. Herbert W. Virgin. All animals were bred and maintained in specific pathogen-free conditions under guidelines approved by the AAALAC and the Vanderbilt Institutional Animal Care and Use Committee. Six to twelve-week-old age- and gender-matched animals were used in all experiments. HMPV (pathogenic clinical strain TN/94-49, genotype A2) was grown and titered in LLC-MK2 cells as previously described (Williams et al., 2005c). For all experiments, mice were anesthetized with intraperitoneal ketamine-xylazine and infected intranasally with 1.5×10^6 PFU of HMPV. Serum was collected from WT and IFNAR^{-/-} mice by submandibular venipuncture and was used in a plaque reduction assay to determine HMPV neutralizing antibody titers as previously described (Williams et al., 2005c). The median of triplicate values for % HMPV neutralization for each animal of each genotype were plotted as a function of log₂-transformed serum dilution factor and analyzed in a sigmoidal dose response curve to determine IC₅₀. Nasal turbinates (NT) and lungs were collected for analysis and tissue viral titers measured by plaque titration as previously described (Williams et al., 2005c). For histopathology, the left lung was removed and inflated with 10% buffered formalin, paraffin-embedded, stained, and

analyzed using a formal scoring system in a group-blinded fashion by an experienced lung pathologist as previously described (Erickson et al., 2012).

Pulse Oximetry. To measure breath distension, mice were anesthetized using an isoflurane:oxygen (2%:98%) (2 liters/min) mixture (VetEquip). Mice were secured on their backs and were given constant anesthesia during the procedure via a nosecone attachment. The right leg and thigh were shaved, and a thigh sensor was secured to the right thigh of each mouse and covered with a dark cloth to reduce ambient light. A pulse oximeter (MouseOx; Starr Life Sciences Corp.) was used to measure arterial O₂ saturation, heart rate, pulse rate, pulse distension, and breath distension every 0.1 seconds (MouseOx software, version 4.0). Each mouse was monitored until sufficient data was collected in which all parameters were successfully measured, and only these data were used in analyses (1-2 minutes per mouse). Breath distension for each animal was calculated by averaging all measurements for each mouse per condition per genotype.

Real-time RT-PCR. 200 µL of undiluted lung homogenate from infected or uninfected IFNAR^{+/+} and IFNAR^{-/-} mice was lysed with an equal volume of RLT lysis buffer (Qiagen) and frozen at -20°C. Samples were thawed and RNA extracted using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Applied Sciences) on a MagNA Pure LC using the Total NA External Lysis protocol and stored at -80°C. Real-time RT-PCR was performed in 25µL reaction mixtures containing 5µL of extracted RNA on an ABI StepOnePlus Real-Time PCR System (Life Technologies/Applied Biosystems) using the AgPath-ID One-Step RT-PCR kit (Life Technologies/Applied Biosystems). For PD-L1, IFNγ, IL-2, IL-12, IL-4, and IL-10 gene expression, exon-spanning primers and probes were used according to the manufacturer's instructions (Applied Biosystems/Ambion). All

values were normalized to the housekeeping gene HPRT. Cytokine transcript levels were low or undetectable in uninfected IFNAR^{-/-} and WT mice. Therefore, experimental samples are reported as fold change in HMPV-infected WT mice compared to HMPV-infected IFNAR^{-/-} using the $\Delta\Delta\text{Ct}$ method (Livak and Schmittgen, 2001). Samples with cycle threshold (Ct) values less than 40 were considered positive.

Statistical Analyses. Data analysis was performed using Prism v4.0 (GraphPad Software). Groups were compared using unpaired t-test or one-way ANOVA with Tukey post-hoc test for multiple comparisons. P <0.05 was considered significant by convention.

CHAPTER IV

Type I IFN signaling primes an efficient T_{CD8} response by expanding lung dendritic cells

Work presented in this chapter also published in the Journal of Virology (Hastings et al., 2015)

Introduction

Type I IFN signaling is thought to be integral to the early immune response through the induction of anti-viral effector molecules (Barber, 2001; Biron, 2001; Sadler and Williams, 2008), and this pathway can modulate the adaptive immune response by contributing to both clonal expansion and maintenance of memory T cells as well as priming and differentiation of antigen presenting cells (APCs) (Havenar-Daughton et al., 2006; Kolumam et al., 2005a; Le Bon and Tough, 2008; Luft et al., 1998). In addition, it has been shown that type I IFN can protect CD8⁺ T-cells from NK cell cytotoxicity, by a perforin-dependent mechanism (Xu et al., 2014). This NK cell-mediated death was demonstrated to depend on signaling through the natural cytotoxicity triggering receptor 1 (NCR1) ligand, which is down regulated on CD8⁺ T cells in response to IFNAR signaling (Crouse et al., 2014).

Recent data indicate that HMPV infection generates functionally impaired virus-specific CD8⁺ T cells in the lungs as a result of signaling through the inhibitory receptor programmed death 1 (PD-1) (Erickson et al., 2012). PD-1, along with other inhibitory receptors, has been shown to be highly upregulated in both cancer and chronic viral infections (Fourcade et al., 2010; Jin et al., 2010a; Zhou et al., 2011), but little is known about the role of PD-1 in acute respiratory viral infections. The ligand for PD-1, programmed death ligand 1 (PD-L1), is expressed on professional APCs as well as

primary infected lung epithelial cells and is thought to be induced in an IFN-dependent manner (McNally et al., 2013; Muhlbauer et al., 2006).

In this study, we used an established model of HMPV infection to demonstrate that genetic ablation of the IFN α receptor (IFNAR^{-/-} mice) diminished the HMPV-specific CD8⁺ T cell response. We found that although IFNAR-deficient animals were able to clear the virus after infection, and developed significantly higher antibody titers, they displayed lower overall disease and lung inflammation than wild-type animals. Despite similar PD-1 expression and lower PD-L1 expression levels in IFNAR^{-/-} mice during HMPV infection compared to wild-type mice, HMPV-specific CD8⁺ T cells were more impaired in IFNAR^{-/-} than WT. T cell Ig- and mucin-domain-containing molecule-3 (TIM-3) was significantly upregulated on HMPV-specific CD8⁺ T cells in the IFNAR^{-/-} animals. Further investigation into the specific APC subsets in the lung during HMPV infection showed that alveolar macrophages do not rely on IFNAR signaling for expansion or the expression of PD-L1, but significantly fewer dendritic cells (DCs) were found in IFNAR^{-/-} mice during HMPV infection. Both DCs and interstitial macrophages upregulated PD-L1 in an IFNAR-dependent manner, while alveolar macrophages expressed high levels of the inhibitory ligand, compared to other lung APC subsets, in both infected and uninfected WT and IFNAR^{-/-} mice.

Results

Lack of type I IFN signaling leads to higher serum nAb titers, and does not change CD8+ T cell or CD11c+ APC infiltration during HMPV infection

The cytokine data presented in chapter III suggested a deficiency in the adaptive cellular immune response to HMPV infection in IFNAR^{-/-} mice. To test the hypothesis that IFNAR signaling promotes adaptive immune responses to HMPV, we assessed

neutralizing antibody (nAb) titer and examined immune cells known to be important in the host response to respiratory viruses in the lungs of WT and IFNAR^{-/-} mice. Twenty-one days post-HMPV infection, serum was collected from previously infected WT and IFNAR^{-/-} mice, and plaque neutralization assays were performed. IFNAR^{-/-} mice possessed significantly higher nAb than WT (Figure 4-1), but, as indicated in Figure 3-1, both groups were immune to reinfection.

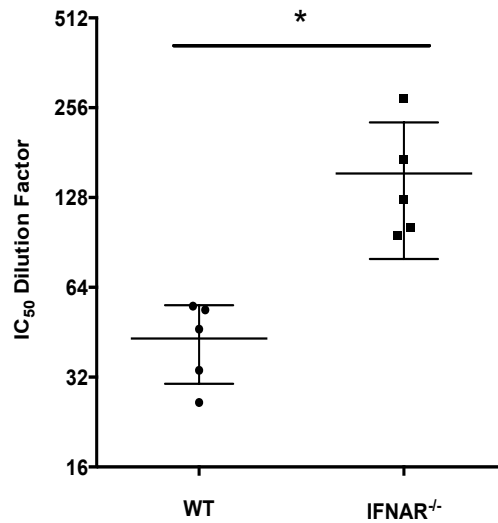


Figure 4-1. Lack of type I IFN signaling leads to higher serum nAb titers. WT C57BL/6 mice and IFNAR knockout mice were infected with 10⁶ PFU of HMPV and 21 days post-infection animals were bled and serum was harvested. *In vitro* neutralization assays were performed in triplicate on LLC-MKC cells with increasing dilutions of previously infected mouse serum to determine IC₅₀. Median values of % neut. for an N = 5 for each group were plotted as a function of the serum dilution factor to obtain best fit curves, and the IC₅₀ for each curve was calculated. Groups were compared using one-way ANOVA with Tukey post-hoc test. Error bars represent SEM. * = P < 0.05.

Next, we examined the infiltration of immune cells to the infected lung. Ten days post-HMPV infection, at the peak of the adaptive response, the total number of lymphocytes in the lungs of WT and IFNAR^{-/-} mice was not significantly different (Figure 4-2A). CD11c⁺ high lung cells, which are important for induction of innate and adaptive immune responses via viral antigen presentation, were significantly increased in both the IFNAR^{-/-} and WT mice 10 days post infection compared to uninfected controls. However,

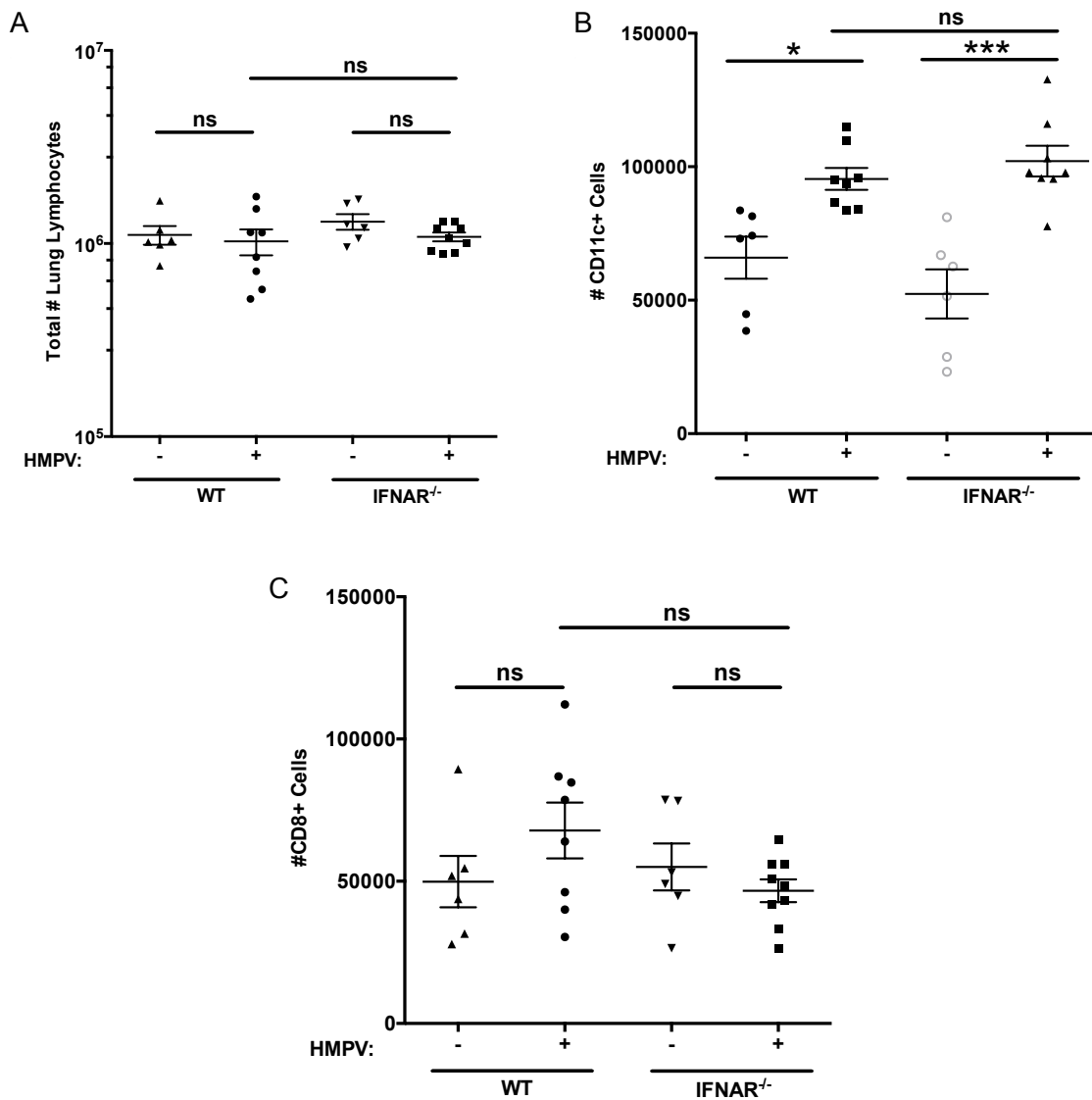


Figure 4-2. Type I IFN signaling does not change CD8⁺ T cell or CD11c⁺ dendritic cell infiltration during HMPV infection. Lungs from WT and IFNAR^{-/-} mice 5 (A) or 10 days (B,C) after HMPV infection, or uninfected control animals, were harvested and analyzed by flow (A) Cells were gated on the small lymphocyte population, and the infiltration of these cells is shown as total number of lung lymphocytes. Specific staining of (B) CD11c and (C) CD8 allowed for the enumeration of CD8⁺ T-cells and CD11c⁺ dendritic cells. Total lung cell values, used to calculate number of cells, were measured by hemocytometer. Groups were compared using unpaired t test or one-way ANOVA with Tukey post-hoc test where appropriate. N = 4-15 mice per group with at least two independent experiments. Error bars represent SEM. * = P < 0.05, ***=P<0.002.

no statistically significant difference in CD11c⁺ high cell numbers was noted between the IFNAR^{-/-} and WT mice (Figure 4-2B). CD8⁺ T cells were not significantly different in HMPV-infected mice when measured as a percentage of the total lymphocyte population after HMPV infection in WT or IFNAR^{-/-} animals (Figure 4-2C). These data suggest that IFNAR signaling is not crucial for the recruitment of adaptive immune cells to the site of HMPV infection.

