Role of Neutrophils in the Modulation of Host Responses to Human Respiratory Pneumovirus Infection

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ROLE OF NEUTROPHILS IN THE MODULATION OF HOST RESPONSES TO HUMAN RESPIRATORY PNEUMOVIRUS INFECTION

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Pathobiological Sciences

by

Nagarjuna Reddy Cheemarla M.S.
Wright State University, Dayton, Ohio 2013
August 2018
This dissertation is dedicated to the memory of my brother Anirudha Cheemarla, whom I still miss everyday. You have been my inspiration.
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ABSTRACT

Neutrophils are the most abundant leukocytes (50% to 70%) in humans and are the first immune cell population recruited to the sites of infection. They are known to act as the first line of innate immune defense against invading pathogens, and more recently, to playing a crucial role in orchestrating adaptive immune responses. The role of neutrophils in the respiratory viral infections till date remains unclear and controversial. Previous studies demonstrated both beneficial as well as pathogenic role for neutrophils in respiratory viral infections, especially influenza.

Previous findings reported an early and high influx of neutrophils into the airways early after respiratory pneumovirus infection. Nevertheless, the role of these cells in respiratory pneumovirus induced host responses remains largely unclear. Human metapneumovirus (HMPV) and Human respiratory syncytial virus (HRSV) belong to the family Pneumoviridae, which are significant human respiratory pathogens and causing acute lower respiratory tract infection with similar clinical symptoms in the young, elderly and immune-compromised individuals.

In understanding the role of neutrophils in these infections, we used in vivo mouse models of HMPV or HRSV infection. Depletion of neutrophils during HMPV infection in mice led to increased inflammatory responses and disease outcome, proving the beneficial role of these cells during HMPV infection. On the other hand, RSV infection in neonatal mice exposed to cigarette smoke in-utero exhibited exacerbated lung pathology characterized by an increased neutrophil recruitment into the airways, suggesting a harmful role of neutrophils when recruited in excessive levels. Overall, our studies indicate that neutrophils play a key role in orchestrating
host inflammatory responses during respiratory pneumovirus infection and that, a fine balance in the recruitment of neutrophils is essential during respiratory viral infection.
CHAPTER 1
INTRODUCTION

1.1. Introduction

Human metapneumovirus (HMPV), belongs to the Paramyxoviridae family and represents the first human member of the genus Metapneumovirus. HMPV is a leading respiratory viral pathogen causing acute respiratory tract infection (ARTI) in young children, the elderly and immunocompromised individuals [1]. HMPV was first isolated in the Netherlands in 2001 from respiratory specimens of young children suffering with acute respiratory tract illness [2] and represents a major respiratory pathogen worldwide. Epidemiological studies show that HMPV is responsible for 5%–15% of pediatric hospitalizations for respiratory tract infections [3,4,5,6,7]. It induces clinical syndromes ranging from mild disease to more severe disease, with high fever, wheezing, severe cough, difficulty in breathing, tachypnea, bronchiolitis and pneumonia [8,9,10].

hHMPV is an enveloped, negative sense single-stranded RNA virus (Figure 1.1). Based on phylogenetic analysis, HMPV is classified into four genetic lineages, named A1, A2, B1 and B2 that divide into the A and B antigenic subgroups that belong to one serotype [11, 12]. HMPV genome size is approximately 13,000 nt as it varies depending on the strain. Examples of the subgroup A indicate that the strain CAN97-83 is 13,335 nt and NL/00/1 is 13,350 nt, and for the subgroup B: CAN98-75 is 13,280 nt and NL/1/99 is 13,293 nt [11,13]. The HMPV sequence includes eight genes encoding nine proteins: nucleocapsid (N), phosphoprotein (P), matrix (M), second matrix (M2-1, M2-2), fusion (F), small hydrophobic (SH), attachment (G) and

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RNA-dependent RNA polymerase (L). The gene order in HMPV is represented as 3′-N-P-M-F-M2- SH-G-L-5′ (Figure 1.1). The attachment (G) and small hydrophobic (SH) genes are found to be highly variable while a high level of sequence conservation has been observed for the fusion (F) gene [13]. The G protein is a transmembrane surface glycoprotein, which initiates the virus-host cell membrane attachment and so considered as a key player in viral replication. The fusion (F) protein is required for the fusion of virus with host cell membrane and is capable of being accessed by neutralizing antibodies. The nucleocapsid (N), phosphoprotein (P) and RNA-dependent RNA polymerase (L) proteins along with M2 protein are involved in RNA synthesis [11,14,15].
Figure 1.1. Model structure and proteins encoded by Human Metapneumovirus (HMPV). (a) HMPV model structure indicating viral proteins encoded by (b) the viral genome.

Several animal models including mouse (see Table 1), cotton rat [16,17,18,19], hamster [20,21,22], ferret [20] and nonhuman primate models [20,23,24] have been established to date to study the immunopathology occurring after HMPV infection. Among them, the mouse model has provided considerable knowledge towards our understanding of the HMPV-host interaction.
Thus this review focuses on the current knowledge of the immunity and immunopathology induced by HMPV in the experimental mouse model of infection.

**Table 1.** Different conditions for mouse infection with HMPV

<table>
<thead>
<tr>
<th>Mice Strain</th>
<th>Mice Age</th>
<th>(Group) Strain</th>
<th>Virus Dose</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>F 6–8 week-old</td>
<td>(A) NL 00-01</td>
<td>$3.3 \times 10^5$ PFU</td>
<td>[25]</td>
</tr>
<tr>
<td>BALB/c</td>
<td>F 4–6-week-old</td>
<td>(A) C-85473</td>
<td>$1.5 \times 10^5$–$10^6$ TCID$_{50}$</td>
<td>[16,26–32]</td>
</tr>
<tr>
<td>BALB/c</td>
<td>F 6–8-week-old</td>
<td>(A) C4-CJP05</td>
<td>$10^6$ PFU</td>
<td>[33]</td>
</tr>
<tr>
<td>BALB/c</td>
<td>F 4–6-week-old</td>
<td>(B) CAN98-75</td>
<td>0.8–1 × $10^6$ PFU</td>
<td>[29,34,35]</td>
</tr>
<tr>
<td>BALB/c</td>
<td>F 5–7 week-old</td>
<td>(A) NL/1/00</td>
<td>$10^6$–$10^7$ PFU</td>
<td>[20,36]</td>
</tr>
<tr>
<td>BALB/c</td>
<td>F 6–7 week-old</td>
<td>(B) NL/1/99</td>
<td>$10^7$ PFU</td>
<td>[36]</td>
</tr>
<tr>
<td>BALB/c</td>
<td>F 6–10 week-old</td>
<td>(A) CAN97-83</td>
<td>$10^6$–$10^7$ PFU/TCID$_{50}$</td>
<td>[30,37–41]</td>
</tr>
<tr>
<td>BALB/c</td>
<td>F 5–6 week-old</td>
<td>(A) CZ0107</td>
<td>$10^6$ PFU</td>
<td>[42]</td>
</tr>
<tr>
<td>BALB/c</td>
<td>M 19 month-old</td>
<td>(A) CAN97-83</td>
<td>$2 \times 10^7$ geq</td>
<td>[43]</td>
</tr>
<tr>
<td>BALB/c</td>
<td>F 8–10 week-old</td>
<td>(A) D03-574</td>
<td>$2 \times 10^5$ PFU</td>
<td>[44]</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>6–10 week-old</td>
<td>(A) CAN97-83</td>
<td>$10^6$–$10^7$ PFU</td>
<td>[38,45–49]</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>F 6–12 week-old</td>
<td>(A) TN/94-49</td>
<td>$0.6$–$1.5 \times 10^6$ PFU</td>
<td>[50–53]</td>
</tr>
<tr>
<td>DBA/2</td>
<td>5–6 week-old</td>
<td>(A) TN/94-49</td>
<td>$10^5.9$ PFU</td>
<td>[17]</td>
</tr>
<tr>
<td>SCID</td>
<td>F 6–8 week-old</td>
<td>(A) NL/1/00</td>
<td>$6.5 \times 10^6$ PFU</td>
<td>[54]</td>
</tr>
</tbody>
</table>

PFU = Plaque Forming Units; geq = genome equivalents; TCID$_{50}$ = 50% tissue culture infective dose.

### 1.2 HMPV Infection in Mice

The experimental mouse model of HMPV infection has been established in several mouse backgrounds using different HMPV strains at diverse inoculum concentrations, as shown in Table 1.

Intranasal inoculation of mice with HMPV induces pulmonary inflammation characterized by interstitial inflammation and/or peribronchiolar and perivascular cellular infiltration [30,35,39,49], body weight loss with a peak of 15%–25% [16,25,32,34,41], altered respiratory function characterized by a significant increase in airway obstruction on day 5 after HMPV infection that could persist until day 21 [30], and lung viral titers that peak between day 3 to day 14 after HMPV infection [16,25,30,41].
However, some variations can be observed depending on the different experimental conditions. For instance, intranasal inoculation of BALB/c mice with HMPV CAN98-75 resulted in a biphasic lung viral replication with peaks at day 7 and day 14 [34,35] while infection of BALB/c mice with any other HMPV strain led to a one-peak only of viral titer on or before day 5 after infection (Table 2). Based on the data from the reports included in Table 2, BALB/c mice appear to be more permissive than C57BL/6 mice. Although, shedding of infectious virus beyond the recovery phase has been rarely reported [34], detection of HMPV transcripts have been found at day 154 [30] and 180 [34] after infection, suggesting that HMPV could persist in the lung of infected animals since HMPV infection has been characterized as a localized infection affecting just the airways but no other organs [35].
Table 2: Mouse susceptibility and permissibility to HMPV.