Efficient development of a functional HMPV-specific CD8⁺ T cell response requires type I IFN signaling

Given diminished respiratory tract pathology in IFNAR^{-/-} mice (Chapter II) but similar recruitment of inflammatory cells to the lungs (Figure 4-2) upon HMPV infection, we wondered whether the functionality of the cells that reach the lung in IFNAR^{-/-} mice differed compared to their WT counterparts. To test this hypothesis, we first quantified the virus-specific component of the adaptive immune response. To do this, we performed MHC tetramer staining for the immunodominant HMPV epitope F₅₂₈₋₅₃₆ (F528) to enumerate HMPV epitope-specific cells. IFNAR^{-/-} mice had significantly fewer F528-specific CD8⁺ T cells in the lungs compared with WT animals (Figure 4-3A). Next, we examined the functionality of these HMPV-specific cells using *ex vivo* peptide stimulation and intracellular cytokine staining (ICS) of lung lymphocytes collected 10 days post-infection. A significantly lower fraction of HMPV-specific CD8⁺ T cells from IFNAR^{-/-} mice produced IFN γ as compared to those from WT mice (Figure 4-3B). Based on previous studies showing that PD-1 signaling contributes to T cell impairment (Erickson et al., 2012), we quantified PD-1 expression on both HMPV-specific and total CD8⁺ T lymphocytes. Although PD-1 was upregulated on HMPV-specific CD8⁺ T cells compared to bulk CD8⁺ T cells following viral infection, PD-1 expression on these HMPV-specific T cells was similar between IFNAR^{-/-} and WT animals (Figure 4-3C). These data indicate

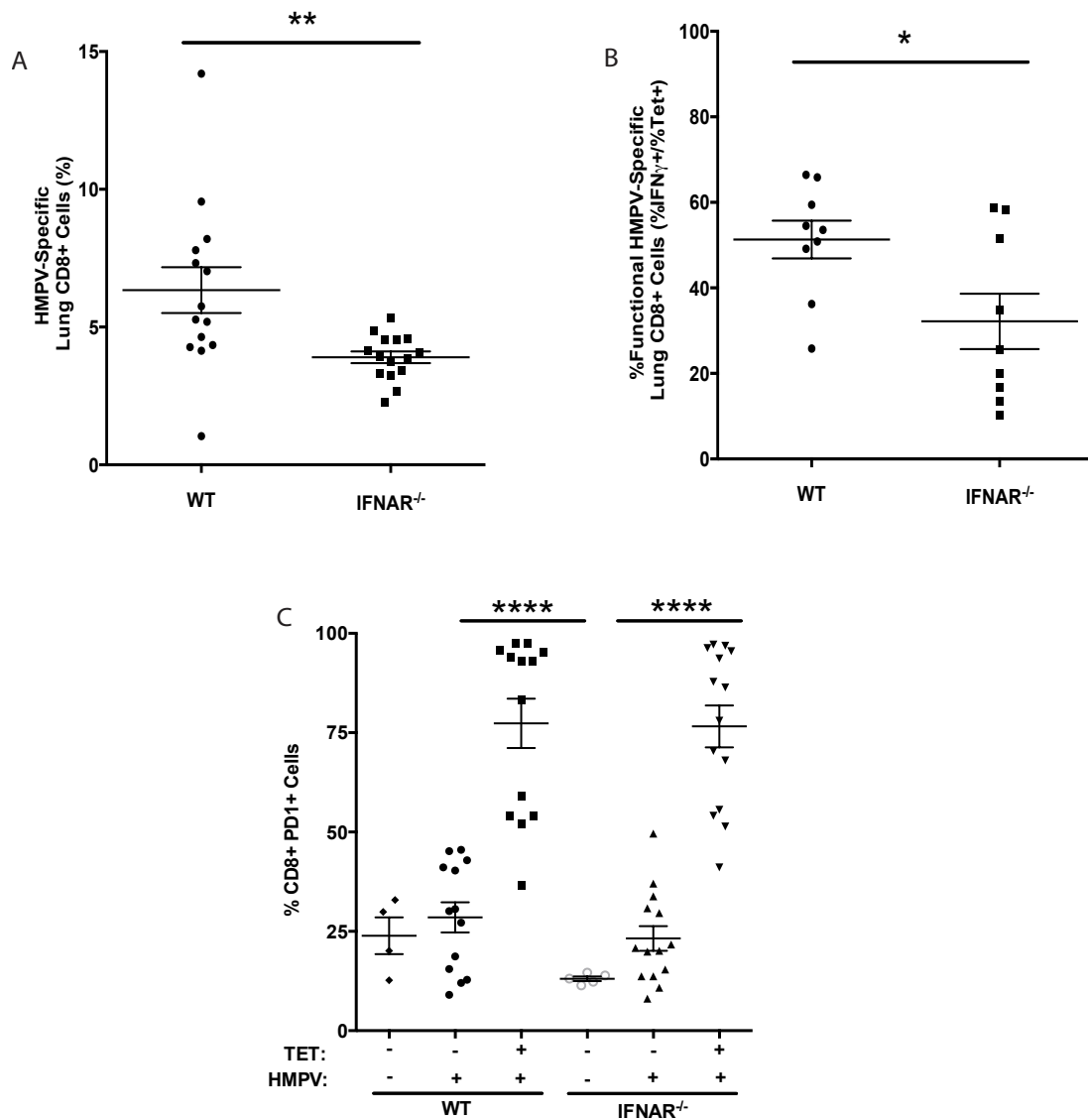


Figure 4-3. Efficient development of a functional HMPV-specific CD8⁺ T cell response requires type I IFN signaling. WT and IFNAR^{-/-} mice were infected with HMPV, and 10 days post-infection lungs were harvested and analyzed by flow cytometry. (A) An HMPV specific tetramer molecule was used to probe for virus specific CD8⁺ T cells in infected lungs for both groups. (B) Lymphocytes were also stimulated with an HMPV peptide in vitro, and intracellular cytokine staining was performed to analyze functionality of HMPV specific T-cells. (C) A specific antibody towards the inhibitory receptor PD-1 was used to examine its expression on bulk CD8⁺ T cells in infected and uninfected animals and HMPV specific CD8⁺ T cells from infected mice. Groups were compared using unpaired t-test or one-way ANOVA with Tukey post-hoc test where appropriate. N = 9-15 mice from at least two independent experiments per group. Error bars represent SEM. * = P < 0.05, ** = P < 0.02, **** = P < 0.0001.

an important role for type I IFN signaling in the development of functional pulmonary CD8⁺ T cells, and show that the absence of IFNAR signaling does not directly affect PD-1 expression on CD8⁺ T cells consistent with prior studies demonstrating TCR signaling as the primary determinant of PD-1 expression (Erickson et al., 2012).

Type I IFN signaling limits the spread of HMPV in lung epithelial cells

To further examine the infected lung epithelium in the mouse model, we developed an assay for the isolation and analysis of lung epithelial cells using flow cytometry. The average number of HMPV-infected lung cells in IFNAR^{-/-} and WT mice was analyzed using a monoclonal antibody to identify EPCAM⁺ lung epithelial cells, and a polyclonal anti-HMPV sera to identify HMPV⁺/EPCAM⁺ cells (Figure 4-4A). IFNAR^{-/-} animals had a significantly higher percentage of HMPV⁺/EPCAM⁺ lung epithelial cells on day 5 post-infection (Figure 4-4B). No viral antigen was detected by flow cytometry in epithelial cell populations at 10 days post infection or in CD11c⁺ high lung dendritic cells (DCs) at either 5 or 10 days post infection (data not shown). These data show that type I IFN is important in controlling not only replication as shown in figure 1, but also viral spread in the lung.

Expression of PD-L1 is driven by, but not dependent on, type I IFN signaling in lung epithelial cells and CD11c⁺ cells

Since the expression of PD-1 was not different on virus-specific IFNAR^{-/-} T_{CD8} compared to WT, we wondered whether the differences in CTL functionality we observed could be due to altered expression of the PD-1 ligand, PD-L1. Prior work has indicated that the promoter of PD-L1 contains IFN regulatory elements, and that IFN signaling can promote PD-L1 expression on epithelial and endothelial cells (Eppihimer et al., 2002; Lee et al., 2006b). We therefore quantified the expression of PD-L1 in respiratory

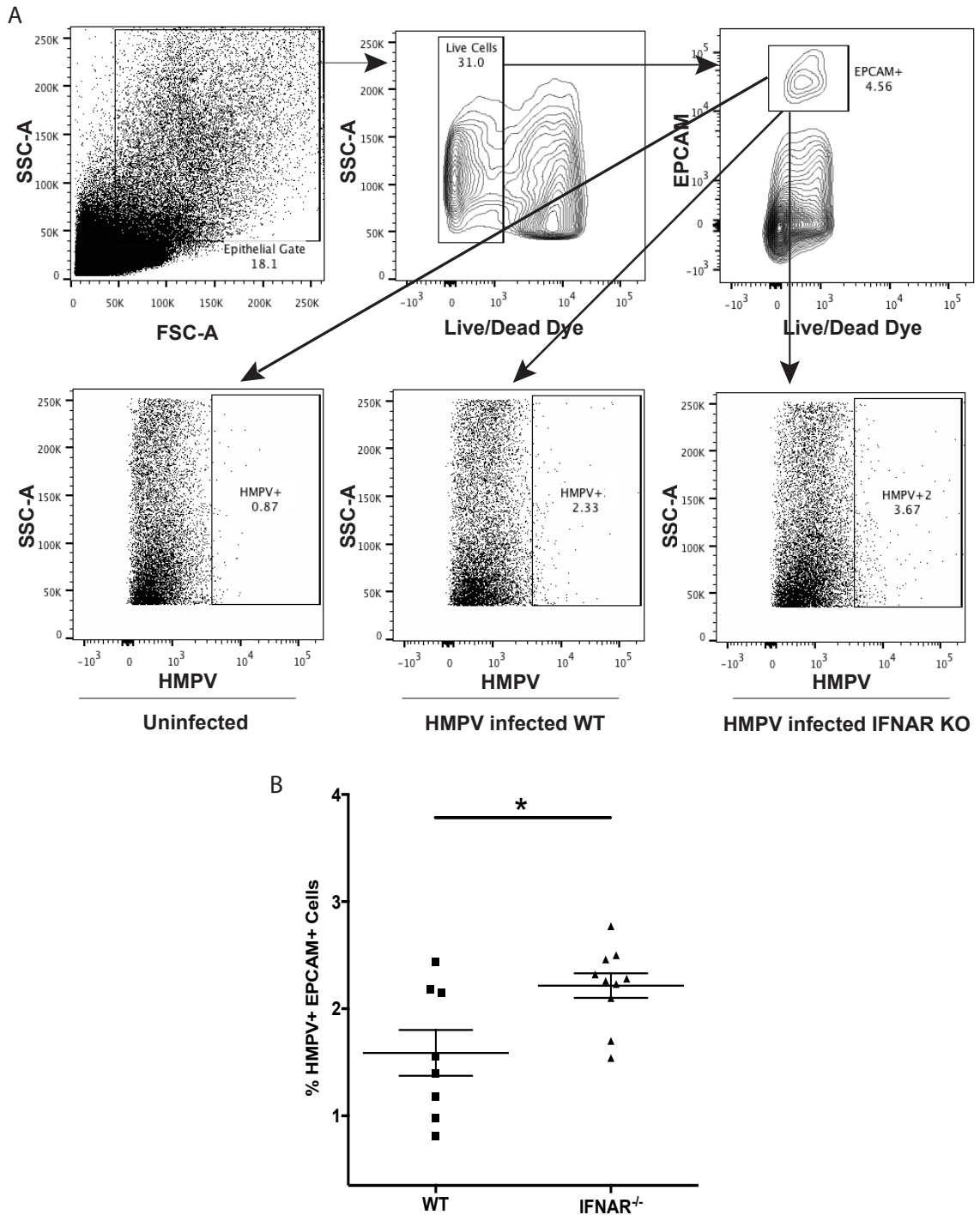


Figure 4-4. Type I IFN limits the spread of HMPV in lung epithelial cells. (A) Cells were stained for EPCAM and HMPV. Dead cells were excluded using an amine reactive dye. All analysis was done directly *ex vivo*. For analysis of HMPV+ EPCAM+ cells background staining of HMPV for uninfected mouse lung cells was determined and subtracted from experimental values. For analysis of PD-L1 expression, cells were probed with an isotype control antibody and this background was subtracted from experimental values. (B) At day 5 after HMPV infection, lungs were harvested and stained for EPCAM positive lung epithelial cells. These cells were then probed for HMPV

antigen with a polyclonal antibody and fluorescent secondary, and % HMPV-infected EPCAM+ cells for each group was measured using flow cytometry. N = 4-10 mice per day per group with at least two independent experiments. Groups were compared with unpaired Student's t test or two-way ANOVA. * $P < 0.05$.

epithelial cells and CD11c high cells on day 5 after HMPV infection. PD-L1 was significantly upregulated on both virus antigen-positive and virus antigen-negative lung epithelial cells in both WT and IFNAR^{-/-} mice (Figure 4-5A). However, PD-L1 expression was significantly lower in IFNAR^{-/-} animals as compared to WT animals. By 10 days post-infection, no differences were observed between the levels of PD-L1 expression in lung epithelial cells in HMPV infected WT compared to IFNAR^{-/-} mice (Figure 4-5B) or in total PD-L1 transcript levels in the lungs of these animals (Figure 4-6B), although the PD-L1 levels on epithelial cells were still significantly higher in both groups than uninfected mice. These results indicate that while IFNAR signaling can lead to increased expression of PD-L1, it is not an exclusive regulator of this ligand, and other signaling pathways can upregulate PD-L1.

PD-L1 expression on bulk CD11c⁺ lung cells 5 days post- HMPV infection in both WT and IFNAR^{-/-} mice increased slightly compared to uninfected animals, but did not reach significance, and no difference was detected between groups (Figure 4-5C). On day 10 post-infection, PD-L1 expression on CD11c⁺ cells was indistinguishable between infected and uninfected animals in both WT and IFNAR^{-/-} mice (Figure 4-5D). Later analyses into the subsets of CD11c⁺ cells in the lung, found in figures 4-8-10), demonstrate that, in fact, this population of cells is heterogeneous, and the discrete APC subsets in this gate have significant differences in their baseline expression of PD-L1 and the upregulation of this inhibitory ligand upon HMPV infection.

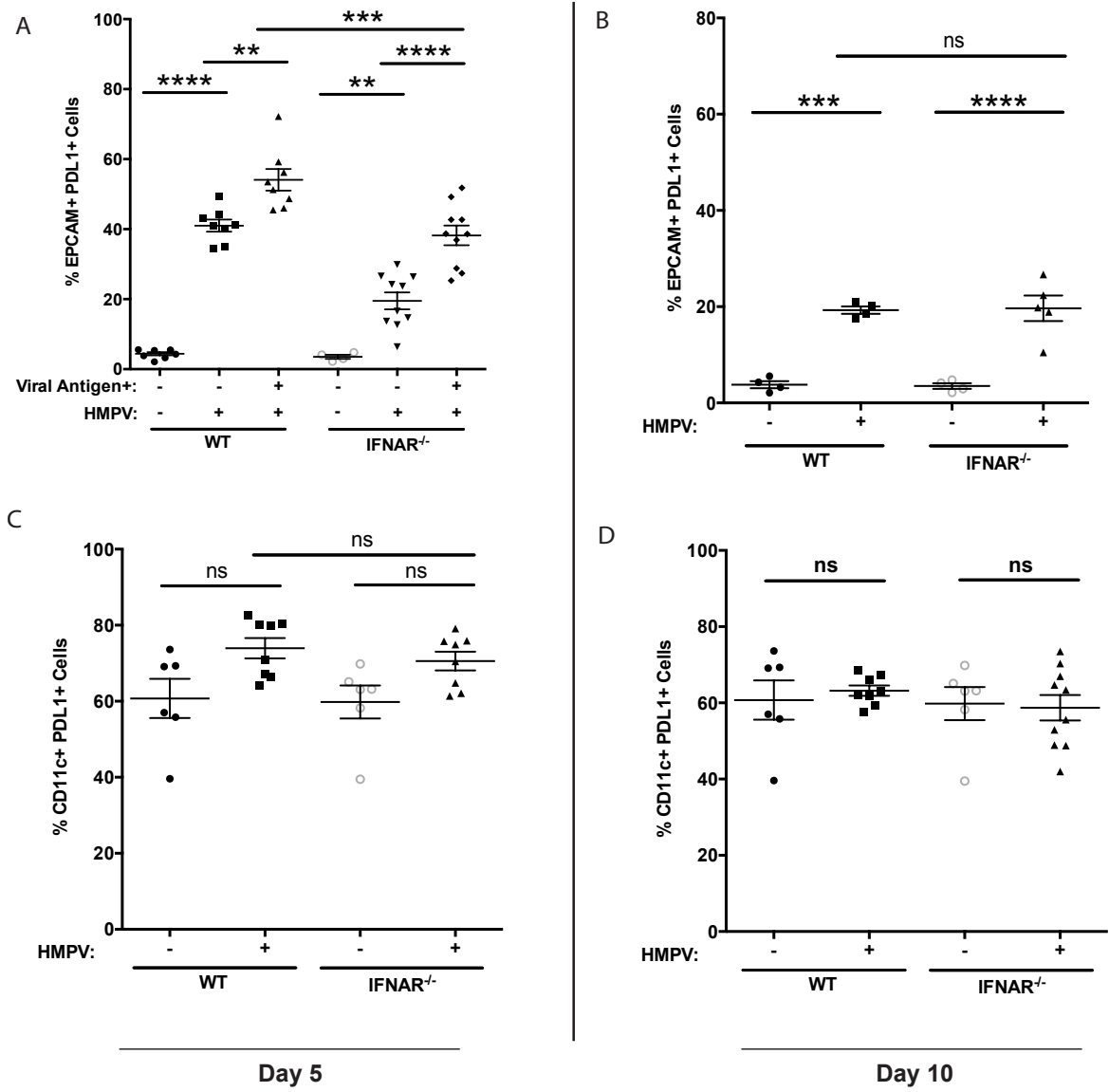


Figure 4-5. Expression of PD-L1 is driven by, but not dependent on, type I IFN signaling in lung epithelial cells and CD11c+ dendritic cells. WT and IFNAR^{-/-} mice were infected with HMPV, and after (A,C) 5 or (B,D) 10 days post-infection lungs were harvested, along with uninfected control (HMPV-), and analyzed by flow cytometry. Specific staining of (A,B) EPCAM and (C,D) CD11c allowed for the analysis of EPCAM+ lung epithelium and CD11c+ dendritic cells. EPCAM+ cells at day 5 were also probed for HMPV antigen with a polyclonal antibody and fluorescent secondary to enable gating of infected (HMPV Antigen +) and uninfected (HMPV Antigen -) cells. A specific antibody towards the inhibitory ligand PD-L1 was used to measure its expression on all epithelial and dendritic cell populations at each time point. Groups were compared using unpaired Student's t test or one-way ANOVA with Tukey post-hoc test where appropriate. N = 4-9 mice per experimental group representative of at least two independent experiments. Error bars represent SEM. * = P < 0.05, ** = P < 0.02, *** = P < 0.002, **** = P < 0.0001.