<table>
<thead>
<tr>
<th>Mice Strain</th>
<th>Virus Strain</th>
<th>Virus Inoculum</th>
<th>Peak Viral Titer</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>NL/1/00</td>
<td>$3.3 \times 10^5$ PFU</td>
<td>Day 4 ($\log_{10} 2.37$ PFU/g)</td>
<td>[25]</td>
</tr>
<tr>
<td>BALB/c</td>
<td>CAN97-83</td>
<td>$10^7$ TCID$_{50}$</td>
<td>Day 4 ($10^5$ TCID$_{50}$/g)</td>
<td>[41]</td>
</tr>
<tr>
<td>BALB/c</td>
<td>C85473</td>
<td>$1.5 \times 10^5$ TCID$_{50}$</td>
<td>Day 6 ($10^4$ TCID$_{50}$/lung)</td>
<td>[26]</td>
</tr>
<tr>
<td>BALB/c</td>
<td>C85473</td>
<td>$1 \times 10^8$ TCID$_{50}$</td>
<td>Day 5 ($7 \times 10^6$ TCID$_{50}$/lung)</td>
<td>[30]</td>
</tr>
<tr>
<td>BALB/c</td>
<td>C85473</td>
<td>$1 \times 10^8$ TCID$_{50}$</td>
<td>Day 5 ($1.92 \times 10^7$ TCID$_{50}$/g)</td>
<td>[16]</td>
</tr>
<tr>
<td>BALB/c</td>
<td>C85473</td>
<td>$5.8 \times 10^5$ TCID$_{50}$</td>
<td>Day 5 ($10^5$ TCID$_{50}$/g)</td>
<td>[32]</td>
</tr>
<tr>
<td>BALB/c</td>
<td>NL/1/00</td>
<td>$1.5 \times 10^5$ PFU</td>
<td>Day 5 ($5.1 \times 10^5$ PFU/g)</td>
<td>[55]</td>
</tr>
<tr>
<td>BALB/c</td>
<td>D03-574</td>
<td>$2 \times 10^5$ PFU</td>
<td>Day 4 ($10^{3.6}$ PFU/lung)</td>
<td>[44]</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>CAN97-83</td>
<td>$5 \times 10^6$ PFU</td>
<td>Day 5 ($10^{4.9}$ PFU/g)</td>
<td>[46]</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>TN/94-49</td>
<td>$1 \times 10^6$ PFU</td>
<td>Day 5 ($4.7 \log_{10}$ PFU/g)</td>
<td>[53]</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>CAN97-83</td>
<td>$1 \times 10^7$ PFU</td>
<td>Day 5 ($10^{4.1}$ PFU/g)</td>
<td>[47]</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>TN/94-49</td>
<td>$6 \times 10^5$ PFU</td>
<td>Day 5 ($10^{4.2}$ PFU/g)</td>
<td>[51]</td>
</tr>
</tbody>
</table>

1.3 Lung Antiviral and Inflammatory Response

1.3.1 Innate Immunity

Innate immune responses to viral infections in the lung serve as the first line of defense and it is activated upon recognition of the pathogen by immune cells in the respiratory tract. The cellular barrier constituting neutrophils, macrophages, natural killer (NK) cells and dendritic cells (DC) play a key role in the innate immune responses, which is triggered by the recognition of pathogen associated molecular pattern (PAMP) by cell receptors called pattern recognition receptors (PRRs) expressed in most cells of the respiratory tract. These pattern recognition receptors are broadly classified into membrane bound Toll-like receptors (TLRs), C-type lectin receptors (CLR), cytoplasmic RIG-I-like receptors (RLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) [56]. The recognition of viral PAMPs by the cellular PRRs initiate the activation of signaling pathways leading to the production of
cytokines and chemokines by the cells in the respiratory tract, that in turn regulate the inflammatory and immune responses in the infected host.

1.3.2 Pattern Recognition Receptors and Signaling Pathways

We have recently demonstrated the importance of the RLR helicase melanoma differentiation-associated gene 5 (MDA5) in the type I (α/β) and type III (λ) interferon (IFN) production by HMPV infection [45]. In a model of MDA5-deficient mice (C57BL/6J background) infected with HMPV CAN97-83, the lack of MDA5 resulted in a decreased viral clearance, enhanced disease severity and pulmonary inflammation, and was necessary for the production of IFN-α/β and IFN-λ2/3. Moreover, MDA5 regulated the production of cytokines and chemokines in response to HMPV, demonstrating the critical role MDA5 plays in the control of HMPV-induced disease [45]. Downstream of the MDA5 signaling pathway, this helicase interacts with the adaptor molecule IFN-promoter stimulator 1 (IPS-1) at the mitochondrial membrane in order to induce the expression of cytokines [57]. In that regard, studies in neonatal IPS-1 deficient mice (C57BL/6 background) have shown that the absence of IPS-1 led to an increased viral load and decreased production of IFN-β and IFN-λ2/3 at day 1 after HMPV infection [58], indicating that IPS-1 contributes to the antiviral response and HMPV clearance. Moreover, similar IFN response to HMPV infection in the absence of IPS-1 has been reported in adult mice [59]. Thus, these findings confirm the key role for the MDA5 and IPS-1 signaling pathway in the antiviral response against HMPV infection.

On the other hand, data of HMPV infection in C57BL/10ScSnJ Toll-like receptor 4 (TLR4) deficient mice have shown that absence of TLR4 resulted in a decreased inflammatory response, disease severity, as well as IFN-α/β and cytokine production [48]. In line with those data, the lack of myeloid differentiation protein response 88 (MyD88), an essential adaptor
molecule for TLR’s (except TLR3), led to a reduced lung inflammation and disease severity compared to wild type mice. The absence of MyD88 also impaired the production of cytokines and chemokines and the recruitment of DC, CD4 and CD8 T cells into the lungs of infected mice [47]. Collectively, these studies indicate that TLR4 and MyD88 are key molecules that regulate the HMPV-induced pulmonary inflammation and disease pathology in mice.

Signaling via PRRs ultimately leads to the activation of the transcription factors interferon (IFN) regulatory factors (IRFs), which induce the expression of the interferons and cytokine responses. Data in C57BL/6 mice have demonstrated that the expression of both IRF3 and IRF7 were necessary for the production of IFN-α/β [45]. In agreement with these results, in HMPV-infected C57BL/6 neonatal mice, both IRF3 and IRF7 were necessary for the expression of IFN-α4 and IFN-β. Moreover, the absence of both IRF3 and IRF7 exacerbated the Th1, Th2, and Th17 lymphocyte responses as well as the recruitment of neutrophils, eosinophils, NK and NK T cells in response to HMPV infection [58]. Similarly, the production of IFN-λ2/3 after HMPV infection was regulated by the expression of IRF-7 in adult [49] and neonatal [58] mice. However, the expression of IRF-3 was necessary for the production of IFN-λ2/3 in neonatal mice [58] but it was dispensable when the IFN-λ2/3 was induced by HMPV in adult mice [49], suggesting that the activation of the IFN-λ response by HMPV in adult and young mice is differentially regulated by IRF-3 and IRF-7 expression. Interestingly, HMPV has also been reported to inhibit the IFN responses [39,49]. Studies in BALB/c mice have demonstrated that HMPV infection inhibits the poly-ICLC- (synthetic dsRNA, TLR3/RIG-I/MDA5 agonist) and CpG-ODN- (TLR9 agonist) induced IFN-α production [39], suggesting that HMPV infection is able to inhibit the activation of RLRs and TLRs in vivo. In addition, recent data have shown that
HMPV G protein inhibits the production of IFN-λ2/3 in BALB/c mice after HMPV infection, at least through the interference with the RIG-I/MDA5 pathway [49].

Based on the reported observations described above, HMPV-induced immune response is regulated by the activation of selected PRRs. It appears that HMPV infection activates TLRs to induce an inflammatory response while it subverts RLRs to alter the antiviral responses via the inhibition of interferons. This immune subversion is attributed to the expression of HMPV G protein. Taken together, experimental evidence demonstrates that HMPV is able to activate and subvert antiviral signaling pathways, likely through different mechanisms. However, unresolved pathways involved in activation or subversion of HMPV induced immune response, need further elucidation. A detailed understating of HMPV induced recognition and signaling cascades is crucial to developing effective therapeutics and vaccine strategies.

1.3.3 Cytokine Production

HMPV is known to induce in humans a profile of cytokines distinct to other respiratory viruses such as respiratory syncytial virus (RSV), and influenza virus [60]. Although very scarce, studies comparing HMPV and RSV infection are clinically relevant as RSV is the closest related human paramyxovirus to HMPV [11]. In fact, symptoms between RSV and HMPV are indistinguishable, ranging from mild cold-like symptoms to more severe clinical manifestations like bronchiolitis or severe pneumonia that require hospitalizations [3,7,61]. However, some aspects of the immune response elicited by these two viral pathogens are distinct. This was demonstrated by the analysis of nasal washes from hospitalized infants showing that HMPV infection induced significantly lower amounts of proinflammatory cytokines including IL-12, IL-6, IL-8, TNF-α and IL-1β compared to RSV infection [60], suggesting that HMPV is a poor inducer of inflammatory cytokines compared to RSV in infected infants. In line with these data,
research in the mouse model resembled the observation in human studies. Using BALB/c mice infected with HMPV (CAN97-83) and compared to RSV (A2) side-by-side, HMPV induced a weaker response of proinflammatory cytokines (IL-1α, IL-1β, IL-6, TNF-α, G-CSF) and regulatory cytokines (IL-10, IL-12p70, IL-17). However, HMPV induced a stronger response of IFN-α, GM-CSF, IL-18, CXCL1 (KC) and a sustained production of IL-12p40 [37]. In contrast to this work, a study conducted in BALB/c mice using a clinical HMPV isolate (D03-574) induced significantly higher levels of TNF-α, IL-6 and MCP-1 compared to RSV (A2) at day 4 and 7 post infection [44]. The discrepancies between these two studies in mice could be due to the use of different virus strains and virus stock preparations.

The effect of HMPV on the IFN response has been further confirmed since experimental observations indicated that HMPV induced a stronger response of IFN-β and IFN-λ2/3 when compared to RSV infection in BALB/c mice [49]. However, levels of IFN-γ were induced similarly by HMPV and RSV-infected BALB/c mice [44]. Additional data have also demonstrated the capacity of HMPV to induce several cytokines in the lung, where a significant induction of CCL2 (MCP-1) and CXCL1 (KC) on day 1 and IFN-γ, CCL5 (RANTES), CCL3 (MIP1α), and IL-4 on day 5 after HMPV infection has been observed [16, 25]. Overall, these findings suggest that HMPV infection induces a unique profile of cytokines and chemokines in the lung of infected mice.

The regulatory effects that lung cytokines and chemokines exert in HMPV-induced disease are still largely unexplored. In that regard, IL-12p40, an induced cytokine during HMPV infection that remains sustained after the resolution of the disease [37] has been shown to be critical to control disease severity by regulating cytokine production, inflammatory response and mucin production in the lung. Using IL-12p40/-/ mice infected with HMPV, showed an
increased goblet cell formation, increased mucin gene expression in the airways and decreased lung function. IL-12p40 was found to specifically regulate the expression of IFN-γ, IL-6, CXCL10 (IP-10), CCL11 (eotaxin), CXCL1 (KC, IL-8 homolog) and CCL2 (MCP-1) in mice infected with HMPV [46]. Furthermore, the level of expression of inflammatory cytokines after HMPV infection appears to be altered in aged animals. For instance, TNF-α levels were decreased ~7-fold in 19 moth-old HMPV-infected mice when compared to 4–6 week-old animals [43] while IL-6 was increased in 18–19 month-old mice when compared to 6–8 week old mice [62]. Also, HMPV infection alters the cytokine response to opportunistic bacterial infection in the lung. Prior HMPV infection exacerbated the levels of TNF-α, IFN-γ, IL-1α, IL-1β, IL-6, IL-12p40, IL-12 p70, IL-9, IL-10, IL-13, KC, G-CSF, GM-CSF, MCP-1 and MIP-1α in Streptococcus pneumoniae-infected mice and predisposed those animals to severe pneumococcal infection [26]. The described cytokine patterns induced by HMPV infection are crucial to understanding the underlying mechanisms in activation of the innate and adaptive immune responses as well as the initiation and resolution of the inflammatory response and lung viral clearance. However, the role of these cytokine pathways in promoting and modulating inflammation and host immune responses in HMPV infection are still largely unknown. The use of genetically modified mice will represent a critical tool to answer these relevant questions.

1.3.4 Dendritic Cells

Dendritic cells (DC) are professional antigen-presenting cells within the immune system. Respiratory tract dendritic cells are present within airway epithelium, submucosa and associated lung parenchymal tissue under resting conditions [63]. Their strategic localization at the site of pathogen entry makes them particularly susceptible to initial viral invasion. After detection, uptake and degradation of viruses, DC initiate immune responses via the secretion of interferon
(IFN), chemokines and proinflammatory cytokines, as well as the upregulation of a variety of costimulatory molecules and receptors, a process globally known as cell maturation. After maturation, DC efficiently present antigens and initiate adaptive immune response by migrating into lymph nodes (LN) to activate the virus-specific T cell response [32]. To date, there have been at least three major subsets of murine lung DC described. These include plasmacytoid DC (pDC), the myeloid DC (also known a conventional DC, cDC), and the interferon-producing killer dendritic cells (IKDC). DC have been reported to participate in the innate and adaptive immune response to HMPV infections, indicating their critical role in the antiviral immunity to this virus. Dendritic cells are susceptible to HMPV infection in vitro [64] and in vivo [40,65]. In fact, HMPV activates mouse lung DC, and induces the upregulation of costimulatory molecules and the secretion of several cytokines including IL-6, IFN-α, IFN-β and TNF-α [40]. HMPV infection also induced the recruitment of pDC and IKDC which peaked by day 8 after infection. The predominant subset recruited to the lung corresponded to cDC, and this remained the highest subset for at least 18 days, beyond the acute phase of infection. CD103+ cDC substantially decreased until three weeks after infection and returned to basal levels by week 8. Differential production of cytokines by murine lung pDC and cDC infected with HMPV was also observed. More interestingly, HMPV infection reduced the capacity of lung cDC to stimulate T cell responses [40], which is in line with some reports in vitro using human DC that indicate that HMPV alters their capacity to activate T cells [64,66].

1.3.5 Alveolar Macrophages

Alveolar macrophages (AMs) are known to be the first line of defense against respiratory pathogens [67]. They reside in the pulmonary alveolus and survey the exposed airways to contribute to the innate host defense against inhaled insults [68]. They are essential source of
immunomodulatory cytokines for host responses against lung infections and their depletion results in impaired host response [67,69,70]. In fact, recent work has demonstrated that AMs differentially control the antiviral response and airway inflammation in HMPV infection when compared with RSV [69]. Using a BALB/c mouse model, AMs were depleted using clodronate liposomes (L-CL2MBP) prior to HMPV infection. Depletion of AMs altered the HMPV-induced disease since there was a reduced body weight loss, lung viral titer, decreased lung inflammation and airway hyperresponsiveness (AHR). Moreover, the recruitment of CD4+ T lymphocytes was significantly decreased following AM depletion. AMs are sources of pro inflammatory cytokines and chemokines. In line with this, depletion of AMs resulted in significantly lower level of cytokines including IL-1α, IL-1β, TNF-α, IL-6, GM-CSF, G-CSF, CCL4, IFN-α and IFN-β. However, their depletion also induced an increased release of CCL3, CCL5, and IL-12p40 after HMPV infection [69]. Thus, the results of this study indicate that the presence of alveolar macrophages regulate and contribute to the HMPV-induced disease.

1.3.6 Natural Killer Cells

Another component of the innate immune system are the natural killer (NK) cells, which are lymphocytes that respond to malignant tumors and intracellular pathogens including viruses. Studies conducted by Alvarez et al. demonstrated that NK cells have a leading role in controlling HMPV viral clearance [36]. Depletion of NK cells with anti-CD49b/Pan-NK cell monoclonal antibody in BALB/c mice resulted in increased lung viral titers on days 7, 28 and 60 after infection compared to NK cell competent mice. In contrast, work reported by Wen S. et al. in C57BL/6 mice have demonstrated that NK cells do not contribute to HMPV clearance [51]. Lung NK cell numbers in infected mice were, however, increased as early as day 1 after HMPV infection and peaked on day 3 compared to mock infected mice. Moreover, HMPV infection
induced activation of lung NK cells, as indicated by the upregulation of CD69. However, depletion of NK cells using the anti-NK1.1 antibody did not result in changes in lung viral titers, lung histopathology, or the numbers of CD4+ and CD8+ T lymphocytes. Suggesting that, NK cells do not play a significant role in the host responses against HMPV, and that the clearance of the viral infection requires different set of immune components in vivo. The discrepancies between these two studies could be attributed to the use of different experimental conditions, as detailed above. Thus, further work to fully define the role of NK cells in HMPV infection is warranted.

1.4 Adaptive Immunity

Cell mediated immunity serves as an important barrier in the multi-step paradigm of immune responses to pathogenic mechanisms. These responses function mainly by activation of cytotoxic T-lymphocytes to induce apoptosis of virus-infected cells or by activating T helper cells to stimulate other immune cells such as macrophages, B cells and NK cells and aid in the production of distinct cytokine profiles to induce intercellular communication. Experimental evidence with clone-specific induction of cytotoxic T cells [38] and experimental models with T cell depletion studies [34,38,41] demonstrate the essential role of T lymphocytes in immune surveillance and protection in HMPV infection.

Characterization of the T cell response against this virus has indicated that HMPV results in an accumulation of virus-specific cytotoxic CD8+ T cells (CTL) in the lungs 7 days after infection but not in regional lymph nodes or spleen. However, a strong memory response can be recalled from the spleen at 21 days post infection [71]. Though both CD4+ and CD8+ T cells act synergistically and play an indispensable role in both inflammatory responses and anti-viral immunity, they have been found to induce different profile of cytokines after HMPV infection.
During primary infection, depletion of either of the two T cell subsets, or in fact both of them, caused reduced inflammation and body weight loss in HMPV-infected mice but were required for viral clearance. These data suggest that primary HMPV infection induces lung disease mediated, in large extent, by T cells while T cells are also necessary for the clearance of primary HMPV infection [41]. Regarding the regulation of the T cell response during HMPV infection, a recent study by Hastings et al., showed that type I IFN signaling is essential for the development of functional HMPV specific CD8 T cells in the lungs using IFN-α receptor deficient C57BL/6 mice [53]. Moreover, in aged mice, CD4+T cells appear to play an important role in the exacerbated HMPV-induced disease. As demonstrated in 18-19 month-old BALB/c mice which showed a significant increased numbers of IL-4-producing CD4+ T cells but no change in the CD8+ T cell numbers when compared with younger mice [62], suggesting a Th2 skewing response in older mice after HMPV infection.

As for the role of T cells in HMPV reinfection, concurrent depletion of both CD4+ and CD8+ T cells led to a decreased airway hyperresponsiveness (AHR) [41]. However, depletion of CD4+ T cells alone during HMPV reinfection, unlike in CD8+ T cell-depleted mice, led to a defective antibody response. Nevertheless, CD4+ T cell-depleted mice had undetectable infectious virus after HMPV challenge and were protected from clinical disease, indicating that protection can be provided by an intact CD8+ T cell response [41]. Interestingly, recent observations indicate that the CD8+ T cell response is impaired during HMPV infection and reinfection and that phenomenon appears to be regulated by the expression of the inhibitor receptor programmed death-1 (PD-1) and programmed death ligand-1 (PD-L1) [52,72,73]. These findings indicate that a defective CD8+T cell response contributes to HMPV reinfection.
Whether this effect characterizes the commonly observed HMPV reinfection in humans warrants future research.

The understanding of the T cell response induced by HMPV vaccine candidates has found their initial steps using the mouse model. The induction of CD8+ cytotoxic T cells by peptide immunization in mice has proven to be protective against HMPV challenge in reducing viral load and lung histopathology [38]. Likewise, immunization with Bacillus Calmette-Guerin (BCG) strains expressing HMPV-phosphoprotein effectively induced a protective response which was mediated by a Th1 T cell response [42]. Immunization with HMPV F-bearing virus-like particles (VLP) was also able to stimulate an HMPV specific CD8+ T cell response and protected lungs from infection after HMPV challenge [50]. Further experimental studies in non-human primates and/or clinical trials are warranted in order to validate the immunological observations in the mouse model towards vaccine development.