Functional impairment of HMPV specific CD8⁺ T cells is not due to Treg infiltration but rather corresponds to expression of the inhibitory receptor TIM3

IFNAR signaling has been shown to affect the development and recruitment of CD4⁺ regulatory T (Treg) cells, but the data are unclear as to whether that effect is positive or negative (de Andres et al., 2007; Golding et al., 2010; Namdar et al., 2010; Srivastava et al., 2014a). Tregs possess a myriad of anti-inflammatory properties, including suppressing virus-specific CTLs during LRI (Fulton et al., 2010). We therefore quantified Treg infiltration into infected lungs of IFNAR^{-/-} and WT mice at day 5 post-HMPV infection. We found significant infiltration of Tregs in the lungs of infected WT mice but not in IFNAR^{-/-} animals (Figure 4-6A). These results help explain the lower IL-10 transcript level we observed in IFNAR^{-/-} mice compared to WT mice (Chapter II), and suggest that IFNAR signaling is important for the infiltration of Tregs during HMPV infection. However, recruitment of Tregs does not appear to be responsible for the impaired CD8⁺ T cell phenotype.

We analyzed HMPV-specific CD8⁺ T cells 10 days post-infection for the expression of the other known inhibitory receptors TIM-3, LAG-3, and 2B4. A significant upregulation of 2B4 (Figure 4-7A) was observed on HMPV-specific CD8⁺ T cells, although there were no differences between WT and IFNAR deficient mice. No significant upregulation of LAG-3 (Figure 4-7B) was observed on HMPV-specific CD8⁺ T cells in either IFNAR^{-/-} or WT animals. However, we found that the percentage of TIM-3⁺ HMPV-specific CD8⁺ T cells was significantly higher in IFNAR^{-/-} compared with WT mice (Figure 4-7C). There were significantly more TIM3⁺ HMPV-specific CD8⁺ T cells in both IFNAR^{-/-} and WT mice than either naïve CD8⁺ T cells from uninfected animals or non-specific CD8⁺ T cells from HMPV-infected WT mice (Figure 4-7C). These data indicate that upregulation of the inhibitory receptor TIM-3 is associated with an exhausted

phenotype observed in HMPV-specific CD8⁺ T cells in the context of an IFNAR deficiency.

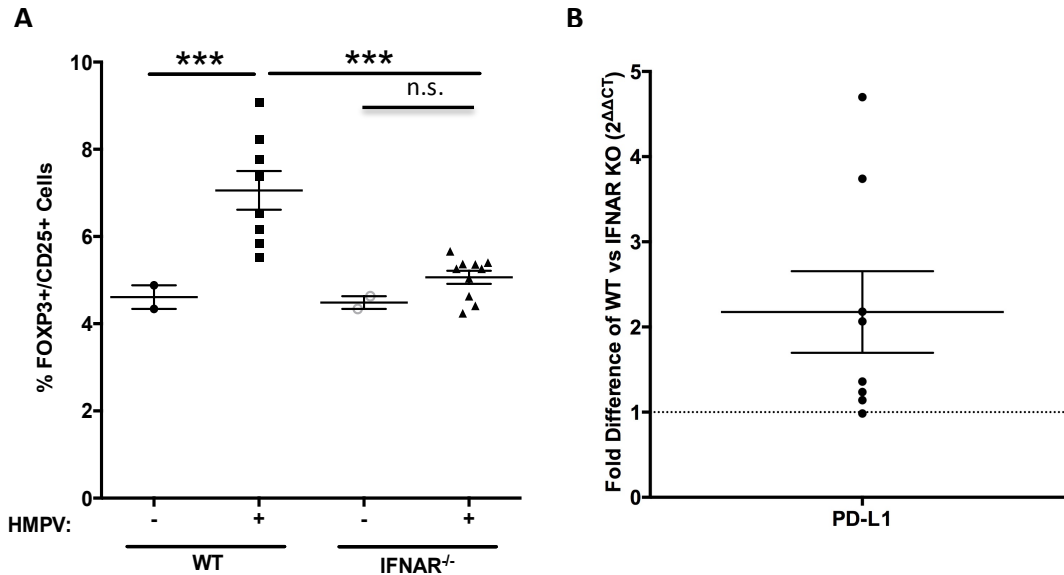


Figure 4-6. Functional impairment of HMPV specific CTLs is not due to Treg infiltration or total PD-L1 transcript. WT and IFNAR^{-/-} mice were infected with HMPV, and after (A) five or (B) seven days post-infection lungs were harvested and analyzed by flow cytometry or RT-PCR. (A) To identify CD4⁺ regulatory T cells, specific antibodies towards CD4, FOXP3, and CD25 were used in uninfected and infected mice. (B) Lungs were processed and RT-PCR was used to measure PD-L1 transcript levels in WT and IFNAR^{-/-} mice. Data are expressed as a 2^{ΔΔCT} analysis between IFNAR^{-/-} and WT groups. Groups were compared using one-way ANOVA with post-hoc Tukey test. N = 4-7 mice per experimental group with two independent experiment. Error bars represent SEM. ***=P<0.002.

Subsets of lung APCs are differentially affected by IFNAR signaling during HMPV infection

Five distinct categories of antigen presenting cells located in the mouse lung have been classified based upon their surface marker expression (Figure 4-8) as well as their ability to present viral epitopes to CD8⁺ T cells during infection (Misharin et al., 2013). Both alveolar macrophages and dendritic cells (DCs) (Figure 4-8, top right) express high levels of CD11c but can be distinguished from each other by the higher

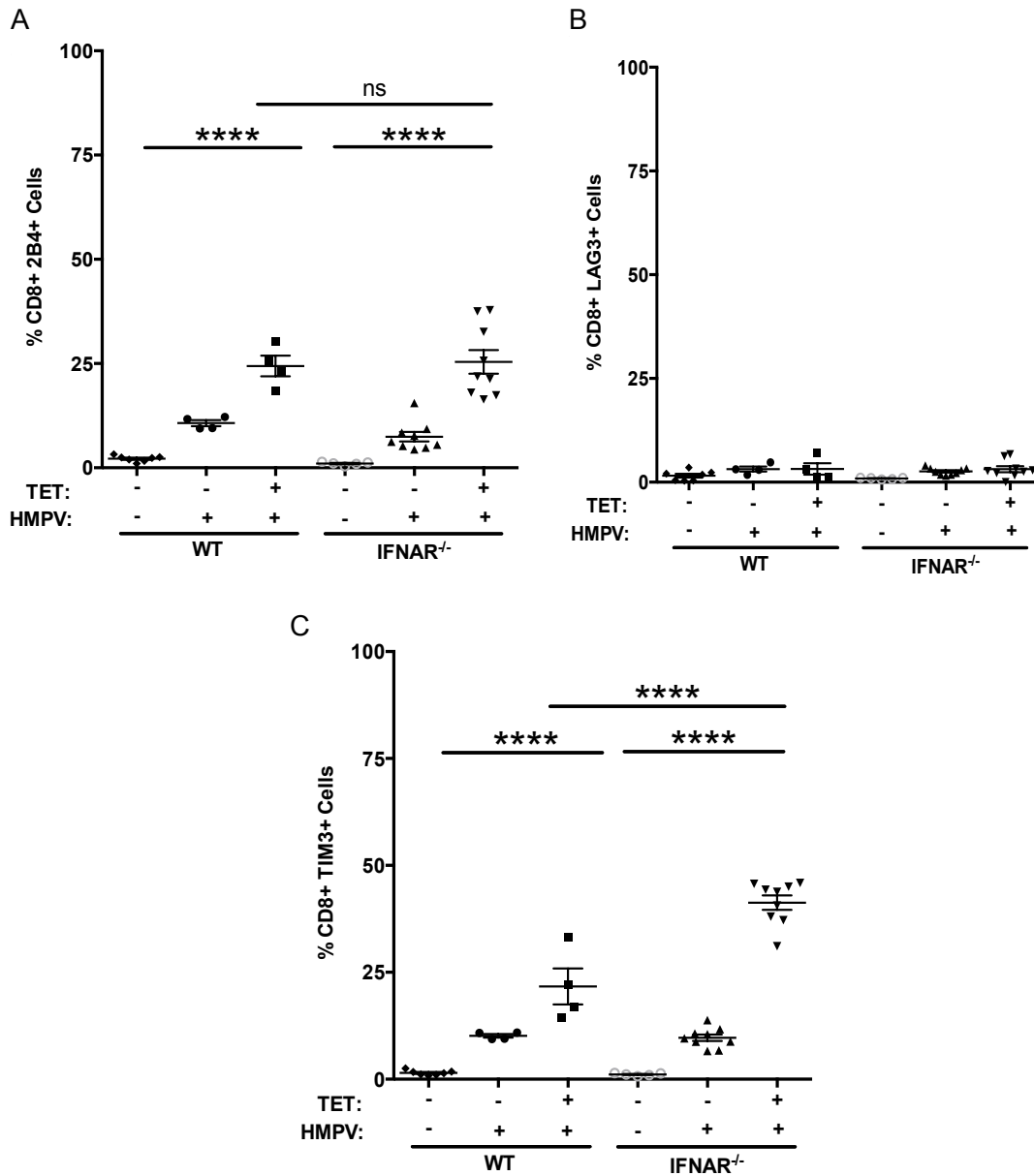


Figure 4-7. : Impairment of HMPV specific CTLs does not correspond with the expression of 2B4 or LAG3, but rather with expression of inhibitory receptor TIM3. WT and IFNAR^{-/-} mice were infected with HMPV, and after ten days post-infection lungs were harvested and analyzed by flow cytometry. Specific staining of bulk CD8+ T cells in uninfected and infected mice and HMPV specific CD8+ T-cells in infected animals with antibodies toward the inhibitory markers (A) 2B4, (B) LAG3, and (C) TIM3 was used to determine %inhibitory receptor positive cells in HMPV specific, as well as non-specific, CD8+ T-cells. Groups were compared using one-way ANOVA with post-hoc Tukey test. N = 4-5 mice per experimental group with two independent experiment. Error bars represent SEM. ***=P<0.002, **** = P < 0.0001.

level of MHC class II expression in the DC subset (Kirby et al., 2006). DCs in the lungs can also be broken down into two groups, CD11b⁺ and CD11b⁻ DCs (Figure 4-8, bottom right), which have also been shown to express CD103 (Furuhashi et al., 2012).

Plasmacytoid dendritic cells (pDCs) express low levels of both CD11c and MHC class II (Figure 4-8, top right). Interstitial macrophages (Figure 4-8, bottom left) in the lung do not express CD11c, but do express high levels of CD11b and moderate levels of MHC class II.

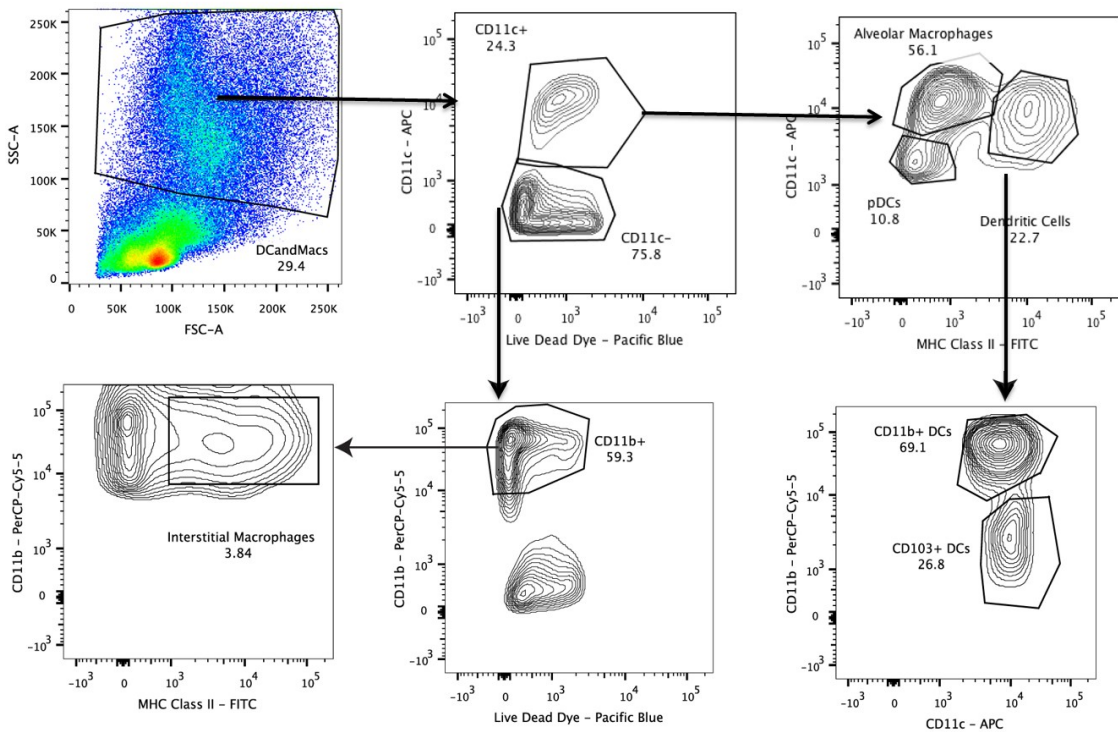


Figure 4-8. Gating strategies for dendritic cell and macrophage analysis. Cells were stained with CD11c, CD11b, and MHC Class II. Dead cells were excluded using an amine reactive dye. All analyses were done directly *ex vivo*. For analysis of PD-L1 expression, cells were probed with an isotype control antibody and this background was subtracted from experimental values.

During HMPV infection both WT and IFNAR^{-/-} mice display a significant increase in alveolar macrophages compared to uninfected animals in each group (Figure 4-9A), but the number of DCs proved to be IFN-dependent, as the number of pDCs as well as conventional DCs in the lungs of infected IFNAR^{-/-} animals equaled the number in

uninfected mice and was significantly fewer than HMPV-infected WT mice (Figure 4-9C and D). In addition, significantly more CD11b⁺ DCs were present in the lungs of HMPV-infected WT mice compared to uninfected WT or IFNAR^{-/-} mice and infected IFNAR^{-/-} mice (Figure 4-9E). No significant differences were seen in the number of CD11b⁻ DCs (Figure 4-9F) or interstitial macrophages between uninfected and infected or WT and IFNAR^{-/-} (Figure 4-9B). These data show that type I IFN is crucial for the recruitment and/or proliferation of DCs in infected mouse lungs, but that alveolar macrophages are unaffected by a lack of IFNAR signaling during HMPV infection.

IFNAR signaling affects the expression of PD-L1 on DCs and interstitial macrophages, but alveolar macrophages constitutively express high levels of PD-L1 regardless of the presence of IFNAR

To determine the ability for various subtypes of lung APCs to signal through the inhibitory PD1:PDL1 pathway, we analyzed the fraction of cells expressing the inhibitory ligand PD-L1 during HMPV infection. We found that nearly all alveolar macrophages in uninfected and infected WT and IFNAR^{-/-} mice expressed PD-L1 (Figure 4-10A). In contrast, the percentage of cells expressing PD-L1 on all DC subsets, as well as interstitial macrophages, during HMPV infection was significantly lower in infected IFNAR^{-/-} mice (Figures 4-10B-E). In DCs and interstitial macrophages of WT mice a significant increase in the fraction of PDL1⁺ cells was observed in HMPV-infected compared to uninfected mice, but upregulation of PD-L1 appears to be IFNAR dependent, as IFNAR^{-/-} mice displayed no difference in PD-L1 expression on these cells during infection (Figures 4-10B-E).