1.5 Conclusions

The experimental mouse model represents a valuable tool for in vivo research on HMPV infection and has provided important information regarding the HMPV-induced disease and detailed aspects of the immune response induced by HMPV infection. Although, inherent limitations are observed in the mouse model when data are extrapolated to the natural human infection, due to the availability of several gene deficient mice strains and multiple murine specific antibodies, it provides a valued experimental small animal model that allows answering critical questions that are necessary to our better understanding of the immune response and disease pathogenesis of HMPV.
1.6 References


CHAPTER 2
NEUTROPHILS REGULATE THE LUNG INFLAMMATORY RESPONSE VIA γδ T CELL INFILTRATION IN AN EXPERIMENTAL MOUSE MODEL OF HUMAN METAPNEUMOVIRUS INFECTION

2.1 Introduction

Neutrophils play an essential role in host defense against pathogens and represent the most abundant leukocytes in human circulation [1, 2]. They play a critical role to protect against microbial infections through multifaceted killing mechanisms [1] and to regulate innate as well as adaptive immune responses [3]. Upon stimulation, neutrophils quickly migrate from circulation to sites of damage and initiate crosstalk between resident and recruited immune cells, including dendritic cells, T cells, B cells, macrophages, and NK cells, primarily through secretion of cytokines and chemokines and also antigen presentation through MHC molecules [4–6]. Neutrophils have been implicated in the antiviral immune response, as they represent an early and abundant cell population recruited to sites of infection [7–10]. However, their role in viral infections remains largely unknown, particularly in the respiratory tract where their effect remains controversial. Studies of influenza infection in mice revealed that neutrophil depletion exacerbates lung injury and development of severe disease [8, 11]. Furthermore, neutrophil depletion before influenza infection, neutrophil depletion alters the magnitude of influenza-specific CD8+ T cells and cytokine production, causing defective viral clearance [12]. These findings confirm a beneficial and protective role of neutrophils during influenza infection in mice. On the other hand, experimental evidence indicates that excessive recruitment

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of neutrophils to the airways during influenza infection in mice contributes to acute lung injury by the formation of neutrophil extracellular traps [2].

Taken together, these contrasting findings warrant the need for further research on the role of neutrophils in respiratory viral infections. HMPV is an enveloped, negative-sense ssRNA paramyxovirus causing clinical syndromes, ranging from mild illnesses, such as the common cold, to more severe symptoms, such as bronchiolitis and pneumonia [13, 14]. HMPV is recognized as a significant global respiratory pathogen in young [15, 16], elderly [17, 18], and immunocompromised individuals [19–21]. HMPV infection accounts for 5–15% of pediatric hospitalizations with lower respiratory tract infections [22–26]. By 5 yr old, most children have been infected by HMPV, and by the age of 25, virtually all adults have been exposed to the virus [26, 27]. Research studies conducted over the last decade demonstrate that HMPV induces a spectrum of immune responses, some of which contribute to the antiviral defense, whereas others mount an evasion strategy by the virus for effective infection [28, 29]. However, several gaps in the understanding of immune responses to HMPV resulted in limited success with the development of effective vaccines and specific therapeutics against this clinically important viral infection. Studies in mice have shown that neutrophils predominate in the airways early during HMPV infection [30, 31]. However, it remains unknown whether these cells play a protective or harmful role in the lung of infected mice, particularly in the context of immune response regulation. Therefore, in the present work, we investigated how neutrophils shape HMPV-induced pathogenesis. We demonstrate that neutrophil depletion, achieved using mAb 1A8, which binds specifically to Ly6G$^{\text{high}}$ neutrophils [8, 32–36], led to increased pulmonary inflammation and severe clinical disease (as indicated by an enhanced body weight loss and clinical illness). However, neutrophils during HMPV infection did not contribute to lung viral
clearance. Interestingly, neutrophil depletion during HMPV infection altered the recruitment of lung γδ T cells and thus, that these cells contributed to HMPV-induced pathogenesis. Taken together, these findings demonstrate that neutrophils play a protective and regulatory role in HMPV-induced immune responses.

2.2 Methods

2.2.1 Virus

HMPV (strain CAN97-83) was grown and titrated in LLC-MK2 cells (ATCC, CCL7) in the presence of trypsin (Worthington Biochemical, Lakewood, NJ, USA), as described previously [37–39].

2.2.2 Infection of mice

BALB/c mice were purchased from Envigo (Indianapolis, IN, USA). δ-TCR KO (B6.129P2-Tcrdtm1Mom/J) and WT mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). All animals were housed in specific pathogen-free conditions in accordance with the Louisiana State University Institutional Animal Care and Use Committee. Eight- to 12-wk-old mice were used in all experiments. Mice were anesthetized with a sub-lethal dose of ketamine-xylazine and infected with 50 ml HMPV via the i.n. route, diluted in PBS at a final administration dose of 1 × 10⁷ PFUs. Mock-infected animals received 50 ml sterile PBS (herein, referred to as mock).

2.2.3 Clinical illness score

A standardized 1–5 grading system was used to score mice for disease severity and illness, as described previously [37, 40]. In brief, mice were assessed daily for visual signs of clinical disease, including inactivity, ruffled fur, and hunched posture. In addition, daily
determination of body weight was observed to monitor the progression of disease over the experimental period.

2.2.4 Depletion of neutrophils

Mice were injected i.p. with 300 mg rat anti-mouse Ly6G (Clone 1A8; BioXCell, West Lebanon, NH, USA) at the time of HMPV infection and every 48 h thereafter. All depletion doses were administrated in sterile PBS. The anti-mouse Ly6G (1A8) antibody specifically and efficiently depletes neutrophils in mice, as previously reported [8, 32–36]. Control animals received an equivalent dose of purified rat IgG (Sigma-Aldrich, St. Louis, MO, USA). Depletion of neutrophils in the airways was confirmed by flow cytometry.

2.2.5 Mouse sample collection

Mice were euthanized by an i.p. injection of ketamine and xylazine and exsanguinated via the femoral vessels, as previously reported [37, 39, 41]. To collect the BALF, lungs were flushed twice with 1 ml PBS, as previously described [37, 39]. Cell-free supernatants were stored at -75°C for cytokine analysis. For histologic analysis, lungs of mice were perfused and fixed in 10% buffered formalin and embedded in paraffin. Lung tissue sections were stained with H&E and visualized by bright-field microscopy using the high-throughput slide-scanner NanoZoomer with a 203 objective (numerical aperture of 0.75) and the NanoZoomer digital pathology view software (both from Hamamatsu Photonics, Hamamatsu City, Japan), as previously reported [39]. For viral gene-expression experiments, lung tissue was snap frozen in liquid nitrogen and stored at -75°C until analysis. For lung cell analysis, lungs were collected and kept on ice until their digestion with collagenase to obtain single-cell suspensions, as previously described [42]. For viral titration, total lungs were collected and snap frozen in liquid nitrogen and stored at -75°C until analysis.
2.2.6 Pulmonary inflammation

For histologic analysis, a blind analysis and scoring for cellular infiltration were performed, as previously described [39]. In brief, inflammatory infiltrates were scored by enumerating the layers of inflammatory cells surrounding the vessels and bronchioles. The number of abnormal perivascular and peribronchial spaces divided by the total perivascular and peribronchial spaces was the percentage reported as pathology score.

2.2.7 Flow cytometry

BALF and lung single-cell suspensions were incubated with anti-CD16/CD32 (Clone 2.G2) antibody at 4°C for 30 min before staining with the following specific antibodies: anti-Gr-1 (Clone RB6-8C5), anti-CD11b (Clone M1/70), anti-CD8a (Clone 53-6.7), anti-CD4 (Clone RM4-5), anti-CD3e (Clone 145-2C11), anti- B220 (Clone RA3-6B2), anti-CD19 (Clone 1D3), anti-γδ TCR (Clone GL3), and anti-MHC class II (Clone 2G9); all from BD PharMingen (San Jose, CA, USA), and anti-CD11c (Clone N418) and anti-F4/80 (Clone BM8); from eBioscience (San Diego, CA, USA). Cells were incubated for an additional 30 min at 4°C and washed with PBS/1% BSA before fixing them with 1% paraformaldehyde. Cells were analyzed using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) and FlowJo software (version 7.6.3; Tree Star, Ashland, OR, USA).

2.2.8 Cytokines and chemokines

Quantification of cytokine and chemokine release in BAL samples was determined using the Milliplex MAP Mouse Cytokine 32 Plex assay (EMD Millipore, Billerica, MA, USA), according to the manufacturer’s instructions. The assay range of sensitivity of is 3.2–10,000 pg/ml.
2.2.9 Lung viral replication

Homogenized lung tissue samples were tested for viral titer. Serial 2-fold dilutions of the supernatant were tested by plaque assay on LLC-MK2 cells under methylcellulose overlay. Plaques were visualized 6 d later by HRP staining, as previously reported [38, 39].

2.2.10 Real-time qRT-PCR

RNA from lung tissues was extracted using the RNeasy Plus kit (Qiagen, Hilden, Germany). Viral gene expression was determined using specific primers and probes (Integrated DNA Technologies, Coralville, IA, USA). qRT-PCRs were run on a 7900HT Fast Real-Time PCR System, following the manufacturer’s suggested cycling parameters (Thermo Fisher Scientific, Waltham, MA, USA). The comparative cycle threshold method was used to quantitate the expression of target genes, and results were normalized to the endogenous GAPDH and expression levels of transcripts from mock-infected tissues.

2.2.11 Statistical analysis

Statistical significance for the comparison of 2 sets of values was performed using a Student’s t-test (two-tailed, two-sample equal variance). A one-way ANOVA was used to compare 3 or more sets of values, followed by a Tukey-Kramer test to correct for multiple comparisons using GraphPad InStat 3 (GraphPad Software, La Jolla, CA, USA). P # 0.05 was considered statistically significant.

2.3 Results

2.3.1 HMPV induces a robust response of neutrophils

To define the importance of neutrophils in HMPV infection, we first assessed the infiltration of neutrophils in mice infected with HMPV at d 1, 4, and 7 after infection. BAL samples were collected, and cytospin analysis was performed in Wright Giemsa stained
preparations. Analysis of cytospin preparations revealed a predominant neutrophil infiltration in the airways in the infected mice compared with mock-infected ones (Fig. 2.1A). Furthermore, a more specific identification of neutrophils was assessed by flow cytometry analysis, where neutrophils were identified as F4/802/Gr-1+/CD11b+. Our flow cytometry data indicate that in the alveolar spaces, the prevailing cells after d 1 of HMPV infection were neutrophils (Fig. 2.1B). Quantification of the neutrophils infiltrated into the alveoli indicate that HMPV induced a significant influx of these cells into the airways as early as d 1 p.i. and decreased over time; however, up until d 7 p.i., the percentage (Fig. 2.1C) and number (Fig. 2.1D) of neutrophils remain increased (0.88 ± 0.2 x10^5) above the basal numbers found in mock-infected mice (0.03 ± 0.2 x10^5).
Figure 2.1. Neutrophil recruitment following inoculation of mice with HMPV. BALB/c mice were infected with 107 PFU of HMPV. BALF was collected at different time points, and neutrophils were quantified using flow cytometry. (A) Differential cell images stained by Wright-Giemsa showing cellular influx in BAL on d 1 from mock and HMPV-infected mice (original magnification, 403). (B) Flow cytometry analysis of neutrophil influx in BAL at d 1 after infection. (C) Percentage of neutrophils in BAL at d 1, 4, and 7 after infection. (D) Total number of neutrophils in BAL at d 1, 4, and 7 after infection. Mean ± SEM are shown; n = 6 mice/group. Control mice were mock infected with PBS. *P < 0.05, **P < 0.01.

2.3.2 Efficient depletion of neutrophils in vivo during HMPV infection

To investigate further the role of neutrophils in HMPV infection, we specifically depleted neutrophils in mice [8, 32–35] using multiple doses of the anti-Ly6G mAb (Fig. 2.2A). Mice were treated i.p. with 300 mg anti-Ly6G (Clone 1A8) on d 0, 2, 4, and 6 of infection. Likewise, control mice were treated with 300 mg rat IgG2a isotype antibody control. The efficiency of neutrophil depletion was confirmed by flow cytometry analysis at d 7 p.i. in BAL (Fig. 2.2C) and
l lung (Fig. 2.2D) samples. We observed that treatment of mice with the anti-Ly6G antibody resulted in a significant reduction in HMPV-induced neutrophil infiltration in the alveolar spaces of .95% compared with those treated with the isotype antibody (Fig. 2.2C). Likewise, we observed a significant depletion of neutrophils in the lungs of infected mice, as the numbers of neutrophils in HMPV-infected mice were reduced from $33.6 \pm 2.1 \times 10^5$ in the competent mice to $7.1 \pm 1.1 \times 10^5$ in the depleted mice. After depletion, the number of neutrophils in the lung of HMPV-infected mice resembled the amount of neutrophils found in the mock-infected ones of $7.9 \pm 1.1 \times 10^5$ (Fig. 2.2D). These results indicate that the neutrophil depletion strategy was efficient.
Figure 2.2. Efficiency of neutrophil depletion in vivo during HMPV infection. BALB/c mice were treated with isotype (IgG) or anti-Ly6G antibody before and p.i. with HMPV. (A) Treatment plan for depletion of neutrophils. (B) Depletion of neutrophils in BAL was confirmed by flow cytometry on d 7 after infection. Total neutrophils in infected mice after depletion in (C) BAL and (D) lung; n = 8 mice/group. Control mice were mock infected with PBS. ***p < 0.001.

2.3.3 Depletion of neutrophils delays disease recovery and exacerbates lung inflammation

Once the conditions for neutrophil depletion were established, and the depletion efficiency was confirmed, we determined the role of neutrophils in the clinical disease and inflammation after HMPV infection. Mice were treated with anti-Ly6G or isotype control antibodies, according to the neutrophil depletion strategy mentioned above (Fig. 2.2A). After HMPV infection, mice were monitored daily for signs of disease and changes in body weight.
Uninfected mice treated with either anti-Ly6G mAb or isotype control did not display any signs of sickness or weight loss over the 7 d monitoring period, indicating that depletion of neutrophils alone does not lead to clinical illness (Fig. 2.3A and C). On the other hand, in HMPV-infected mice, depletion of neutrophils resulted in an enhanced disease severity compared with undepleted mice (Fig. 2.3A). This was more evident at d 3 p.i. and was statistically significant at d 6 and 7. Signs of HMPV-induced disease peaked between d 2 and 3 in both groups of infected mice. However, competent mice fully recovered by d 6, whereas depleted mice remained sick at least by d 7, the last time point tested. An evident difference between the competent and depleted mice after 7 d of HMPV infection is depicted in Fig. 3B. In line with these observations, depletion of neutrophils showed an enhanced body weight loss that was statistically significant by d 5 and continued through d 7 (Fig. 2.3C). Furthermore, we investigated whether the signs of disease were concomitant with other measurable parameters in the infected mice, such as lung inflammation. For that purpose, lung tissues were collected and fixed on d 7 after infection (peak of inflammation in HMPV infection [30, 39]), and lungs were processed as described in Methods. Lung tissue sections were stained with H&E and analyzed for abnormal cellular infiltration in perivascular and peribronchial spaces. Results shown in Fig. 2.3D revealed significantly increased lung inflammation, represented as pathology score, in depleted mice infected with HMPV (33%) compared with competent mice after HMPV infection (20.7%). Together, these data demonstrate that neutrophils are necessary to reduce illness and inflammation during HMPV infection.
Figure 2.3. Neutrophil depletion enhances body weight loss and augments disease severity and lung pathology in HMPV infection. BALB/c mice were treated with isotype or anti-Ly6G antibody before and p.i. with HMPV. (A) Infected mice were scored for illness based on disease severity. (B) Signs of sickness in isotype or anti-Ly6G treated and HMPV-infected mice on d 7 after infection. (C) Body weight loss. (D) Pathology score from lung sections obtained at d 7 after HMPV infection. Means ± SEM are shown; n = 8 mice/group. *P < 0.05, **P < 0.01, ***P < 0.001.
2.3.4 Neutrophil depletion does not alter lung HMPV titers

Next, we examined whether the exacerbated HMPV-induced disease and inflammation observed in the depleted mice were a consequence of an altered lung viral replication. Mice were treated with isotype or anti-Ly6G antibody and infected with HMPV, as described above. Lung tissues were collected at d 4 and 7 after HMPV infection. Sampling times were designed based on the lung viral titer, which peaks between d 4 and 5 after HMPV infection but is almost cleared by d 7 [30, 39]. By testing d 4 after infection, we tested the peak of the viral titer; by testing d 7, we sought to determine whether there was any delay in the lung virus clearance as a consequence of neutrophil depletion. The presence of virus was assessed by quantifying the HMPV N gene expression by qRT-PCR and the yield of infectious virus by plaque assay. Our results shown in Fig. 2.4A indicate that there was no difference in the viral gene expression at d 4 and day 7 after HMPV infection. This observation was confirmed by plaque assay, where no change in the viral titers was observed between the depleted or competent mice infected with HMPV at d 4 p.i. (Fig. 2.4B).
2.3.5 Depletion of neutrophils increases the release of lung cytokines and chemokines after HMPV infection.

To delineate further the effect of neutrophils in the regulation of HMPV-induced inflammatory response, the concentration of cytokines and chemokines was measured in neutrophil-depleted mice and compared with those from neutrophil-competent mice. Mice were infected i.n. with HMPV, and BAL samples were obtained from each group at d 7 after infection. The level of cytokines and chemokines in BAL was measured using a multiplex cytokine detection system. As shown in Fig. 2.5, depletion of neutrophils led to an increased response of inflammatory and immunoregulatory cytokines after HMPV infection. We observed a 2-fold increase in the production of CCL11 (eotaxin), whereas IL-12p40, CXCL9 (monokine induced by IFN-g), CXCL2 (MIP 2), IL-1b, TNF, and IL-17 were incremented 1.4-fold in depleted mice compared with competent ones. The most up-regulated chemokines after neutrophil depletion...
were CCL2 (MCP-1) and CXCL1 (KC, IL-8 homolog), which were increased 3-fold compared with nondepleted mice.

Figure 2.5. Pulmonary inflammation in neutrophil depleted mice infected with HMPV. BALB/c mice were treated with isotype (IgG) or anti-Ly6G antibody before and p.i. with HMPV or mockinfected with PBS. BAL samples from each group of mice were assessed for cytokine/chemokine production by a multiplex cytokine detection system; n = 8–18 mice/group. *P < 0.05, **P < 0.01, ***P < 0.005.
2.3.6 Neutrophils regulate lung γδ T cell recruitment during HMPV infection

As described above, depletion of neutrophils induced an exacerbated inflammatory response in the lung after HMPV infection (Fig. 2.3D). Therefore, we also sought to characterize the cell subsets infiltrated in the lung affected by neutrophil depletion. For that, lungs were harvested at the peak of inflammation (d 7 p.i.). Lung single-cell suspensions were prepared from mock-infected, neutrophil-depleted, and undepleted mice with HMPV infection. The infiltration of myeloid cells, such as macrophages and dendritic cells, as well as the lymphocyte response, including B and T cells (CD4+, C8+, and γδ), was analyzed. As shown in Fig. 2.6, the depletion of neutrophils did not impact the total number of macrophages or dendritic cells (Fig. 2.6A). Likewise, the total number of CD4+ T, CD8+ T, and B cells (Fig. 2.6B) was also not affected upon neutrophil depletion. Interestingly, the depletion of neutrophils had a significant impact on the infiltration of γδ T cells in the lung. We observed an increased number of these cells after HMPV infection in the neutrophil-depleted mice.
Figure 2.6. Neutrophil depletion alters lung T cell responses during HMPV infection. BALB/c mice were treated with isotype (IgG) or test antibody (anti-Ly6G) before and p.i. with HMPV. Lung single-cell suspensions were stained for (A) myeloid populations (macrophages, dendritic cells) and (B) lymphocyte populations (CD4+, CD8+, B cells, and γδ T cells). Means ± SEM are shown; n = 4–15 mice/group. ***P < 0.001.