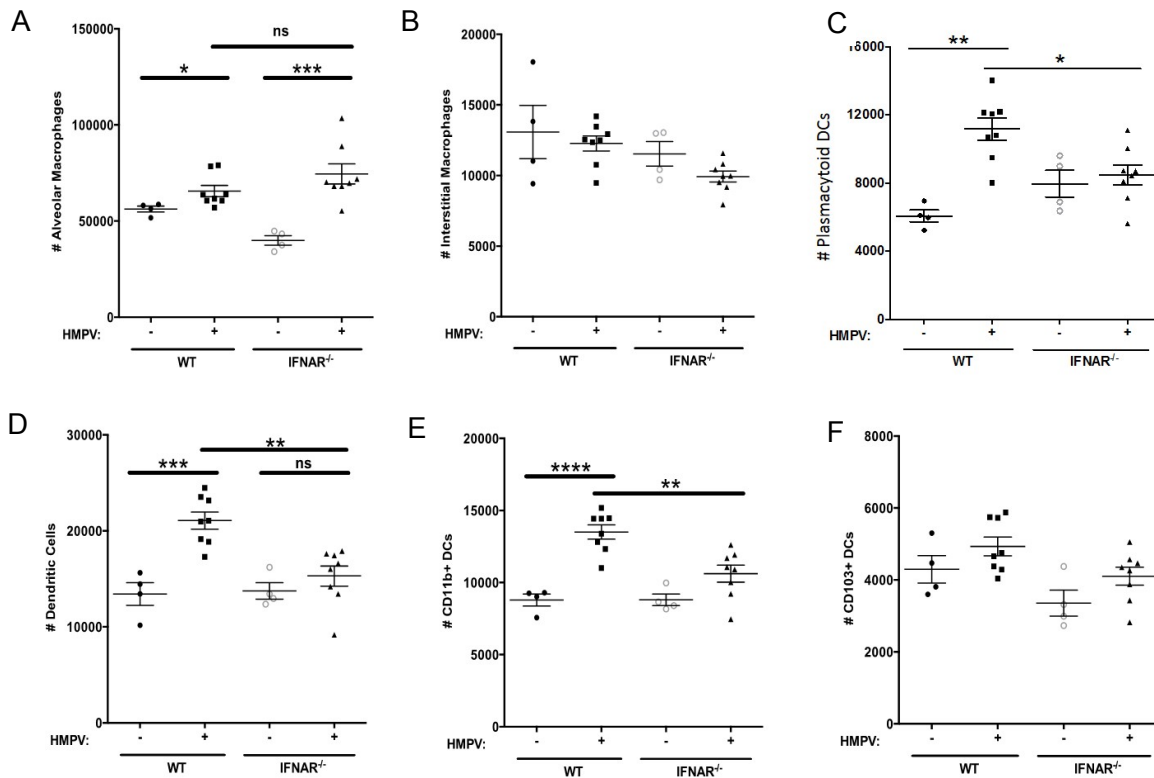


Figure 4-9. Subsets of lung APCs are differentially affected by type I IFN signaling during HMPV infection. WT and IFNAR^{-/-} mice were infected with HMPV, and after five days post-infection lungs were harvested and analyzed by flow cytometry. Cells were gated according to the gating strategy outlined in figure 4-8 as (A) CD11c⁺ MHCII^{mid} alveolar macrophages, (B) CD11c⁻ MHCII^{mid} CD11b⁺ interstitial macrophages, (C) CD11c^{lo} MHCII^{lo} plasmacytoid dendritic cells (D) CD11c⁺ MHCII^{high} dendritic cells, (E) CD11c⁺ MHCII^{high} CD11b⁺ dendritic cells and (F) CD11c⁺ MHCII^{high} CD11b⁻ dendritic cells. Total lung cell values, used to calculate number of cells, were measured by hemocytometer. Groups were compared using unpaired Student's t test or one-way ANOVA with Tukey post-hoc test where appropriate. N = 4-9 mice per experimental group representative of at least two independent experiments. Error bars represent SEM. * = P < 0.05, ** = P < 0.02, *** = P < 0.002, **** = P < 0.0001.

Additionally, the level of PD-L1 expression on alveolar macrophages as measured by mean fluorescence intensity was significantly higher than the other APC subsets (Figure 4-11). Type I IFN appears to be indispensable for PD-L1 expression on DCs and interstitial macrophages during HMPV infection, but my data suggest that alveolar macrophages constitutively express this inhibitory ligand at high levels in both uninfected and infected lungs.

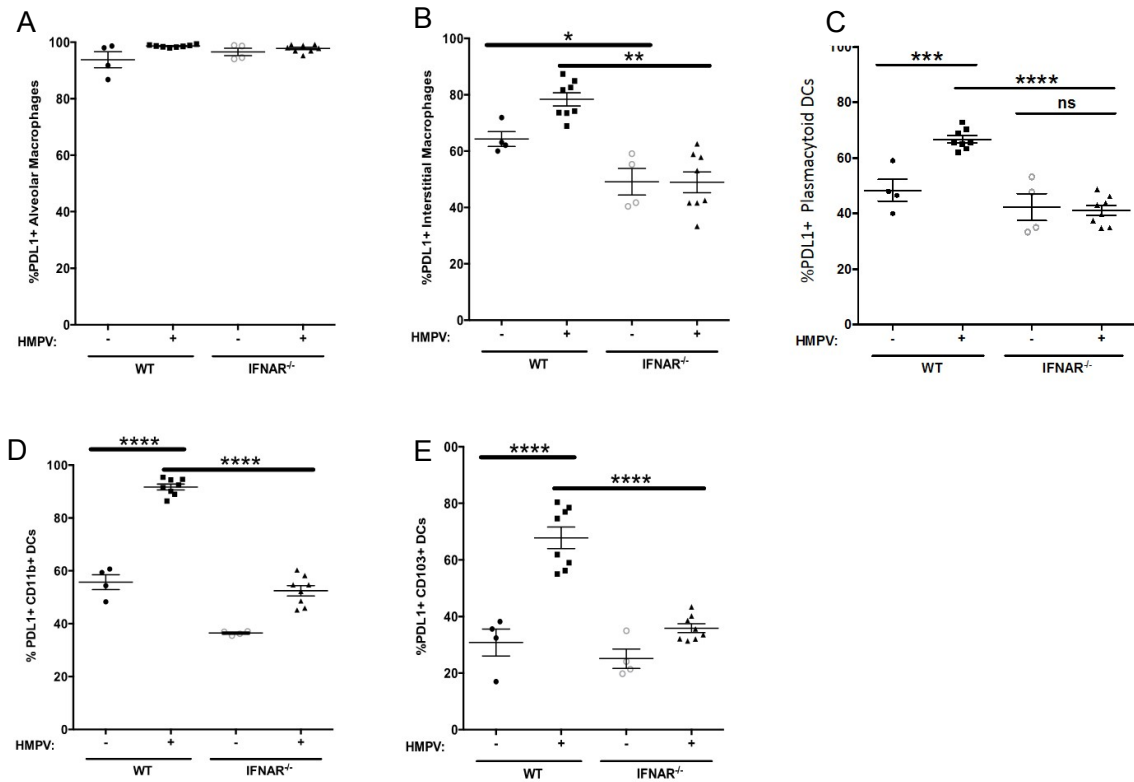


Figure 4-10. Type I IFN signaling drives the expression of PD-L1 on DCs and interstitial macrophages, but alveolar macrophages constitutively express high levels of PD-L1. WT and IFNAR^{-/-} mice were infected with HMPV, and after five days post-infection lungs were harvested and analyzed by flow cytometry. (A) CD11c⁺ MHCII^{mid} alveolar macrophages, (B) CD11c⁺ MHCII^{high} CD11b⁺ dendritic cells, (C) CD11c⁺ MHCII^{high} CD11b⁻ dendritic cells, and (D) CD11c⁻ MHCII^{mid} CD11b⁺ interstitial macrophages were probed with a specific antibody for the inhibitory ligand PD-L1. An isotype control antibody was used to draw gates to measure the percentage of cells expressing this marker. Groups were compared using unpaired t test or one-way ANOVA with Tukey post-hoc test where appropriate. N = 4-9 mice per experimental group representative of at least two independent experiments. Error bars represent SEM. * = P < 0.05, ** = P < 0.02, **** = P < 0.0001.

Elimination of IFNAR in hematopoietic or non-hematopoietic cells leads to less pathogenesis, while IFNAR expression on bone marrow-derived cells is important for a potent T_{CD8} response

In order to assess the importance of type I IFN signaling in the lung tissue compared to the hematopoietic compartment, we irradiated WT and IFNAR^{-/-} mice and performed reciprocal bone marrow transfers between the two groups. In this way, we

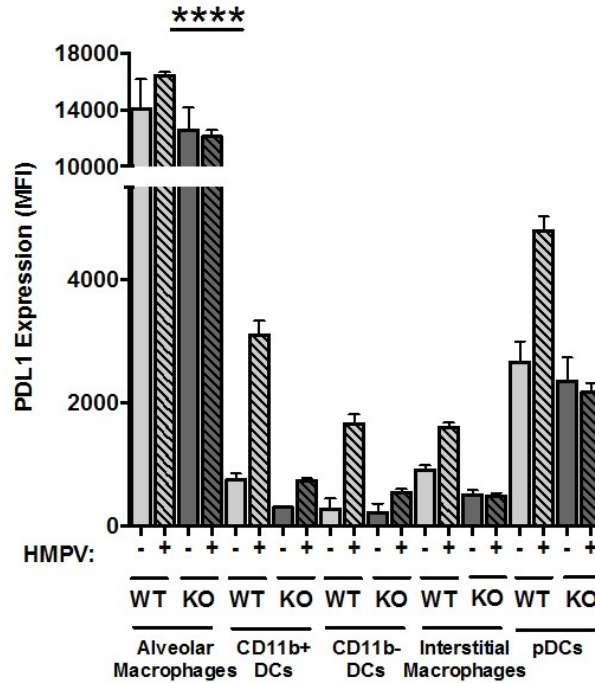


Figure 4-11. Alveolar macrophages express higher levels of PD-L1 than other APC subsets regardless of IFNAR signaling. WT and IFNAR^{-/-} mice were infected with HMPV, and after five days post-infection lungs were harvested and analyzed by flow cytometry. PD-L1⁺ (A,F) CD11c⁺ MHCII^{mid} alveolar macrophages, (B,F) CD11c⁻ MHCII^{mid} CD11b⁺ interstitial macrophages, (C,F) CD11c^{lo} MHCII^{lo} plasmacytoid dendritic cells, (D,F) CD11c⁺ MHCII^{high} CD11b⁺ dendritic cells, and (E,F) CD11c⁺ MHCII^{high} CD11b⁻ dendritic cells was measured with a specific antibody for the inhibitory ligand. (F) An isotype control antibody was used to normalize its expression level as measured by MFI on four subtypes of lung APCs. Groups were compared using unpaired Student's t test or one-way ANOVA with Tukey post-hoc test where appropriate. N = 4-9 mice per experimental group representative of at least two independent experiments. Error bars represent SEM. * = P < 0.05, ** = P < 0.02, **** = P < 0.0001.

derived mice that expressed IFNAR on either the bone-marrow derived cells only or only on non-hematopoietic cells. When these mice were infected with HMPV, neither mice lacking IFNAR on tissues nor those with type I IFN in bone marrow-derived cells reached the level of lung dysfunction, as measured by breath distension, observed in WT mice (Figure 4-12). The highest amount of pathogenesis occurs at day 9, suggesting that type I and type II IFN contributes to this disease state. The elimination of type I IFN signaling in either cell compartment is capable of ameliorating the level of pathogenesis seen in WT animals, so we analyzed the functionality of T_{CD8} response in both of these models.

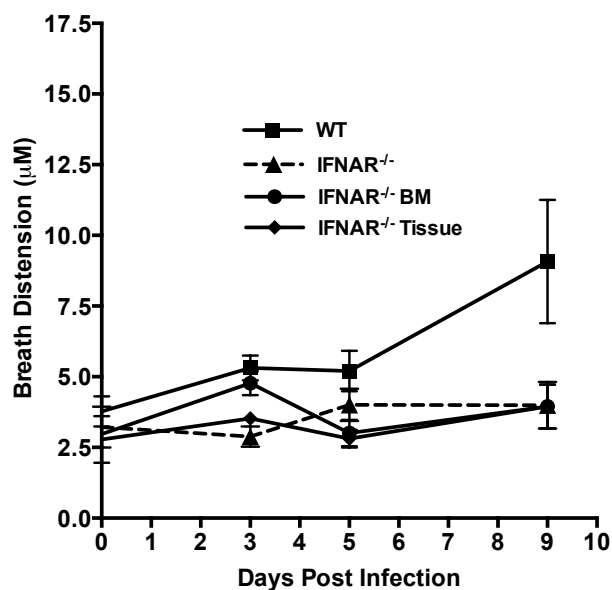


Figure 4-12. Expression of IFNAR on both hematopoietic and non-hematopoietic cells contributes to lung dysfunction. Breath distension of IFNAR^{+/+}, IFNAR^{-/-} mice, IFNAR^{-/-} BM or IFNAR^{-/-} tissue was measured using pulse oximetry following infection HMPV. N = 3 mice per day per group with one experiment.

The magnitude of the HMPV-specific T_{CD8} response is restored to more WT levels in mice reconstituted with IFNAR^{+/+} bone marrow as measured by the percentage of HMPV F528-specific TET⁺ in infected lungs at day 10 (Figure 4-13A) or by the absolute number of these cells (Figure 4-13B). We treated these same cells with the F528 peptide for 6 hours, and measured their ability to express IFN γ by intracellular cytokine staining. We found that similar to the size of the HMPV-specific response, the functionality of this response also relies on type I IFN signaling in hematopoietic cells (Figure 4-13C).

The expression of PD-L1 increases significantly on CD11b⁺ and CD11b⁻ DCs on day 10 of HMPV infection, regardless of type I IFN expression

On day 10 post HMPV-infection, we measured frequency of the APC subsets and observed that, unlike day 5, there was no differences between most groups (Figure

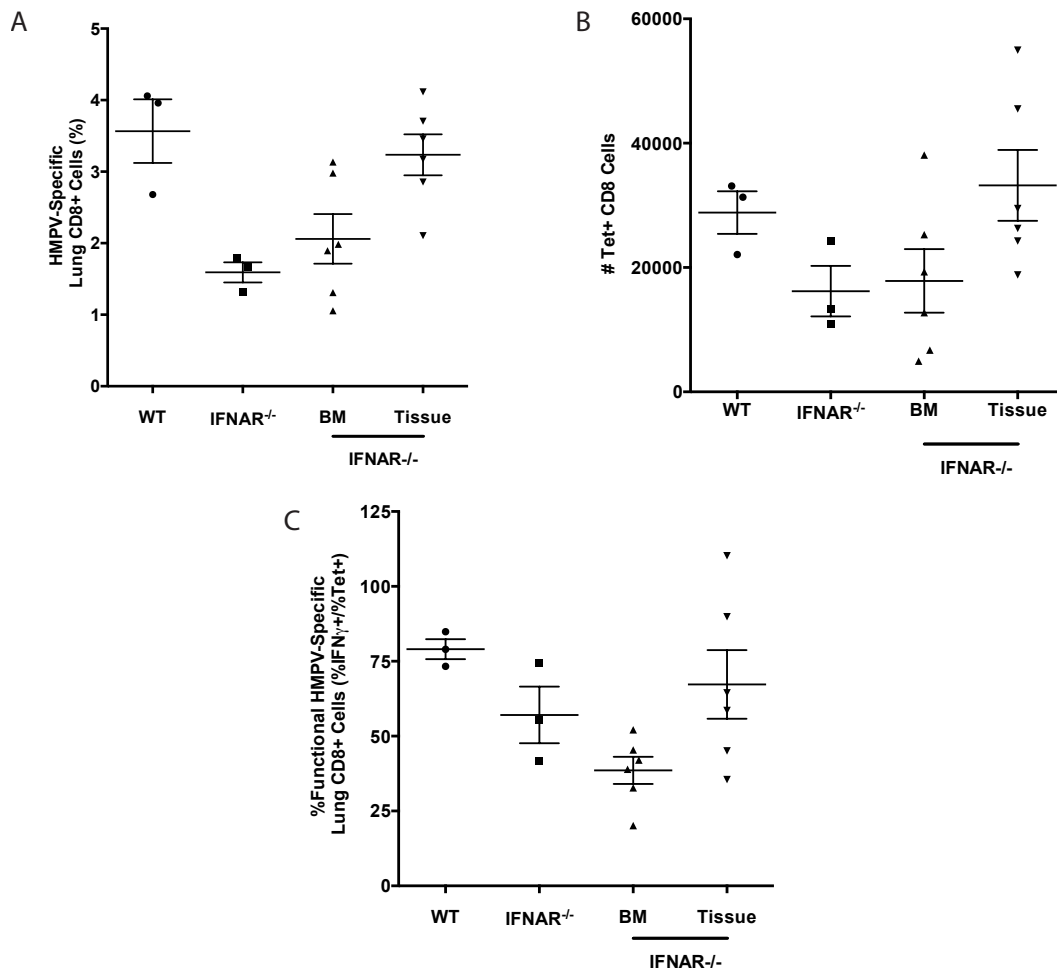


Figure 4-13. Type I IFN signaling in bone marrow-derived cells is crucial for a robust and functional T_{CD8} response. (A) An HMPV specific tetramer molecule was used to probe for virus specific CD8+ T cells in infected lungs for all groups. (B) Lymphocytes were also stimulated with an HMPV peptide in vitro, and intracellular cytokine staining was performed to analyze functionality of HMPV specific T-cells. N = 3-6 mice per day per group with one experiment.

4-14A-F), likely due to type II IFN signaling from infiltrating T_{CD8}. There were lower levels of alveolar macrophages in mice reconstituted with IFNAR^{-/-} bone marrow (Figure 4-14A) and a concurrent increase in the level of interstitial macrophages (Figure 4-14B). The expression of PD-L1 on alveolar and interstitial macrophages, as well as on pDCs, was relatively homogenous and not different between groups (Figure 4-15).