2.3.7 HMPV infection induces γδ T cell recruitment to the lung, and their deficiency leads to reduced lung pathology

Based on the above observations, the depletion of neutrophils led to an exacerbated HMPV-induced pathogenesis and also to an increased number of γδ T cells in the lung. We hypothesized, based on these findings, that γδ T cells could contribute to the inflammatory response induced by HMPV. We first determined the effect of HMPV in γδ T cell recruitment. Data shown in Fig. 2.7A indicate that HMPV is capable of inducing an increase in γδ T cell numbers, which were significantly recruited (13.6 x 10^4 ± 1.1) by d 5 after HMPV infection and
remained increased (15.7 x 10^4 ± 1.2) until d 15 after infection, as compared with baseline levels in mock-infected mice (6.7 x 10^4 ± 1.1). To evaluate the contribution of γδ T cells in the inflammatory response induced by HMPV infection, we infected TCR-δ KO mice (mice lacking the δ chain of the TCR) or WT mice with HMPV. At d 7 after infection, lungs were collected, and tissue sections were processed for further evaluation, as described above. Lung histopathological analysis revealed that no airway inflammation was observed in mock-infected WT or KO mice (Fig. 2.7C, left and second to left). However, HMPV infection induced a cellular infiltration in the perivascular and peribronchial spaces of WT mice (Fig. 2.7C, second to right), which was reduced in the TCR-δ KO mice (Fig. 2.7C, right). Quantification of the pulmonary inflammation, represented as pathology score, indicated that inflammation in the respiratory tract of TCR-δ KO mice was significantly decreased compared with WT animals (Fig. 2.7B).
Figure 2.7. Recruitment and role of γδ T cells during HMPV infection. (A) C57BL/6 mice were infected i.n. with HMPV and lungs harvested at d 1, 3, 5, 7, and 15 after infection. Lung single cell suspensions were stained for γδ T cells and quantified using flow cytometry. (B) TCR-δ or WT C57BL/6 mice were infected with HMPV and lung sections obtained at d 7 after infection for histopathology analysis. Lung pathology is represented as percentage pathology score. (C) Representative scans of lung tissue corresponding to the pathology score from the indicated treatment. Arrows indicate cells infiltrating the perivascular spaces. Original scale bars, 0.4X = 5 mm; 20X = 50 mm. *P < 0.05, **P < 0.01.
2.4 Discussion

Neutrophils are the most abundant leukocytes in human circulation and are considered a first line of defense against invading bacterial and fungal infections [43, 44]. Indeed, neutrophils are known to be markedly increased and to protect against these pathogens [45]. However, the role of neutrophils in viral infections remains largely understudied, particularly in the respiratory tract, where they are recruited after viral infections, as shown in experimental models [11, 30, 46] and in humans [9, 10, 47, 48]. In fact, it has been recently reported that HMPV induces neutrophil influx in the airways, as demonstrated by increased infiltration of these cells in nasopharyngeal aspirates from infected infants [9], which underline the importance of these cells in HMPV infection. In the current study, we demonstrated that neutrophils were the predominant cell population in the lungs of infected mice as early as d 1 after HMPV infection, and their infiltration in the lungs remained significantly higher than that of the uninfected control mice at d 7 after infection (Fig. 2.1). These data are in line with those previously reported in HMPV-infected mice, where the number of neutrophils increased during the first 2 d of infection [30, 49] and gradually decreased by d 4 and 7 after infection [30, 50]. Therefore, these findings led us to speculate that neutrophils play a role in HMPV infection.

To investigate that hypothesis, we used a model of neutrophil depletion using several doses of the anti-Ly6G mAb (1A8; Fig. 2.2), which specifically depletes neutrophils [8, 32]. In this model, we observed that depletion of neutrophils led to an increased disease severity and inflammation, as demonstrated by an increase in clinical illness, body weight loss, and exacerbated lung inflammation (Fig. 2.3), which suggests a beneficial role of neutrophils in HMPV infection. These data are in agreement with those reported from mice infected with other respiratory viruses, such as influenza virus, where the depletion of neutrophils under similar
conditions resulted in an enhanced body weight loss, increased pulmonary inflammation, and reduced survival [8, 11]. However, neutrophil depletion did not have an effect on HMPV lung titers and viral clearance, as there was no significant difference between the depleted and competent mice in the viral gene expression and infectious virus at d 4 and 7 after HMPV infection (Fig. 2.4). A similar effect has been observed in the close related human paramyxovirus RSV infection, where depletion of neutrophils in mice did not alter the lung viral titers in the infected animals but resulted in altered levels of mucus and cytokines, such as TNF and IL-13 [51]. Therefore, it appears that the increased disease severity and pulmonary inflammation observed in the depleted mice were not results of higher lung viral titers in the depleted mice and that neutrophils regulate the inflammatory response to HMPV infection independently of viral load. In that regard, it has been reported that a high HMPV viral load can be associated to greater disease severity on infected children [52], but recent findings indicate also that viral load does not have a significant impact on disease severity in children infected with HMPV [53]. Thus, further research is needed to define the controversial relationship between viral load and HMPV-induced disease severity and progression. Increased production of inflammatory cytokines in a moderate fashion results in a beneficial and effective mechanism to fight infections; however, an exacerbated or excessive inflammatory response is harmful to the host and could lead to tissue damage. We observed that depletion of neutrophils significantly exacerbated the production of cytokines and chemokines after HMPV infection. Increased levels of IL-1b, TNF, IL-17, CXCL2, CCL11, IL-12p40, CXCL1, CXCL2, and CXCL9 were detected, which correlate with the exacerbated pulmonary inflammation observed in the depleted mice. Data obtained in neutrophil depleted mice infected with RSV indicate, however, that neutrophil depletion reduced the amount of TNF after d1 of RSV infection [51]. This discrepancy may be attributable not only
to the different virus used but also to the day that the samples were collected. TNF production after HMPV infection peaks as early as d 1 after infection, but RSV induces higher levels of TNF than HMPV [54]. In the present work, we analyzed the production of cytokines and chemokines at d 7 after infection, as that was the day we also observed an increased pulmonary peribronchiolar inflammation. The role of neutrophils in regulating the recruitment and activation of other cells in the immune system, such as T cells, is well documented [3, 36, 55]. The heterogeneity of the neutrophil population is responsible for their different effects on T cells, as recent reports support the existence of several neutrophil subsets [56–58], including after respiratory viral infections [10]. Thus, a subset of mature neutrophils can activate the immune system and clear the infection, whereas other subsets of neutrophils can induce immunosuppression or are inefficient in antimicrobial immune activities [56, 59]. In this work, we observed a significant increase in lung γδ T cells when mice were treated with the anti-Ly6G, suggesting a suppressive effect of pulmonary neutrophils on γδ T cells after HMPV infection. In that regard, the suppression of γδ T cells by human neutrophils has recently been demonstrated in different in vitro models and is known to be achieved by several mechanisms [60–62]. Interestingly, in our model of HMPV infection, the effect of neutrophils appeared to be selective to γδ T cells, as we observed no difference in the number of other immune cells (CD4 T cells, CD8 T cells, B cells, macrophages, and dendritic cells) in the lungs of infected animals when compared between the competent and depleted mice. Studies in pulmonary experimental infection with the opportunistic fungal pathogen Cryptococcus neoformans have also revealed that depletion of neutrophils led to increased numbers of γδ T cells in the lung, which in turn, were responsible for the increased production of IL-17 after neutrophil depletion [43], indicating that neutrophils inhibit γδ T cell function. Likewise, in our work, we also observed significantly
higher levels of IL-17 and increased numbers of \( \gamma \delta \) T cells in the lungs of neutrophil-deficient mice, which coincided with increased levels of CXCL1, likely produced by lung \( \gamma \delta \) T cells [63]. This suggests that neutrophils may indeed exert a regulatory effect on \( \gamma \delta \) T cells after HMPV infection. Future studies are needed to demonstrate specifically a suppressive subset of neutrophils during HMPV infection. \( \gamma \delta \) T cells are a subset of nonconventional T lymphocytes that play a critical role in linking the innate and adaptive arms of the immune system [64]. Different subsets of \( \gamma \delta \) T cells are widely distributed in the lung [65], and pulmonary \( \gamma \delta \) T cells contribute to the mucosal immunity and protection of the lungs [66, 67]. In our model of HMPV-infected mice, we observed that pulmonary \( \gamma \delta \) T cells are increased after d 5 of infection and that accumulation remained high by d 15 after HMPV infection. Compared with HMPV infection, reported experiments in RSV-infected mice indicate that the accumulation of \( \gamma \delta \) T cells peaks in lower numbers by d 4 after infection, and it returns to basal levels by d 15 after infection [66]. Moreover, after HMPV infection, TCR-\( \delta \) KO mice exhibited a decreased pulmonary inflammation compared with WT mice, indicating that these cells play an important role in the inflammatory response induced by HMPV. Similar results were found in RSV-infected mice [66], as well as in a model of respiratory bacterial infection (Staphylococcus aureus) [68], where \( \gamma \delta \) T cells also contributed to the inflammatory response in the lungs. In conclusion, the current data represent the first evidence that neutrophils have a critical regulatory role in HMPV-induced pathogenesis. Our results indicate that neutrophils contribute to the control of disease severity and pulmonary inflammatory response by regulating the accumulation of \( \gamma \delta \) T cells to the site of infection in the HMPV-infected mice. Whereas the experimental mouse model of HMPV infection has intrinsic limitations, it represents a valuable experimental small animal model that allows exploring relevant aspects of the HMPV-induced pathogenesis and immune
responses. Future studies to define the mechanism of regulation between neutrophils and γδ T cells, as well as to investigate further the role of γδ T cells in HMPV infection are warranted. Overall, these findings enhance our understanding of the HMPV-induced pathogenesis and highlight the importance of neutrophils in regulating the fine line between disease and protection to HMPV infection.

2.5 References


CHAPTER 3
ROLE OF GAMMA DELTA (γδ) T CELLS IN HMPV INFECTION

3.1 Introduction

HMPV is a *pneumovirus* causing symptoms, varying from mild illness such as common cold to severe conditions such as pneumonia [1, 2]. Innate immune cells, especially neutrophils have been known to play a key role in γδ T cells induced airway inflammation during HMPV infection [3]. Apart from innate immune system response in HMPV infection, gamma delta TCR carrying T cells, also known as γδ T cells, also play an essential role in mucosal host defense towards viral infection [4]. In fact, we have recently reported that depletion of neutrophils leads to an increased number of γδ T cells in the lung of mice infected with HMPV and that γδ T cells control lung inflammatory response in the infected mice [3], as described in Chapter 2. Those findings led to us to hypothesize that γδ T cells play a critical role in HMPV-induced pathogenesis. Therefore, in this chapter I focused on the study of the effect of γδ T cells on disease parameters such as lung viral replication and the role of these cells on other cells of the innate and adaptive immune systems.

3.2 Materials and methods

3.2.1 Virus

HMPV (strain CAN97-83) was grown and titrated in LLC-MK2 cells (ATCC, CCL7) in the presence of trypsin (Worthington Biochemical, Lakewood, NJ, USA), as described previously [12–14].

3.2.2 Infection of mice

BALB/c mice were purchased from Envigo (Indianapolis, IN, USA). δ-TCR KO (B6.129P2-Terdtm1Mom/J) and WT mice were obtained from The Jackson Laboratory (Bar Harbor, ME,
USA). All animals were housed in specific pathogen-free conditions in accordance with the Louisiana State University Institutional Animal Care and Use Committee. Eight- to 12-wk-old mice were used in all experiments. Mice were anesthetized with a sublethal dose of ketamine-xylazine and infected with 50 ml HMPV via the i.n. route, diluted in PBS at a final administration dose of 1 x 10⁷ PFUs. Mock-infected animals received 50 ml sterile PBS (herein, referred to as mock).

3.2.3 Mouse sample collection

Mice were euthanized by an i.p. injection of ketamine and xylazine and exsanguinated via the femoral vessels, as previously reported [12, 14, 16]. To collect the BALF, lungs were flushed twice with 1 ml PBS, as previously described [12, 14]. Cell-free supernatants were stored at 75°C for cytokine analysis. For viral gene-expression experiments, lung tissue was snap frozen in liquid nitrogen and stored at 75°C until analysis. For lung cell analysis, lungs were collected and kept on ice until their digestion with collagenase to obtain single-cell suspensions, as previously described [17]. For viral titration, total lungs were collected and snap frozen in liquid nitrogen and stored at 75°C until analysis.

3.2.5 Flow cytometry

Lung single-cell suspensions were incubated with anti-CD16/CD32 (Clone 2.G2) antibody at 4°C for 30 min before staining with the following specific antibodies: anti-Gr-1 (Clone RB6-8C5), anti-CD11b (Clone M1/70), anti-CD8a (Clone 53-6.7), anti-CD4 (Clone RM4-5), anti-CD3e (Clone 145-2C11), anti- B220 (Clone RA3-6B2), anti-CD19 (Clone 1D3); all from BD PharMingen (San Jose, CA, USA), and anti- anti-F4/80 (Clone BM8); from eBioscience (San Diego, CA, USA). Cells were incubated for an additional 30 min at 4°C and washed with PBS/1% BSA before fixing them with 1% paraformaldehyde. Cells were analyzed
using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) and FlowJo software (version 7.6.3; Tree Star, Ashland, OR, USA).

3.2.6 Cytokines and chemokines

Quantification of cytokine and chemokine release in BAL samples was determined using the Milliplex MAP Mouse Cytokine 32 Plex assay (EMD Millipore, Billerica, MA, USA), according to the manufacturer’s instructions. The assay range of sensitivity is 3.2–10,000 pg/ml.

3.2.8 Real-time qRT-PCR

RNA from lung tissues was extracted using the RNeasy Plus kit (Qiagen, Hilden, Germany). Viral gene expression was determined using specific primers and probes (Integrated DNA Technologies, Coralville, IA, USA). qRT-PCRs were run on a 7900HT Fast Real-Time PCR System, following the manufacturer’s suggested cycling parameters (Thermo Fisher Scientific, Waltham, MA, USA). The comparative cycle threshold method was used to quantitate the expression of target genes, and results were normalized to the endogenous GAPDH and expression levels of transcripts from mock-infected tissues.

3.2.9 Statistical analysis

Statistical significance for the comparison of 2 sets of values was performed using a Student’s t-test (two-tailed, two-sample equal variance). A one-way ANOVA was used to compare 3 or more sets of values, followed by a Tukey-Kramer test to correct for multiple comparisons using Graph Pad InStat 3 (GraphPad Software, La Jolla, CA, USA). P # 0.05 was considered statistically significant.
3.3 Results

3.3.1 Lack of γδ T cells alter lung chemokine/cytokine profile

As we previously observed lack of γδ T cells leading to reduced lung inflammation during HMPV infection, we sought to identify the cells inflammatory mediators behind this mechanism. For this, the concentration of cytokines and chemokines were measured in mock-infected vs HMPV infected WT vs γδ-KO mice. WT or γδ TCR KO mice were infected i.n. with HMPV or PBS (Mock), and BAL samples were obtained from each group at d 7, after infection. As shown in Figure 3.1, we observed a robust increase in chemokine and cytokine levels in the BAL samples from WT mice that were HMPV infected compared to the mock infected WT or KO mice. Even though the levels of chemokines or cytokines in HMPV infected KO mice BAL samples appear elevated compared to the mock-infected controls, they were lower than HMPV-infected WT mice. All the cytokines and chemokines that had changes in their levels were primarily known for their role in neutrophil trafficking and activation. Only IL-6, a pro-inflammatory cytokine, was significantly reduced in the HMPV infected γδ TCR KO mice compared to the HMPV infected WT mice. This is in line with previous studies suggesting an IL-6 mediated activation of γδ T cells in airway inflammation [18].
3.3.1 Role of γδ T cells in viral clearance

To further investigate whether the exacerbated HMPV-induced inflammation in the γδ TCR KO mice was a result of altered viral replication, we performed lung viral gene expression. For this, WT or KO mice were infected with HMPV, as mentioned above. At d 4 or 7, after HMPV infection, lung tissues were obtained and tested for the viral gene expression [15,19]. The presence of virus was analyzed by quantifying HMPV N gene expression using qRT-PCR. As shown in Figure 3.2, we observed a trend of increase in the HMPV N gene expression on d 4, p.i.
in the KO mice compared to WT mice (one-tailed \( P \) value is 0.1), but no changes in the HMPV N gene expression on day 7 after HMPV infection.

![Graph showing relative expression of HMPV N gene on day 4 and day 7](image)

**Figure 3.2.** Lack \( \gamma \delta \) T cells alter lung viral gene expression in HMPV-infected mice. Lung tissues from WT or KO mice infected with HMPV were collected on d 4 and 7 p.i. HMPV N gene relative expression in lung was quantified by qRT-PCR. Means 6 SEM are shown; \( n = 3-6 \) mice/group.

3.3.1 Lack of \( \gamma \delta \) T cells alter lung neutrophil influx

As described above, knockout of \( \gamma \delta \) TCR resulted in decreased proinflammatory cytokines (Fig. 3.1). To see the population of immune cells affected by this change in cytokine/chemokine profile, we pursued characterizing the cell subsets infiltrated in the lung affected by \( \gamma \delta \) TCR-KO. At d 7, p.i., which is peak of inflammation in HMPV infection in mice, we harvested lungs and obtained lung single-cells suspensions from WT or KO mock infected or HMPV infected mice. We then analyzed the infiltration of granulocytic cells, such as neutrophils, as well as lymphocytic response including CD4+ or CD8+ T cells. As shown in Figure 3.3, \( \gamma \delta \) TCR knockout did impact the total number of neutrophils. However, the total number of CD8+ T cells or CD4+ T cells in the lung showed marginal changes. Thus, lack of \( \gamma \delta \) T cells alters lung neutrophilic response.
**Figure 3.3.** γδ T cells regulate lung neutrophilic responses during HMPV infection. WT or KO mice were infected with HMPV. At day 7 after infection, lung single-cell suspensions were stained for neutrophils and lymphocyte populations (CD4+, CD8+ T cells). Means ± SEM are shown; n = 4–15 mice/group. ***P < 0.001.
3.4 Discussion

In this study, we investigated the role of γδ T cells during lung inflammatory response in HMPV infection. With ablation of γδ TCR, we observed a change in IL-6, which from previous studies, is known to regulate neutrophil trafficking [20]. Other studies have also shown that IL-6 signaling activates γδ T cells in airway inflammation [18]. Our study indicates that IL-6 levels are altered in HMPV infection in the absence of γδ T cells (Fig. 3.1). Thus, these findings complement our previous report, where we found that neutrophils regulate lung inflammatory responses via recruitment of γδ T cells during human metapneumovirus infection [3]. Hence we confirm that γδ T cells and neutrophils act interdependently during lung inflammatory response to HMPV infection, which was previously shown with other models of infection.

Based on our previous findings, where it was demonstrated a major contribution of γδ T cells in HMPV-induced lung inflammation [3], we further investigated the effect of γδ T cells in other parameters of HMPV-induced pathogenesis. We assessed the viral replication in the absence of γδ T cells. Experimental observations indicate that HMPV replication is marginally increased at d 4 p.i. and no differences were observed between the animal groups at d 7 p.i., indicating that γδ T cells do not have a major role on HMPV replication and clearance.