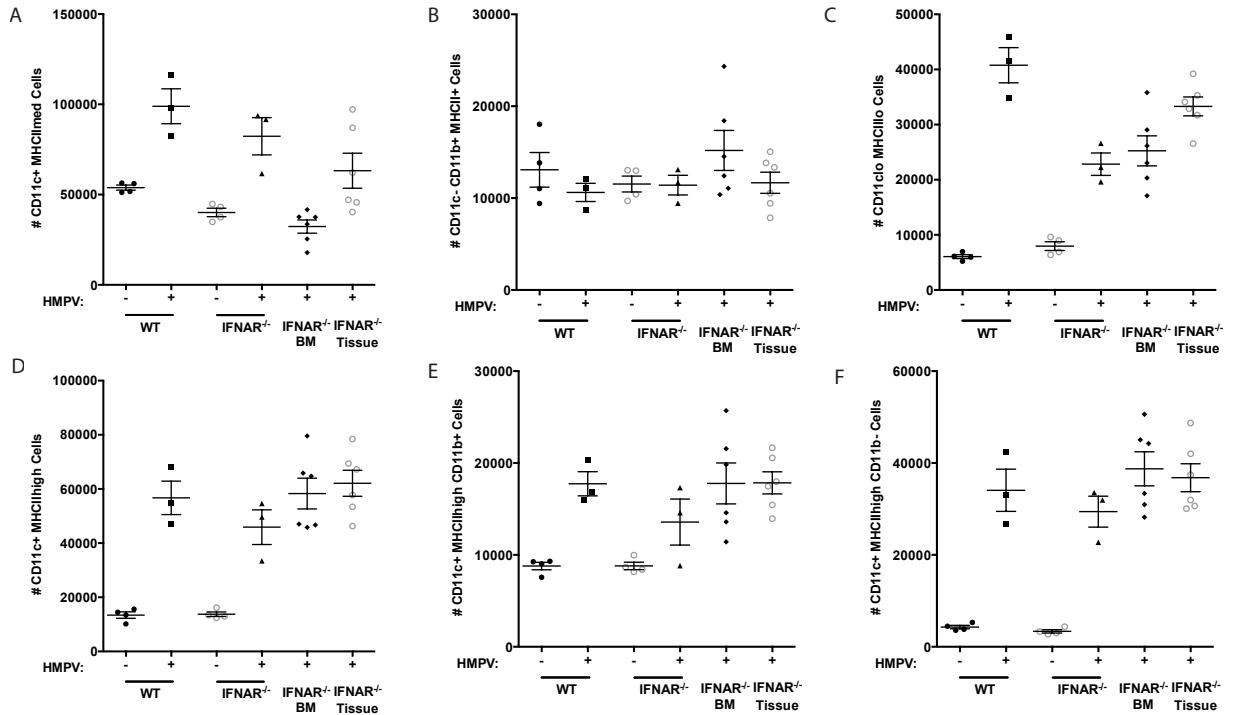


Figure 4-14. Type I IFN signaling is not important to APC expansion at day 10 post HMPV-infection. WT and IFNAR^{-/-} mice as well as the reciprocal bone marrow chimeras were infected with HMPV, and after ten days post-infection lungs were harvested and analyzed by flow cytometry. Cells were gated according to the gating strategy outlined in figure 4-8 as (A) CD11c⁺ MHCII^{mid} alveolar macrophages, (B) CD11c⁺ MHCII^{mid} CD11b⁺ interstitial macrophages, (C) CD11c^{lo} MHCII^{lo} plasmacytoid dendritic cells (D) CD11c⁺ MHCII^{high} dendritic cells, (E) CD11c⁺ MHCII^{high} CD11b⁺ dendritic cells and (F) CD11c⁺ MHCII^{high} CD11b⁻ dendritic cells. Total lung cell values, used to calculate number of cells, were measured by hemocytometer. N = 3-6 with one independent experiment.

Interestingly, when the level of this inhibitory ligand was measured on either CD11b⁺ or CD11b⁻ DCs, a distinct bimodal distribution of PD-L1 expression level was observed (Figure 4-16A and B). The level of PD-L1 expression on the higher expressing subset of cells on either CD11b⁺ or CD11b⁻ DCs was equal or higher than the level seen on alveolar macrophages (Figure 4-17). The ratio of PD-L1^{lo} vs. PD-L1^{high} expressing DCs is distinctly different for each subset, CD11b⁺ DCs displayed approximately equal percentage of each while CD11b⁻ DCs have over 80% of PD-L1⁺ cells in the higher expressing group (Figure 4-16C and D).

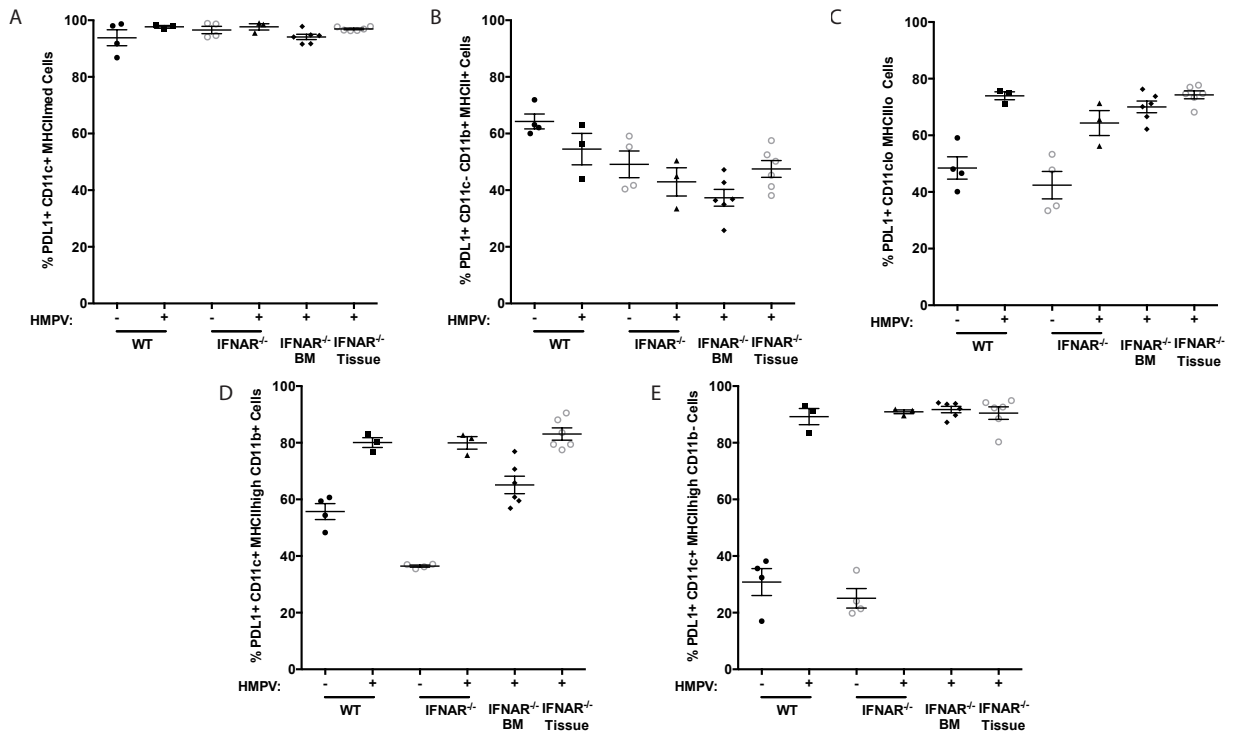


Figure 4-15. Type I IFN signaling does not contribute to PD-L1 expression on lung APC subsets at day 10 HMPV infection. WT, IFNAR^{-/-} mice as well as the reciprocal bone marrow chimeras were infected with HMPV, and after ten days post-infection lungs were harvested and analyzed by flow cytometry. PD-L1⁺ (A) CD11c⁺ MHCII^{mid} alveolar macrophages, (B) CD11c⁻ MHCII^{mid} CD11b⁺ interstitial macrophages, (C) CD11c^{lo} MHCII^{lo} plasmacytoid dendritic cells, (D) CD11c⁺ MHCII^{high} CD11b⁺ dendritic cells, and (E) CD11c⁺ MHCII^{high} CD11b⁻ dendritic cells, was measured with a specific antibody for the inhibitory ligand. N = 3-6 mice per group with 1 independent experiment.

Discussion

IFNAR^{-/-} mice had a significant defect in the number of HMPV-specific CD8⁺ T cells, consistent with previous data suggesting that IFNAR signaling plays a role in the clonal expansion of CD8⁺ T cells (Kolumam et al., 2005a) and in the maturation and priming of APCs (Azuma et al., 2012; Ehlers and Ravetch, 2007; Kadowaki and Liu, 2002; Luft et al., 1998). Virus-specific CD8⁺ T cells become functionally impaired in the lungs during acute viral LRI, including HMPV, and this functional defect is predominantly

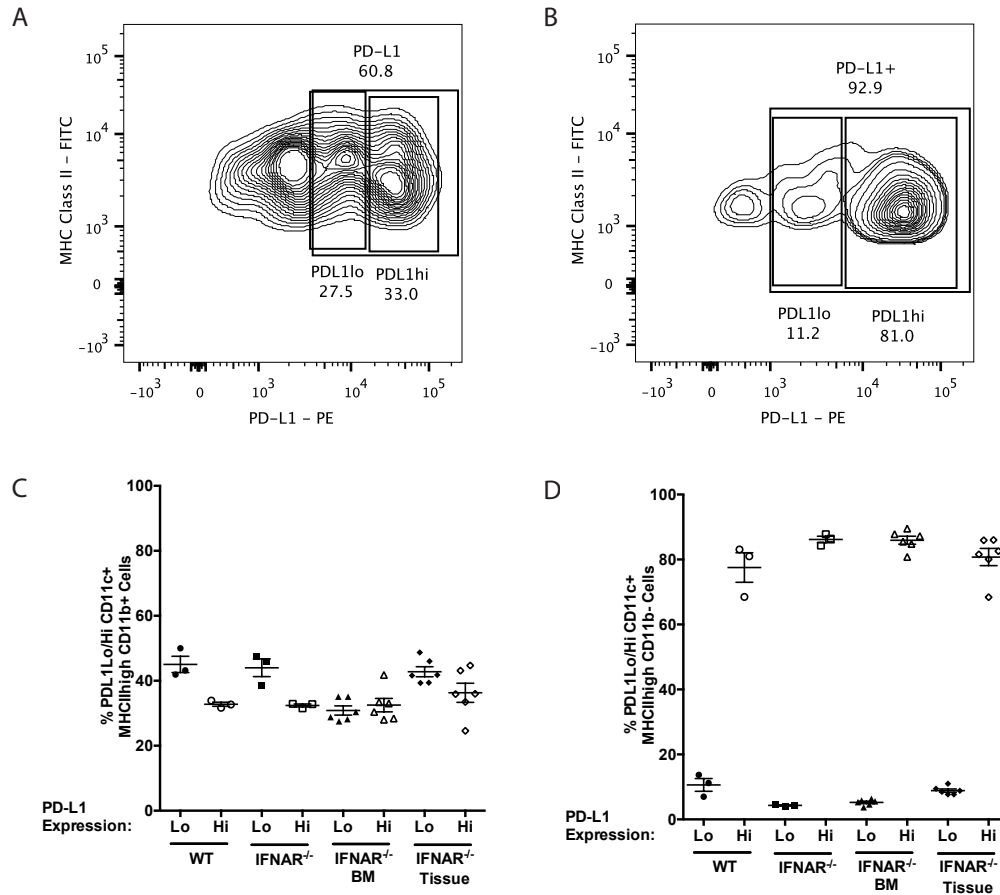


Figure 4-16. Bimodal distribution of PD-L1 expression in conventional DC subset on day 10 HMPV infection. WT, IFNAR^{-/-} and reciprocal bone marrow chimera mice were infected with HMPV, and lungs were harvested at day 10. (A,C) CD11c⁺ MHCII^{high} CD11b⁺ dendritic cells and (B,D) CD11c⁺ MHCII^{high} CD11b⁻ dendritic cells were gated according to strategy in figure 4-8 and probed for PD-L1 level with a specific antibody. N = 3-6 mice with 1 independent experiment.

driven by inhibitory signaling from the PD-1 receptor and its ligand, PD-L1 (Erickson et al., 2012). Because interferons have been identified as inducing expression of PD-L1 (Eppihimer et al., 2002; Lee et al., 2006b; Muhlbauer et al., 2006), we expected to observe restoration of T cell functionality in our IFNAR^{-/-} model. However, we found that the functional impairment of CD8⁺ T cells in IFNAR^{-/-} mice was actually significantly increased. Importantly, this difference in impairment was not associated with increased expression of PD-1. In addition, while PD-L1 expression levels in the lung were upregulated in both WT and IFNAR^{-/-} mice, IFNAR^{-/-} animals exhibited lower PD-L1

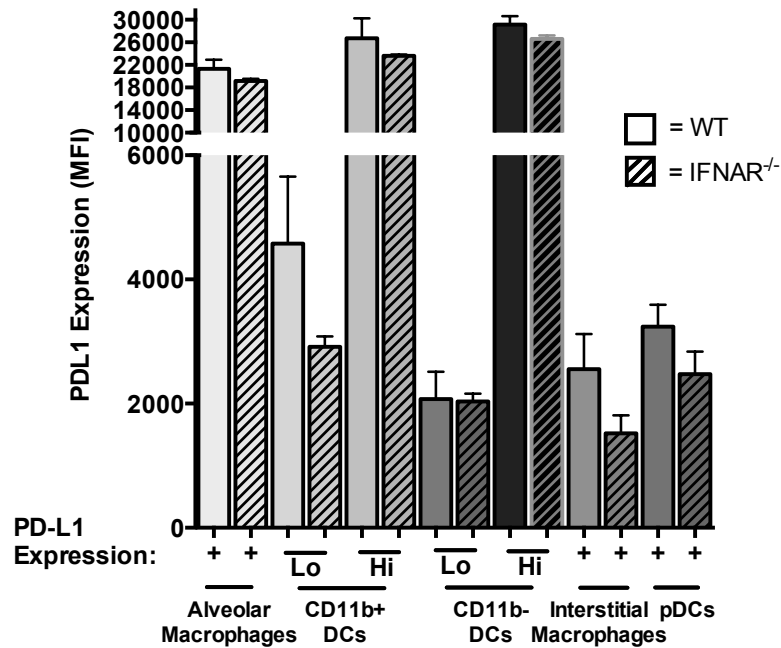


Figure 4-17. Expression of PD-L1 is highest in alveolar macrophage and conventional dendritic cells at day 10 HMPV infection. WT, IFNAR^{-/-} and reciprocal bone marrow chimera mice were infected with HMPV, and lungs were harvested at day 10. Cells were gated according to the gating strategy outlined in figure 4-8 as CD11c⁺ MHCII^{mid} alveolar macrophages, CD11c⁻ MHCII^{mid} CD11b⁺ interstitial macrophages, CD11c^{lo} MHCII^{lo} plasmacytoid dendritic cells, CD11c⁺ MHCII^{high} dendritic cells, CD11c⁺ MHCII^{high} CD11b⁺ dendritic cells and CD11c⁺ MHCII^{high} CD11b⁻ dendritic cells, and probed for PD-L1 with a specific antibody. An isotype control antibody was used to normalize expression as measured by MFI. N = 3-6 mice per group with 1 independent experiment.

levels than WT animals in both lung epithelial and CD11⁺ cells. Thus, although PD-L1 expression on both lung epithelial and CD11c⁺ cells was affected by IFNAR signaling, these differences were not sufficient to explain the reduction in the CD8⁺ T cell functionality observed in IFNAR^{-/-} mice.

To date there are conflicting studies on the impact of IFNAR signaling on Tregs. Some groups have shown a negative impact of IFNAR signaling on the development of Tregs (Golding et al., 2010; Srivastava et al., 2014a), but others have shown that treatment with type I IFN can lead to higher Treg numbers (de Andres et al., 2007; Namdar et al., 2010). Tregs can secrete suppressive cytokines, such as IL-10, to limit

the functionality of CD4⁺ and CD8⁺ T-lymphocytes during respiratory viral infections (Fulton et al., 2010), but we actually observed fewer Tregs and lower IL-10 gene expression in IFNAR^{-/-} mice indicating a positive role for IFNAR signaling in Treg recruitment or proliferation.

Other inhibitory T cell receptors including LAG-3 (Workman and Vignali, 2005), 2B4 (Chlewicki et al., 2008), and TIM-3 (Sakuishi et al., 2011) contribute to CD8⁺ T cell exhaustion during chronic infection, with expression of multiple receptors increasing the exhaustion phenotype (Nirschl and Drake, 2013; Odorizzi and Wherry, 2012). While we saw no differences in LAG-3 and 2B4 expression during HMPV infection, HMPV-specific CD8⁺ T cells from IFNAR^{-/-} mice were significantly more likely to express the TIM-3 receptor. TIM-3 is a member of the Tim family of molecules containing a N-terminal IgV domain, a mucin domain, a transmembrane domain, and a tail extending to the cytoplasm (Kuchroo et al., 2003). This molecule is capable of binding to its ligand, galectin-9, and inducing suppressive signals in both CD4⁺ and CD8⁺ T cells (Cao et al., 2007; Santiago et al., 2007; Zhu et al., 2005). This interaction has been associated with peripheral tolerance (Sabatos et al., 2003). Recent studies have revealed the importance of TIM-3 in cancer (Fourcade et al., 2010; Zhou et al., 2011) and chronic viral disease (Golden-Mason et al., 2009; Jin et al., 2010a; McMahan et al., 2010; Ngiow et al., 2011; Sakuishi et al., 2010; Sehrawat et al., 2010). Our data shows that in the context of respiratory viral infections, the diminished function of HMPV-specific CD8⁺ T cells in IFNAR deficient mice corresponds to increased expression of the inhibitory receptor TIM-3. Since HMPV and other viruses display the ability to downregulate IFNAR signaling, TIM-3 may provide an attractive target for therapeutic intervention during HMPV infection.

Alveolar macrophages and dendritic cells both express the cell surface marker CD11c, and these cells types along with interstitial macrophages are crucial antigen

presenting cells in the lung (Misharin et al., 2013). In WT mice, we observed an increase in both alveolar macrophages and dendritic cells during HMPV infection, but in mice lacking IFNAR signaling there was a significant defect in the amount of dendritic cells, but not alveolar macrophages, in the lung during infection. Analysis of PD-L1 expression in these subsets indicated that alveolar macrophages constitutively express high levels of this inhibitory ligand, while dendritic cells and interstitial macrophages rely on IFNAR signaling for PD-L1 expression. Reflecting this, the total expression level of PD-L1 was significantly lower in DCs and interstitial macrophages from IFNAR^{-/-} than WT mice. Thus, the defect in the number of PD-L1-low DCs during viral infection of IFNAR^{-/-} mice leads to a greater ratio of APCs expressing high levels of PD-L1 (i.e., alveolar macrophages) in infected lungs, potentially explaining the decrease in CD8⁺ T cell functionality observed in IFNAR^{-/-} mice. Further, the decrease in total APCs was consistent with the defect in HMPV-specific CD8⁺ T cell numbers, suggesting a global effect of the lack of type I IFN. Additionally, our data suggest that due to intrinsic differences in PD-L1 expression, dendritic cells produce a more functional CD8⁺ T cell response compared to alveolar macrophages. These data corroborate previous findings on the importance of dendritic cells to the immune response in the respiratory tract (Kim and Braciale, 2009).

Preliminary data on the importance of type I IFN in the hematopoietic versus non-hematopoietic cells presented here show that the contribution of IFNAR in bone marrow derived cells is crucially important for the development of a T_{CD8} response. Type I IFN signaling on tissue did not contribute to this impairment, but it did play a role in the development of disease pathogenesis in this model. This phenomenon is likely due to the lack of IFNAR signaling early in infection protecting IFNAR^{-/-} tissue and IFNAR^{-/-} mice, and the impairment in T_{CD8} expression of type II IFN by IFNAR^{-/-} mice and those

with IFNAR^{-/-} BM. This suggests an additive role in disease pathogenesis for type I and II IFNs.

We observed that on day 10 the number and PD-L1 expression level of APC subsets were not markedly different regardless of IFNAR deficiency. Intriguingly though, plasmacytoid DCs were higher only in WT mice on day 10. These cells have been shown to specifically detect HMPV (Banos-Lara Mdel et al., 2013) and contributes to RSV disease pathogenesis in mice (Smit et al., 2008), so the higher levels of pDCs could be related to the increase in lung dysfunction observed in this group. Conventional DCs exhibited the ability to increase expression of PD-L1 to levels comparable to those found on alveolar macrophages at day 10 post infection, and this upregulation appears to comprise two distinct subsets of cells, one expressing high levels and the other expressing relatively lower levels of PD-L1. In CD11b⁺ cells, these two subsets are found at approximately the same level, but in CD11b⁻ DCs, we showed much higher percentages of PD-L1^{high} cells. As discussed earlier, CD103⁺ DCs, found in the CD11b⁻ group of DCs, have been shown to be particularly adept at generating a functional T_{CD8} response, so this increase in PD-L1 could be directly related to interactions between these cells and functional T_{CD8}.

Cross-presentation of viral peptides by APCs is an essential part of the generation of a specific T_{CD8} response [Yu, 2008 #1080], and IFN signaling is capable of driving cross-presentation in these cells [Belz, 2002 #610]. This defect is also likely a major factor in the observed defect in the development of an HMPV-specific T_{CD8} response in IFNAR^{-/-} mice. It should be noted that these experiments used the C57BL/6 mouse model for HMPV infection. This model is well established as a good model for viral replication and the development and assessment of adaptive immune responses (Erickson et al., 2012; Erickson et al., 2014b), but other groups studying HMPV use the BALB/c mouse model, and some differences do exist (Alvarez and Tripp, 2005; Melendi

et al., 2007). Specifically related to T cell polarization, C57BL/6 mice have a TH1 bias, dominated by the expression of IFN γ , IL-2 and TNF α , while BALBC mice show a TH2 bias, marked by expression of IL-4, IL-5, IL-6, IL-9, IL-10, and IL13 along with a strong antibody response (Gessner et al., 1993; Locksley et al., 1987; Romagnani, 2000). Both of these models have provided valuable information towards understanding human disease, but the differences should be taken into consideration.

Taken together, our data reveal an important role for type I interferon signaling in both the innate and adaptive host control of HMPV as well as in disease pathogenesis. We demonstrate that signaling through IFNAR significantly contributes to increased inflammatory lung disease during HMPV infection, and therapeutics targeting this pathway may provide a way to mitigate disease symptoms. Because IFNAR signaling promotes dendritic cell expansion during early HMPV replication, elimination of this pathway results in a defect in the development of an efficient and effective CD8⁺ T cell response. These findings expand our understanding of the importance of IFNAR signaling in HMPV replication and disease, as well as suggest a mechanism for IFNAR-independent impairment of the CD8⁺ T cell response.

Materials and Methods

Mice and Viruses. C57BL/6 (B6) mice were purchased from The Jackson Laboratory. IFN alpha/beta receptor deficient (IFNAR^{-/-}) B6 mice were kindly provided by Dr. Herbert W. Virgin. All animals were bred and maintained in specific pathogen-free conditions under guidelines approved by the AAALAC and the Vanderbilt Institutional Animal Care and Use Committee. Six to twelve-week-old age- and gender-matched animals were used in all experiments. HMPV (pathogenic clinical strain TN/94-49, genotype A2) was grown and titered in LLC-MK2 cells as previously described (Williams et al., 2005c). For

sucrose purified virus, HMPV TN/94-49 used in previous experiments was purified through a sucrose gradient as previously described (Cox et al., 2014). For all experiments, mice were anesthetized with intraperitoneal ketamine-xylazine and infected intranasally with 1.5×10^6 PFU of HMPV. Serum was collected from WT and IFNAR^{-/-} mice by submandibular venipuncture and was used in a plaque reduction assay to determine HMPV neutralizing antibody titers as previously described (Williams et al., 2005c). The median of triplicate values for % HMPV neutralization for each animal of each genotype were plotted as a function of log₂-transformed serum dilution factor and analyzed in a sigmoidal dose response curve to determine IC₅₀. Nasal turbinates (NT) and lungs were collected for analysis and tissue viral titers measured by plaque titration as previously described (Williams et al., 2005c). For histopathology, the left lung was removed and inflated with 10% buffered formalin, paraffin-embedded, stained, and analyzed using a formal scoring system in a group-blinded fashion by an experienced lung pathologist as previously described (Erickson et al., 2012).

Generation of Bone Marrow Chimera. WT and IFNAR^{-/-} mice were exposed twice to 5 Gy of radiation, with 4 hour rest in between treatments, from a cesium irradiator. Mice were housed in sterile conditions and their water was supplemented with 6.25 mg/mL Sulfamethoxazole and 1.25 mg/mL Trimethoprim. After 24 hours later, bone marrow was harvested from both WT and IFNAR^{-/-} mice as previously described (Erickson et al., 2014a), and 1×10^6 WT and 1×10^6 IFNAR^{-/-} bone marrow cells were injected retro-orbitally. Mice were rested for 8 weeks before HMPV infection.

Flow Cytometry. Cells were isolated from lungs of infected animals as previously described (Erickson et al., 2012). Briefly, lungs were rinsed in R10 medium (RPMI-1640 (Mediatech) plus 10% FBS, 2 mM glutamine, 50 µg/ml gentamicin, 2.5 µg/ml

amphotericin B, and 50 μ M β -mercaptoethanol (Gibco, Invitrogen), and then minced with a scalpel and incubated with 2 mg/ml collagenase A (Roche) and 20 μ g/ml DNase (Roche) for 1 hour at 37°C. Single-cell suspensions of digested lungs were obtained by pressing through a steel screen (80 mesh) and then passing over a nylon cell strainer (70- μ m pore size). Erythrocytes were lysed using Red Blood Cell Lysis Buffer (Sigma-Aldrich). For labeling of HMPV-specific CD8⁺ T cells, single cell suspensions of mouse lungs were incubated with APC-labeled H2-D^b tetramers (0.1-1 μ g/mL), anti-CD8 α (clone 53-6.7, BD Biosciences), and anti-CD19 (clone 1D3, eBioscience) (Erickson et al., 2012). Surface/tetramer staining was performed for 1 hour at room temperature in PBS containing 2% FBS and 50 nM dasatinib (LC Laboratories) (Lissina et al., 2009). For assessment of PD-L1 expression, cells were stained with anti-EPCAM (clone G8.8, Biolegend), anti-CD11c (clone HL3, BD), anti-CD11b (clone M1/70, Tonbo), and anti-MHC Class II (clone 2G9, eBioscience). Cells were also stained for PD-1 (clone J43, BD Biosciences), TIM-3 (clone RMT3-23), LAG-3 (clone C9B7W), 2B4 (clone m2B4 (B6)458.1, Biolegend), PD-L1 (clone MIH5, BD), or an isotype control antibody (hamster IgG2 κ). Staining for HMPV-specific CD8⁺ T cells was normalized to the binding of a cognate APC-labeled H2-D^b tetramer loaded with NP366 flu peptide to CD8⁺ T cells (typically 0.05-0.2% of CD8⁺ T cells). For all cell populations FSC and SSC gating were used to obtain cells of appropriate size and shape.

To identify HMPV-infected cell populations, homogenized lung cell suspensions were stained with polyclonal anti-HMPV guinea pig sera (Williams et al., 2005c) for 1 hour at room temperature (RT), washed, and then stained with an anti-guinea pig FITC secondary antibody (Life Technologies). Lung epithelial cells and CD11c⁺ high dendritic cells were identified using forward/side scatter and stained for EPCAM and CD11c as described above. Gates for HMPV-positive cell populations were set using uninfected

mice and isotype controls (BioLegend and BD). All flow cytometric data were collected using an LSRII or Fortessa (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Intracellular Cytokine Staining (ICS). Lung or spleen lymphocytes were isolated and re-stimulated *in vitro* for 6 hours at 37°C with a non-specific peptide or the indicated synthetic peptide (10µM final concentration) in the presence of an anti-CD107a antibody (clone 1D4B, BD Bioscience). The protein transport inhibitors brefeldin A and monensin (BD Biosciences) were added for the final 4 hours of re-stimulation. Stimulation with PMA/ionomycin (50ng/mL PMA and 2µg/mL ionomycin, Sigma) served as a positive control. After re-stimulation, cells were stained for surface expression of CD3ε (clone 145-2C11), CD8α, and CD19, followed by fixation/permeabilization and staining for intracellular IFNγ (clone XMG1.2) (BD Biosciences) and were analyzed using flow cytometry. Background cytokine levels following re-stimulation were normalized to a non-specific peptide.

Statistical Analyses. Data analysis was performed using Prism v4.0 (GraphPad Software). Groups were compared using unpaired t-test or one-way ANOVA with post-hoc Tukey test for multiple comparisons. P <0.05 was considered significant by convention.

CHAPTER V

Summary and Future Directions

Thesis Summary

PRR detection and type I IFN signaling are conserved throughout the animal kingdom, and represent a significant negative pressure on the life cycle of viruses. These pathogens have evolved a myriad of innate immune evasion mechanisms to escape detection and response by the host, but the HMPV genome lacks homology for the known innate immune evasion genes in the *Paramyxoviridae* family. This thesis

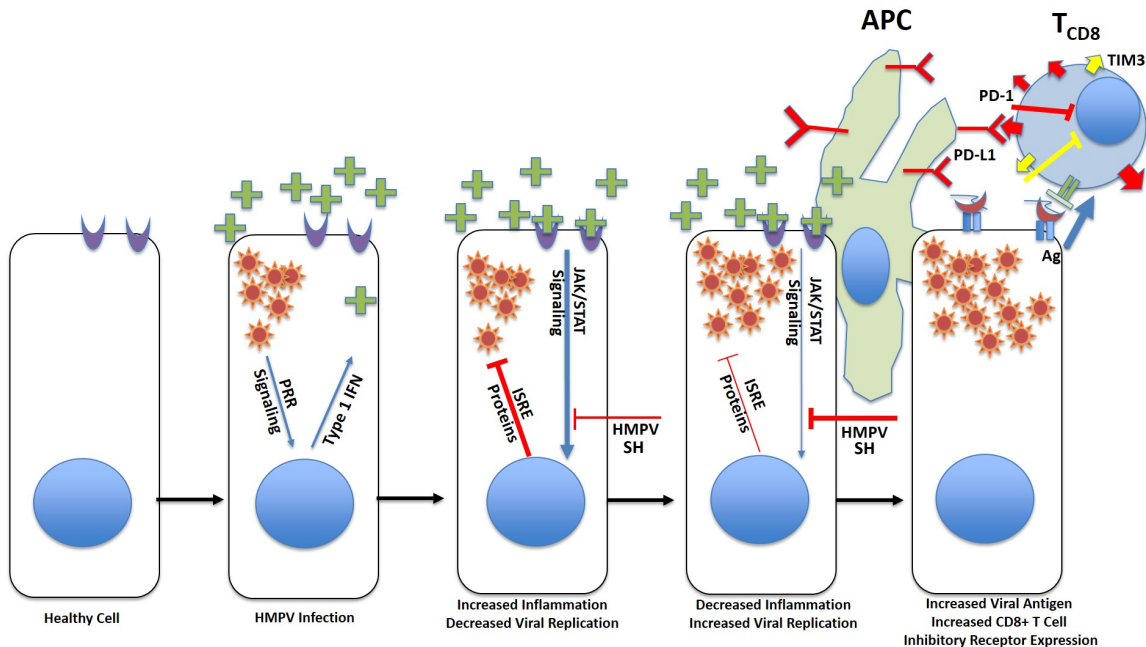


Figure 5-1. Model of type I IFN evasion effect on T_{CD8} response. Respiratory epithelium is infected with HMPV, which induces signals through the detection of viral nucleic acids by PRRs to induce the expression of type I IFNs. These molecules can act in an autocrine and paracrine fashion to upregulate anti-viral peptides, cytokines and chemokines. As viral replication progresses the expression of the SH protein increases and is capable of downregulating signaling through the inhibition of STAT1. This in turn leads to increased viral replication in the infected cell, and higher levels of Ag stimulation of responding T_{CD8} cells inducing higher inhibitory receptor expression. Proximal APCs can interact with T_{CD8} via inhibitory receptor interactions and lead to functional impairment.

uncovers a viral gene product from HMPV that is capable of inhibiting signaling through the type I IFN pathway by targeting STAT1, and elucidates potential repercussions of this evasion on the replication, pathogenesis and adaptive immune response in an animal model.

I first set out in this thesis project using a cell line derived from healthy bronchial epithelium, BEAS-2B, to determine the effects of HMPV infection on cells *in vitro*. Western blot, as well as bulk flow analysis, showed that BEAS-2B cells infected with HMPV upregulated STAT1, but were inhibited in their ability to undergo IFN-induced phosphorylation of this molecule. Curiously, when HMPV-specific antibodies were used to separately analyze both HMPV-infected and uninfected BEAS-2B cells in the same well, both groups were found to significantly upregulate STAT1 and become refractory to further IFN-induced STAT1 phosphorylation. Since we know that type I IFN is expressed at high levels in these cells upon activation of RIG-I, a known PRR for the detection of HMPV (Boehme and Compton, 2004; Manuse and Parks, 2010; Matsumoto et al., 2003; Melchjorsen et al., 2005; Rudd et al., 2005), and is capable of acting in an autocrine and paracrine fashion (Yoneyama et al., 2005), I wanted to investigate whether this expression and subsequent signaling in these cells could lead to this later observed inhibition.

By treating these cells for an extended period with type I IFN and also by inducing the endogenous expression of these molecules through the use of the synthetic viral nucleic acid, poly(I:C), I was able to show that this phenotype is induced by the detection of virus by PRRs and the ensuing type I IFN production during infection of this cell type and not by an HMPV-specific mechanism. These results indicate that cells capable of IFN production are a poor system for the analysis of the phenomenon of innate immune evasion. Fortunately, HMPV is capable of infecting a cell line derived from the kidney epithelium of the African green monkey, Vero cells, and this cell line has a defect

in the production of type I IFN but not the recognition of viruses through PRRs or the signaling through the JAK/STAT pathway (Emeny and Morgan, 1979a; Hasler and Wigand, 1978). Long-term treatment of this cell line with IFN did not result in an upregulation of STAT1, and later treatment of these cells with IFN was sufficient to induce STAT1 phosphorylation. It is intriguing to consider, that lung epithelia *in vivo* most likely has a phenotype that more closely resembles that seen in the BEAS-2B cell line. This suggests that these cells have intrinsic mechanisms to quickly upregulate interferon stimulated genes (ISGs), and indeed recent work has shown that these cells have high basal levels of innate immune pathways (Seng et al., 2014). Previous work showing HMPV can inhibit IFN α , but not IFN γ , were performed in lung epithelial cell lines competent for type I IFN production so these findings might suffer from these confounding effects (Dinwiddie and Harrod, 2008).

Using the Vero cell line and again utilizing an HMPV-specific antibody to label infected cells, I was able to show that infection of these cells with HMPV resulted in significantly lower expression level of STAT1. When these cells were treated with type I IFN, they also showed a significant decrease in their ability to phosphorylate STAT1. This time, the phenotype was not observed in the uninfected cells grown in the same well. This shows that HMPV has the capability to specifically affect the type I IFN pathway, but I was interested in which viral protein was responsible for this phenomenon. To that end, I went about generating overexpression plasmids of the HMPV viral proteins.

In order to maximize the efficiency of translation for the viral proteins, we modified the viral gene sequences for optimal codon usage and GC content in mammalian cells (Graf et al., 2004). Next, with the help of Sherry Wen and Laura Peterson, I cloned these mammalian-optimized viral protein genes into overexpression plasmids for HMPV proteins, as well as GFP-fusion proteins of these viral gene

products. When the SH protein of HMPV was expressed in these Vero cells, reduced expression of the STAT1 protein was seen, and when treated with type I and II IFNs, these cells showed reduced STAT1 phosphorylation as compared to both mock-treated and cells in the same well. Other expression vectors, such as those for the N and P proteins, were able to drive high levels of viral protein in transfected cells, but the expression of SH was restricted to a lower level indicating that toxicity might occur at high expression levels.

To further validate the effect of SH on STAT1, I also generated 293flp cell lines stably expressing HMPV proteins and measured STAT1 expression, phosphorylation and promoter activity downstream of the type I IFN pathway. I observed that the expression of SH in these cells for the multiple passages necessary for selection, resulted in a significant upregulation of STAT1 protein expression, possibly due to compensation over time in these cells. Despite this higher expression, when these cells were treated with type I IFN, less relative STAT1 phosphorylation was observed. By using a luciferase reporter plasmid driven by 6 copies of the ISRE promoter, I was able to show that the SH protein was also capable of blocking signals downstream of STAT1. Interestingly, when these cells were analyzed by forward and side scatter area they appeared larger in both measurements, perhaps showing that SH interacts with membranes of these cells and alters them. This would be reasonable in light of data indicating this protein may be capable of forming pores in the cell membrane (Masante et al., 2014). Taken together these data show that the SH protein of HMPV can downregulate signaling through the type I IFN by affecting STAT1 expression and IFN-induced phosphorylation. Further work to elucidate the mechanism by which SH is able to evade innate immune signaling will be proposed later in this chapter.

The preceding data has demonstrated that HMPV is able to inhibit type I IFN signaling, and inhibitory mechanisms employed by other viral pathogens to target this

same pathway is well described. This led me to ask what the implications of this evasion of the innate immune system were on the development of a strong adaptive response, and particularly a robust T_{CD8} response. In order to accomplish this, I requested a mouse line deficient in the receptor for type I IFN signaling, IFNAR, from Skip Virgin's lab at Washington University in St. Louis. These mice were intranasally infected with HMPV and analyzed for viral replication and pathogenesis, as well as for the development of T_{CD8}. I also examined the expression level of the inhibitory T cell receptors, PD-1, TIM-3, Lag3 and 2B4 on HMPV-specific TCD8 as well as the ligand for PD-1, PD-L1.

I showed that early in infection, day 3 and 5, there was significantly more HMPV replication in the IFNAR deficient animals, but they were able to clear virus with normal kinetics. Intriguingly, while WT mice lost a small but significant amount of weight early in infection, this weight loss was not observed in IFNAR^{-/-} mice. In order to measure disease pathogenesis in these mice throughout viral infection, I used a mouse pulse oximeter and showed that, corroborating the previous experiment, mice lacking IFNAR seemed to be significantly protected from increased breath distension, a measure of breathing effort. This indicates that instead of being necessary for clearance of HMPV, type I IFN signaling could do more to contribute to the morbidity of this virus.

To further investigate whether type I IFN signaling was driving the disease state in these animals, I harvested lung tissue from WT and IFNAR^{-/-} mice to analyze by histology. In WT animals a significant inflammatory infiltrate was observed during HMPV infection on day 5 compared to mock-infected mice, and this disease state was significantly ameliorated by ablation of the IFN α receptor. Expression of multiple inflammatory cytokines was also significantly higher in WT than in IFNAR^{-/-} mice during HMPV infection when measured by RT-PCR, and IFN γ levels were significantly higher by ELISA. Recent work on RSV has shown a similar role for IFNAR in the upregulation of inflammatory cytokines. These data confirm the previous work suggest a role for type I

IFN signaling in the pathogenesis of HMPV, and also show the importance of this pathway in the robust development of an immune response.

Neutralizing Ab levels have proven to be the key mechanism preventing reinfection of WT mice *in vivo*, and IFNAR^{-/-} mice were shown in chapter III to generate protection from subsequent infection. The results in chapter IV showed that, in fact, serum from these IFNAR-deficient animals actually contained significantly more nAbs as measured by neutralizing plaque assay. This phenotype is likely due to increased viral antigen in these animals, but it is also possible that lack of type I IFN leads to a more Th2-type response, capable of producing a more potent nAb response. The total number of lymphocytes was not significantly different between uninfected and HMPV-infected WT or IFNAR KO mice, but both groups upregulated the number of CD11c⁺ cells during infection. Bulk CD8⁺ T cells were higher during HMPV infection in WT mice, but the IFNAR^{-/-} mice did not show a difference in the number of T_{CD8} during infection. Since these cells are crucial for HMPV response, I wanted to also analyze the quality of the T_{CD8} virus-specific response using HMPV-specific tetramer molecules and intracellular cytokine staining.

IFNAR^{-/-} mice showed a significant defect in their ability to generate an HMPV-specific T_{CD8} response. These results were not surprising since previous work has suggested that type I IFN is important in direct and indirect signals to these cells for their proliferation. What was more interesting was that these specific T cells were also less able to respond in a functional manner to peptide stimulation in the ICS assay. Previous studies have shown that impairment of lung T_{CD8} during HMPV infection is driven by the upregulation of the inhibitory receptor PD-1, which is not differentially expressed between WT and IFNAR^{-/-} mice. I originally hypothesized that since this inhibitory receptor interacts with its ligand, PD-L1, which is driven by promoters downstream of IFN signaling, that these cells would actually be more functional instead of less. To

further explore this curious phenomenon, I examined the expression of PD-L1 on both lung epithelial cells and CD11c⁺ cells, which includes subsets of APCs discussed in more detail later.

In order to specifically analyze the PD-L1 expression on infected lung epithelium I used a polyclonal HMPV-specific Ab to probe for HMPV-infected cells expressing the epithelial cell marker, EPCAM. Using this assay I showed that not only are levels of HMPV replication higher in IFNAR^{-/-} mice, but more lung epithelial cells are infected in these animals. The level of PD-L1 was highest in infected WT cells on day 5, but this inhibitory ligand was also increased in infected animals in the absence of type I IFN signaling indicating that IFNAR is sufficient but not necessary for the upregulation of PD-L1 on these cells. It should also be noted that infected cells in both WT and IFNAR^{-/-} mice more highly expressed PD-L1 than the uninfected EPCAM⁺ cells from the infected mouse, showing that signaling within the infected cell is also capable of increasing the levels of the inhibitory ligand. By day 10, the percentage of PD-L1 expressing lung epithelial cells was greatly reduced and no difference was seen between groups. The baseline percentage of PD-L1 expressing CD11c⁺ cells was markedly higher than the lung epithelium, but only a slight, non-significant, increase in these cells was seen at day 5 post-HMPV infection and this difference disappears by day 10.

Since the expression of IFNAR results in higher PD-L1 expression, as expected, the interactions between PD-1 and PD-L1 do not appear to drive the difference in functionality detected between WT and IFNAR^{-/-} animals. CD4⁺ Tregs are also capable of exerting inhibitory actions on T_{CD8}. Recent data from an LCMV model indicates that direct type I IFN signaling on Tregs can suppress proliferation (Srivastava et al., 2014a), but other groups have shown that this signaling can also play a role in FOXP3 maintenance and the development of the suppressive phenotype of this subset of T cells (Lee et al., 2012). Interestingly, in our disease model IFNAR^{-/-} mice displayed a

significant defect in the number of these cells present in the lung during HMPV infection, failing to provide an explanation for T_{CD8} impairment. Other inhibitory receptors besides PD-1 can also contribute to the impairment of T_{CD8} in the lungs, so I next measured the percentage of HMPV-specific T cells that expressed the inhibitory receptors, 2B4, Lag3 and TIM-3. While no difference was observed between WT and IFNAR KO animals during viral infection for 2B4 or Lag3 expression, the percentage of HMPV-specific T_{CD8} cells expressing TIM-3 was significantly higher in mice deficient for type I IFN signaling. These data indicate a possible mechanism through the action of the inhibitory receptor TIM-3 for impairment of the T_{CD8} in the absence of type I IFN signaling.

The CD11c⁺ population analyzed previously contains multiple subsets of APCs, and I wanted to further explore the expansion and PD-L1 expression in each of these. Using a multi-parametric flow gating scheme I was able to distinguish five separate subsets of APCs in the lungs, alveolar macrophages, interstitial macrophages, plasmacytoid DCs, CD11b⁺ DCs and CD11b⁻ DCs. I measured both the expansion of these cells in the lung compartment during HMPV infection, as well as the PD-L1 expression in naïve and HMPV-infected mice.

Alveolar macrophages represented the largest subset of APCs in the lungs of both WT and IFNAR^{-/-} mice at day 5 post-infection, and IFNAR-deficient animals actually had a higher number of these cells as compared to WT during infection. This shows that the expansion of these cells occurs independently of type I IFN signaling. Interstitial macrophages and the three subsets of dendritic cells on the other hand, were significantly lower in IFNAR^{-/-} mice during HMPV infection indicating that these cells require type I IFN for their proliferation or infiltration to the lung early in viral infection. I wanted to know if these cells had varying degrees of suppressive capabilities towards T_{CD8} , so I measured PD-L1 levels in the subsets of APCs in the lung.

The baseline expression level of PD-L1 was found to be strikingly different between these groups of APCs. Alveolar macrophages were found to constitutively express PD-L1 even in uninfected mice, while the percentage of dendritic cells and interstitial macrophages expressing PD-L1 in uninfected mice was much lower. For interstitial macrophages, the number of PD-L1 expressing cells at steady state was also dependent on type I IFN, as IFNAR^{-/-} mice had significantly less than their WT counterparts in uninfected mice. Type I IFN signaling was also shown to be crucial for the increase in PD-L1⁺ cells in the subsets beside alveolar macrophages. IFNAR^{-/-} mice did not show an increase in PD-L1⁺ interstitial macrophages or dendritic cells at day 5 post-HMPV infection. When the level of expression of PD-L1 was measured on cells expressing this inhibitory ligand, it became even clearer that type I IFN is important for the upregulation of PD-L1. In every subset of APC found in the lung, the expression of PD-L1 was higher in infected IFNAR-competent animals than uninfected controls, and this increase in expression was lost in IFNAR^{-/-} mice. I also showed that alveolar macrophages expressed significantly higher levels of the inhibitory ligand on PD-L1⁺ cells than in pDCs, CD11b⁺ and CD11b⁻ DCs, as well as interstitial macrophages. These data indicate that alveolar macrophages may represent a subset of APC that is particularly capable of the inhibition of T_{CD8} response in the lungs. Since the increase in was not dependent on type I IFN signaling, the ratio of PD-L1^{hi} alveolar macrophages compared to the other PD-L1^{lo} APC subsets was increased in IFNAR^{-/-} mice. This difference could contribute to higher levels of impairment observed in these mice, as more T_{CD8}:APC interactions occurred between the T cells and this PD-L1^{hi} population of APC as compared to other subsets, perhaps more capable of generating a functional response.

At day 10, I showed that signals other than type I IFN results in an increase in many APC subsets, as well as potent upregulation of PD-L1 on conventional DC

subsets. This is most likely due to type II IFN signaling by T cells responding to HMPV infection, and shows the benefit for a virus in targeting a component present in both pathways such as STAT1. I also observed that conventional DCs have two distinct expression levels of PD-L1, and the ratio of high PD-L1 expressing to low PD-L1 expressing CD11b⁻ DCs is much higher than CD11b⁺ DCs. This suggests that, as has been suggested (Kim and Braciale, 2009), CD11b⁻ DCs are more capable at driving a functional response and this proximity to highly functional T_{CD8} leads to an upregulation of this inhibitory molecule.

Finally, in order to investigate the importance of type I IFN signaling on hematopoietic compared to non-hematopoietic cells, I generated reciprocal bone marrow (BM) chimeras of WT and IFNAR^{-/-} mice. Preliminary data from these experiments show that the lung dysfunction observed during HMPV infection in WT mice is driven by type I IFN signaling in both compartments, since neither mice with IFNAR^{-/-} tissue nor those with IFNAR^{-/-} BM displayed as severe pathogenesis as the WT animals, as measured by breath distension. The functionality and magnitude of the T_{CD8} response, in contrast, was driven by type I IFN signaling on BM-derived cells. HMPV-specific T_{CD8} was greater in WT and mice with WT BM but IFNAR^{-/-} tissue, as compared to either holistic IFNAR KO or mice reconstituted with IFNAR^{-/-} BM.

The data presented in this thesis contribute to the understanding of how the innate immune system is able to prime a functional anti-viral response to respiratory viruses and what the implications of type I IFN signaling are on disease pathogenesis. Chapter II also outlined a potential mechanism for type I IFN evasion for HMPV via the expression of the SH protein and its inhibition of STAT1. My data reveal that type I IFN signaling in the tissues of the lung make a significant contribution to disease pathogenesis during HMPV infection, and that without this IFNAR signaling in BM-derived cells, the infected host is unable to generate as potent of an anti-viral T_{CD8}

response. Despite this, IFNAR^{-/-} mice are able to clear HMPV with normal kinetics, suggesting that, for critically ill patients, a therapeutic approach targeting the type I IFN response might actually lead to less disease. It will be important to closely study the consequences of loss of type I IFN signaling in repeat infections, but data presented here show that mice lacking this pathway are able to potentially develop a nAb response required for protection. Overall, the results presented in this thesis suggest that the evasion of type I IFN by HMPV results in higher viral replication and less disease, without affecting viral clearance, representing a distinct evolutionary advantage to the virus.

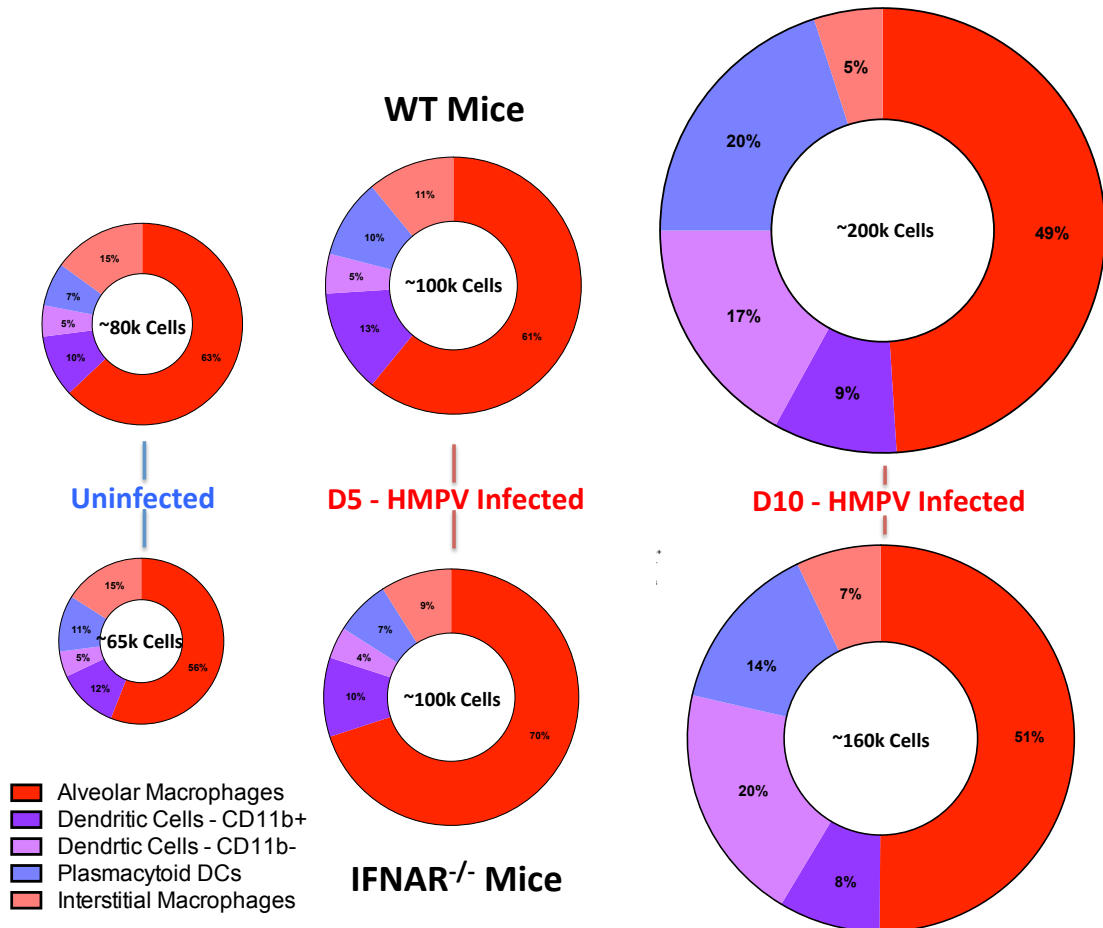


Figure 5-2. Heterogeneous APC populations in the lung upon HMPV infection. The ratio of PD-L1 high alveolar macrophages to other APC populations with lower PD-L1 expression is increased in the absence of type I IFN signaling on day 5, but APC ratios are similar at day 10 although overall APC numbers are markedly increased.

Future Directions

Mechanisms used by HMPV SH protein to inhibit STAT1

While evidence in this thesis shows that HMPV can use the expression of the SH protein to inhibit STAT1 expression and phosphorylation (Chapter II), the mechanism by which this viral protein exerts its inhibitory action is unknown. As discussed earlier, paramyxoviruses use various specific mechanisms to target STAT1, but virtually all require a physical interaction with the host molecule (Andrejeva et al., 2004; Childs et al., 2012; Gainey et al., 2008; Lo et al., 2005). Therefore, it will be interesting to generate an HA- or FLAG-tagged overexpression plasmid of the SH protein to perform IP pull-down assays in vitro, and explore what cellular factors might interact with this protein. Since this interaction could be transient, a technique developed to increase sensitivity of pull-downs between labile binding partners by temporarily cross-linking the interacting proteins together before precipitating the protein complex, called reversible cross-link immuno-precipitation (ReCLIP), could be used (Smith et al., 2011).

In order to analyze the cellular factors potentially interacting with the SH protein, we will first run the IP product on a SDS-PAGE gel and use specific antibodies towards components in the type I IFN pathway, including STAT1, to probe for their presence. This same gel can also be probed with coomassie to non-specifically label proteins, and individual bands differing from controls can be excised from the gel and subjected to shotgun analysis by single-dimension liquid chromatography tandem mass spectrometry (LC-MS/MS) (Smith et al., 2011). The importance of these identified SH binding partners to STAT1 inhibition can then be assessed by using siRNAs to specifically target these proteins.

Once cellular targets of the SH protein are identified, the domain with which this protein interacts with its target should be determined. Since HMPV SH is a type II

transmembrane protein, its N-terminus is thought to be embedded in the membrane, so by progressively deleting the C-terminal tail the motif necessary to interact with host proteins can be identified. SH is also thought to contain multiple N-glycosylation sites so the mutation of these sites will allow us to determine if the glycosylation of SH contributes to its function as an innate immune antagonist. The SH protein of HMPV is only about 60% conserved among the subgroups of this virus (Biacchesi et al., 2003; van den Hoogen et al., 2002), and so far only the version from the A2 subgroup has been analyzed. To determine if this effect is conserved, overexpression plasmids with the SH protein from the A1, B1 and B2 subgroups of HMPV should be generated and tested in these assays.

Many paramyxoviruses hijack cellular proteasomal pathways to degrade important innate signaling molecules (Andrejeva et al., 2002; Ramaswamy et al., 2004; Ramaswamy et al., 2006), but preliminary studies using an irreversible proteasome inhibitory, epoxomicin, indicate that chemical inhibition of the proteasome does not prevent that inhibition of STAT1 (data not shown). Additional work to further test this pathway, perhaps using other proteasome inhibitors or testing a wider concentration of epoxomicin, is necessary to completely eliminate the possibility that SH is using host proteasome to apply its inhibitory action on STAT1.

Microscopy can be used to determine the sub-cellular localization of both the SH protein, as well as STAT1, and to determine if these two molecules co-localize together. Other paramyxoviruses have shown the capability to sequester STAT1 in high molecular weight conglomerates (Rodriguez et al., 2004; Rodriguez et al., 2002), and both the transient SH-GFP overexpression plasmid as well as the stably transfected 293Flp cell line expressing HMPV-SH can be used to assess the effect of SH on STAT1 *in vitro*. Other groups have suggested that HMPV SH, like RSV SH, can form a viroporin (Masante et al., 2014), and these studies could shed more light on how this putative

function might harmonize with the ability for this protein to inhibit STAT1. Finally, the use of the HMPV- Δ SH virus can be used along with expression plasmids of SH mutants to determine if these mutants can rescue wild-type function, and this virus can also be used *in vivo* to look at the generation of adaptive immune responses.

Importance of IFNAR signaling in various immune cell types for T_{CD8} function and HMPV pathogenesis

Experiments outlined in chapter IV began to answer the question of which cell type relies on type I IFN signaling for its part in the generation of a robust and functional T cell response. I generated reciprocal bone-marrow chimeras of WT and IFNAR^{-/-} mice to create mice that expressed the type I IFN receptor only on hematopoietic cells or only in non-hematopoietic cells, and infected them with HMPV to determine how expression in each of these host compartments affects HMPV pathogenesis and T cell response. The results can be found previously, but, in short, type I IFN signaling in bone-marrow derived cells is particularly important for the generation of a specific functional T_{CD8} response. Deletion of INFAR in either cell type ameliorates disease pathogenesis as it is measured by lung dysfunction, in a phenomenon likely related to the expression of type II IFNs.

In order to more accurately deduce the effect of type I IFN deficiency in hematopoietic or non-hematopoietic cells, it will be important to monitor the kinetics of viral replication in these animals. IFNAR^{-/-} mice showed higher replication early in HMPV infection but normal clearance, and titers should be measured in bone-marrow chimeric mice over the course of a normal infection. I hypothesize that type I IFN in the lung tissue plays an important role in early HMPV replication, and that this higher early replication leads to higher nAb response, since viral antigen drives the neutralizing response (Pinschewer et al., 2004), and enhanced clearance as lower PD-L1 and higher

Ag levels early in infection drive a functional response. The presence of APCs in the lungs of mice infected for 10 days with HMPV in these animals is difficult to interpret given the extensive overlap in downstream signaling events between type I and type II IFN (Platanias, 2005), so an analysis of these cells at day 5, before substantial TCD8 infiltration and IFN γ expression, is needed.

The reconstitution of IFNAR $^{-/-}$ mice with bone marrow from WT animals can restore T $_{CD8}$ function, but this assay cannot parse the contribution of type I IFN signaling to impairment in individual cell types within this group. Published data shows that type I IFN signaling directly on T $_{CD8}$ could be important for the development of a strong T cell response (Kolumam et al., 2005b), and also that IFNAR signals are crucial for the proliferation and maturation of APCs, important for a potent T $_{CD8}$ response. To determine the direct contribution of type I IFN signaling in T $_{CD8}$ cells, CD8-Cre (Yao et al., 2013) expressing mice can be bred with IFNAR $^{flox/flox}$ mice to generate a mouse lacking IFNAR specifically on CD8 $^{+}$ T cells and the response to intranasal infection with HMPV should be monitored. To more closely examine the importance of this signaling in the APC subsets, CD11c-Cre (DCs/Alveolar Macs) (Caton et al., 2007) or LysM-Cre (Macrophages) (Clausen et al., 1999) can be used with the IFNAR $^{flox/flox}$ mouse strain to ablate expression of IFNAR on these cells. Subsets of APCs can also be harvested from WT and IFNAR $^{-/-}$ mice at the peak of HMPV infection and used in *ex vivo* T cell activation assays to see if these cells have a differential capacity to prime a functional T $_{CD8}$ response (Kim and Braciale, 2009).

Since we know that type I IFN signaling can play an important role in early immune development, it would be ideal to have a model that developed in a normal manner but then could be manipulated temporally to lose expression of IFNAR. The newly developed CRISPR-Cas9 technology allows for just that. Recently, a mouse has been developed on the C57BL/6 background that constitutively expresses the Cas9

enzyme in all cells, as well as one expressing this enzyme upon Cre-expression (Platt et al., 2014). These mice will also both soon be available through Jackson laboratories. It was shown that by utilizing short-guide RNAs (sgRNA) specific for a gene sequence they were able to efficiently ablate genes even in difficult to target primary cells, like dendritic cell precursors in the bone marrow (BMDCs)(Platt et al., 2014). The Cas9-expressing cells also express EGFP, which allows for easy identification of these cells upon transfer to a second animal.

To determine what the role of IFNAR is on specific cells, the CD8-Cre, CD11c-Cre and LysM-Cre mice could be used in conjunction with the Cas9 mouse to generate mice expressing the enzyme necessary for excision of the gene only in specific cell types. Next, a lentivirus could be injected into mice to transduce the IFNAR sgRNA into cells *in vivo* (Leander Johansen et al., 2005). This would result in a specific loss of IFNAR only in cells expressing Cre, and then HMPV infection of these mice could be used to determine the specific contribution of type I IFN signaling in selected cells to the overall host response. Alternatively, a technique for generating lentiviral vectors capable of targeting specific cell types *in vivo* (Yang et al., 2006) could be used along with Cas9 mice to transduce only specific cells in the immune response with the targeting IFNAR sgRNA. To target IFNAR in lung epithelial cells, an adenoviral vector could also be administered intranasally to deliver sgRNA to lung epithelium (Platt et al., 2014).

Another attractive aspect of this experimental approach is that, it will allow us to probe the importance of IFNAR on the secondary response by first infecting with HMPV and then waiting until mice have developed immunity before experimentally manipulating animals to selectively delete the IFNAR receptor. In order to more accurately study the secondary response *in vivo*, a modification to our infection protocol is needed and that is explored more in the next section.

Generation of memory T_{CD8} in absence of IFNAR

As shown in chapter III, C57BL/6 mice, regardless of their expression of IFNAR, are capable of generating a potent neutralizing antibody response that results in protection from subsequent viral infections (Graham et al., 1991; Prince et al., 1999; Williams et al., 2005c). Because of this, these animals are of limited use for the examination of a memory response, but Blimp-1 and Bcl-6 are markers for effector and central memory, respectively, and a transcriptional analysis of WT compared to IFNAR^{-/-} HMPV-specific T_{CD8} could compare the ratio of expression of these distinct drivers of differentiation and give a clue as to whether a lack of type I IFN signaling drives the T cell response towards a more effector or memory phenotype.

To more fully characterize the secondary responses of T_{CD8} to HMPV, and the impact that type I IFN has on this reactivation, a mouse deficient in B cells, μ MT, has been utilized in our lab to investigate secondary HMPV infection. These mice, while susceptible to re-infection with HMPV, are severely immunocompromised and could have secondary defects due to their complete lack of B cells (Kitamura et al., 1991). Therefore, I propose to instead use a mouse with a transgenic B cell receptor restricted in specificity to a single antigen, such as the model frequently used to study B cell immune tolerance which only makes antibodies specific to hen egg lysozyme (HEL) (Ferry and Cornall, 2004). These animals would be unable to make an HMPV-specific nAb response, but would have a more normal immune response as compared to μ MT. These animals could be crossed with IFNAR^{-/-} mice, either genetically or using bone-marrow chimeras since my data shows that type I IFN in hematopoietic cells drives T_{CD8} functionality, to assess the implications of type I IFN on the generation of a protective secondary T_{CD8} response and the impairment observed during viral infection.

Role of PD-L1^{high} alveolar macrophages in T_{CD8} exhaustion and HMPV pathogenesis

Data presented in chapter IV of this thesis indicate that alveolar macrophages are the most abundant APC found in the lungs of naïve or HMPV-infected mice, and that they constitutively express the inhibitory ligand PD-L1. This raises the possibility that alveolar macrophages act as master inhibitory cells early in viral replication and could contribute to the T_{CD8} impairment observed during acute viral LRI. A recent study in influenza shows that the treatment of mice intranasally with clodronate-loaded liposomes can potentially eliminate airway macrophages, and this leads to higher pathogenesis during viral infection (Tate et al., 2010).

Viral control, disease pathogenesis and T_{CD8} response should be measured in the context of macrophage depletion, as well as the ability for these animals to develop sterilizing immunity. Utilization of LysM-Cre mice and PD-L1^{flox/flox} mice (Keir et al., 2007) to generate mice lacking expression of PD-L1 solely on macrophages could also be used to determine whether this cell type is a major contributor to the inhibitory effect on T_{CD8} in the lung. Using this macrophage-specific KO of PD-L1 and also a CD11c-Cre and PD-L1^{flox/flox} breeding strategy to create mice lacking this molecule on both alveolar macrophages and dendritic cells could provide a good way to determine whether these APC subsets have differential or overlapping roles in T_{CD8} impairment over the course of HMPV infection.

Conclusions and significance

Together, the data generated during my thesis work build a foundation that provides for multiple avenues for future studies. The molecular tools I have created can be used to evaluate the contribution of viral proteins from HMPV on a myriad of cellular processes, and the flow assays I have designed will allow for the further analysis of the

various APC subsets *in vivo* during acute respiratory viral infection. The future directions outlined here will elucidate the mechanism underlying the observed inhibition of STAT1 signaling by the SH protein of HMPV. Additionally, these experiments will enrich the understanding of the role of type I IFN signaling on individual cell types involved in the T_{CD8} response in the lungs during acute viral infection. They will also specifically examine the inhibitory role of alveolar macrophages in the lung during acute viral infection, as well as their contribution to disease pathogenesis. These studies will help to uncover more about the specific immune evasion strategy of HMPV. They will also aid in furthering our understanding of the contribution of the balance of APC subtypes in the lung to T_{CD8} impairment and pathogenesis during HMPV. The ability for HMPV to reinfect individuals, despite the presence of neutralizing antibodies, is a major public health issue and the presence of alveolar macrophages, with a potent ability to inhibit T_{CD8} response, could increase the likelihood of this occurrence. Understanding the importance of the type I IFN response in pathogenesis could also lead to the development of improved therapies for severely ill patients.

The type I IFN response is crucially important for the response of the host to some viruses, and loss of this pathway leads to lethal consequences in these models. For HMPV however, the loss of type I IFN signaling, while leading to significantly more viral replication early in infection, is not catastrophic for the infected animal. The data presented in this thesis suggest that this is likely due to an overlapping role between type I and II IFN in the anti-viral response. HMPV has responded to this dual-attack stratagem of the host by evolving strategies that target STAT1, a molecule common to both signaling pathways. This allows for the virus to replicate to higher levels locally in infected cells while the IFN pathway drives disease pathogenesis, which actually facilitates the expulsion of viral particles from the lung through coughing. The ability for the adaptive response to clear this virus without assistance from type I IFN signaling,

reveals this pathway as one that potentially could be therapeutically manipulated to ameliorate symptoms in the lungs of patients severely ill with HMPV without repercussions to clearance.

In light of my data showing the IFN-induced pathogenesis of HMPV, the phenomenon of Ag-driven T_{CD8} impairment in the lungs during acute viral infection can be seen as a host adaptation to protect the sensitive and crucial tissues of the lung. An over-active T_{CD8} response can be detrimental to the host, and even the highly impaired function observed in virus-specific T_{CD8} in IFNAR^{-/-} mice is sufficient to clear HMPV. The implications of this impairment to the memory response could lead to significant advancements in vaccine development and to the understanding of why viruses like HMPV are able to repeatedly infect individuals despite the presence of neutralizing antibodies.

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