γδ T cells are also known to play an important role in linking innate and adaptive immune responses [24]. However, in our study we found that lack of γδ T cells did not have any effect on CD4+ or CD8+ T cell recruitment in the lung following HMPV infection, suggesting regulatory role of γδ T cells on innate but not adaptive responses. Overall, this study contributes to our understanding of the role that γδ T cells play in lung inflammatory responses during HMPV infection.
3.5 References


CHAPTER 4
HUMAN METAPNEUMOVIRUS ATTACHMENT PROTEIN CONTRIBUTES TO NEUTROPHIL RECRUITMENT INTO THE AIRWAYS OF INFECTED MICE

4.1 Introduction

Human metapneumovirus (HMPV) is a single negative-stranded RNA, enveloped virus classified in the Paramyxoviridae family. Its genome, of 13,335 nt, codes for nine different proteins: the fusion (F), attachment (G), small hydrophobic (SH), nucleocapsid (N), phosphoprotein (P), polymerase (L), matrix (M), and second matrix (M2-1, M2-2) [1]. HMPV was first identified in 2001 from nasopharyngeal aspirates of hospitalized infants [2], and has soon emerged to be a leading respiratory pathogen worldwide infecting infants, elders, and immunocompromized individuals [3]. Epidemiological data indicate that this respiratory virus represents a major respiratory pathogen worldwide. HMPV is responsible for 5 to 15% of pediatric hospitalizations for respiratory tract infections [4,5,6,7]. Indeed, it is second only to Respiratory Syncytial virus (RSV) infection in infants admitted with lower respiratory tract viral infections causing mortality and morbidity [4,8,9,10]. In elderly adults aged ≥65 years old, HMPV accounts for about 4.1% hospitalizations with respiratory tract infections, impacting more severely those subjects with underlying conditions, such as cardiovascular diseases, organ transplantation, or other hematologic malignancies [11,12,13,14]. One hallmark of HMPV infection is that it is characterized by aggravated inflammatory responses leading to bronchiolitis and pneumonia [8]. Currently there is no approved vaccine available to protect from HMPV infection. Inflammatory outcomes during HMPV infection are mediated by virus-induced cytopathology and the secretion of cytokines and chemokines [15,16]. Clinical evidence

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indicates that HMPV induces neutrophil infiltration and associated mediators within the airways of infants with bronchiolitis [17], providing evidence of the neutrophilic inflammatory response in vivo and highlighting the importance of these cells as a potential target of therapeutic intervention for treatment of bronchiolitis in infected children. The increased neutrophil infiltration by HMPV in the airways has been reproduced in the mouse model of infection, including in adult [18,19,20] and aged mice [21], where HMPV infection induces similar neutrophil recruitment into the airways of both age groups of mice [21]. However, the role of HMPV in regulating the recruitment of neutrophils to the lungs remains elusive. On the other hand, the interferon (IFN) response appears to regulate the neutrophil infiltration in some viral [22,23] and bacterial [24] infections, as well as in tumor bearing mice [25,26]. In that regard, HMPV infection induces a robust production of type I interferon in infected mice [27,28], which appears to be regulated by the expression of the HMPV attachment protein (G protein) [28,29]. Therefore, we reasoned that HMPV G protein contributes to the neutrophil recruitment into the airways during HMPV infection through the IFN response. For that, we used an experimental mouse model to quantify IFN-α production, neutrophil recruitment, and chemokine response to a recombinant HMPV lacking the G protein. We found that the lack of the attachment protein increased the production of IFN-α but decreased the production of neutrophil chemoattractants and the recruitment of neutrophils to the alveolar spaces. These findings suggest a key role for HMPV attachment (G) protein in contributing to the inflammatory responses in vivo.

4.2 Materials and methods

4.2.1 Virus Stocks

Recombinant HMPV lacking the attachment G protein (rHMPV-ΔG) and full-length recombinant HMPV (rHMPV) were generated by reverse genetics, as we previously described
The viruses were grown and titrated in LLC-MK2 cells (ATCC, Manassas, VA, USA) in the presence of trypsin (Worthington, Lakewood, NJ, USA). Viruses were sucrose purified and not used beyond passage 5. In some experiments, rHMPV was exposed for 10 min to UV irradiation, as previously reported.

4.3.2 Ethic Statement

Animal care and use were conducted in accordance with the National Institutes of Health and Louisiana State University institutional guidelines. The Louisiana State University Animal Care and Use Committee specifically approved this study under the protocol number: 15-062 (15 October 2015). Mice were housed in a temperature-controlled room with proper darkness-light cycles, fed with a regular diet, and maintained under the care of the Division of Laboratory Animal Medicine facility, Louisiana State University, Baton Rouge, LA. The mice were sacrificed by an intraperitoneal injection of ketamine and xylazine, and exsanguinated via the femoral vessels.

4.2.3 Mice and Infection Protocol

BALB/c mice were purchased from Harlan Laboratories. Female 8- to 12-week-old mice were used in all of the experiments. Mice were anesthetized with a combination of ketamine and xylazine, and infected intranasally with 50μL of hMPV diluted in phosphate-buffered saline. A final administration dose of $5 \times 10^4$ PFU/mouse was used for the recombinant virus infections. Mock-infected mice received 50μL total volume of PBS.

4.2.4 Mouse Sample Collection

Mice were euthanized by intraperitoneal injection of ketamine and xylazine, and exsanguinated via the femoral vessels, as previously described [20,28]. Bronchialveolar lavage (BAL) samples were collected by flushing the lungs twice with 1 mL PBS and centrifuged 3500
rpm for 5 min at 4 °C. Cell-free BAL supernatants were stored at −75 °C until further analysis. For viral gene expression using qRT-PCR, lung tissue was snap frozen in liquid nitrogen and stored at −75 °C until further analysis.

4.2.5 Differential Leukocyte Counts

Bronchialveolar lavage (BAL) fluid was used for differential leukocyte counts using cytospin methods. Cytospin samples were subsequently prepared from BAL cells and dyed with Wright-Giemsa staining and subjected to differential leucocyte counts under a light microscope. A total of 200 cells per slide were counted. The total cell numbers were enumerated from BAL cell counts obtained by trypan blue exclusion.

4.2.6 Detection of Cytokines and Chemokines

Levels of cytokines and chemokines in BAL fluid were determined with the Milliplex MAP™ 32-Mouse-Plex cytokine detection system (Millipore, Billerica, MA, USA), according to the manufacturer’s instructions. The panel included the following cytokines: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL 10, IL-12 p40, IL-12 p70, IL-13, IL-15, IL-17, M-CSF, G-CSF, GM-CSF, IFN-γ, TNF, CXCL1, CXCL2, CXCL5, CXCL9, CXCL10, CCL2, CCL3, CCL4, CCL5, CCL11, LIF, and VEGF. The range of sensitivity of this assay is 3.2 to 10,000 pg/mL. Cell-free BAL supernatants were also tested for the production of type I (IFN-α) interferon using ELISA tests according to the manufacturer’s instructions (PBL Assay Science, Piscataway, NJ, USA).

4.2.7 Real-Time qRT-PCR

Lung tissue stored at −75 °C was used for viral gene expression using qRT-PCR, as described previously [13]. Briefly, RNA was extracted from the lung tissue using RNeasy Plus kit (Qiagen, Hilden, Germany) and viral gene expression was determined using specific primers
and probes (Integrated DNA Technologies, Coralville, IA, USA) on a 7900HT Fast Real-Time PCR following manufacturer’s instructions. Expression of target genes was quantified using the comparative cycle threshold method and results were normalized to the endogenous GAPDH with expression levels normalized to transcripts from mock-infected mice.

4.2.8 Statistical Analysis

Statistical significance was calculated by unpaired t test and one-way ANOVA to ascertain the differences between the animal groups, followed by a Tukey-Kramer test to correct for multiple comparisons using Graph Pad InStat 3 (GraphPad Software, La Jolla, CA, USA).

4.3 Results

4.3.1 Inhibition of IFN-α Responses by HMPV G Protein In Vivo

Previous studies in vitro have identified HMPV G protein as a negative regulator of the IFN response [28,29]. In order to determine the effect of the HMPV G protein in the IFN response in vivo, BALB/c mice were infected with rHMPV, rHMPV-ΔG or mock infected. After 24 h of infection, BAL supernatants were collected and tested for the IFN production by ELISA. As shown in Figure 4.1a, the lack of the attachment glycoprotein resulted in a significant increase in the production of IFN-α. We observed that mice infected with rHMPV-ΔG induced a 1.3-fold increase in the production of IFN-α as compared to full-length rHMPV infected mice. However, no difference in viral gene expression was noted when compared between rHMPV and rHMPV-ΔG after 24 h of infection (Figure 4.1b), confirming that the infection of mice with rHMPV and rHMPV-ΔG was comparable. Thus, validating the inhibitory effect of G protein on the IFN response. Furthermore, in order to confirm that the observed production of IFN-α by rHMPV was due to the viral infection, a group of mice were inoculated with UV-inactivated rHMPV and the production of IFN was determined. Our data show that the inoculation of mice
with UV-inactivated rHMPV failed to induce any production of IFN-α showing that IFN-α production was dependent on the rHMPV replication (Figure 4.1a). Together, these results demonstrate that HMPV-G protein regulates IFN-α responses in vivo.

**Figure 4.1.** Human Metapneumovirus (HMPV) G protein regulates interferon responses in vivo. (a) BALB/c mice were infected with rHMPV-ΔG or rHMPV and Bronchialveolar lavage (BAL) collected at day 1 after infection. Interferon-α (IFN-α) responses were determined by ELISA in BAL collected at day 1 after infection; (b) HMPV viral gene expression in infected mice. BALB/c mice were infected with rHMPV-ΔG or rHMPV and lung tissue collected at day 1 after infection. Expression of HMPV N was done by qRT-PCR. n = 4–10 mice/group. Mean ± SEM are shown. **p < 0.01, ***p < 0.005.

4.3.2 HMPV G Protein Contributes to Neutrophil Recruitment

To determine whether the HMPV attachment glycoprotein plays a role in the recruitment of neutrophils, BAL samples were collected 24 h after inoculating mice with rHMPV, rHMPV-ΔG or PBS, and differential cell analysis was performed. We focused on the neutrophil recruitment at 24 h based on previous observations that indicate that neutrophil recruitment peaks at day 1 after HMPV infection [20]. As shown in Figure 4.2a, analyses of cytospin preparations revealed a significant decrease in the total number of neutrophils recruited to alveolar spaces in mice infected with rHMPV-ΔG (1.0 × 10⁵ ± 0.2) when compared with those with rHMPV infection (3.1 × 10⁵ ± 0.3), indicating that G protein contributes to the recruitment
of neutrophils. Also, the levels of recruitment of neutrophils in rHMPV-ΔG \((1.0 \times 10^5 \pm 0.2)\) were comparable to mice infected with UV light inactivated rHMPV \((0.8 \times 10^5 \pm 0.3)\). On the other hand, there was no evident change in the recruitment of monocytes/macrophages (Figure 4.2c) or lymphocyte population (Figure 4.2b), suggesting that G protein contributes mainly to the recruitment of neutrophils to the alveolar spaces.

**Figure 4.2.** HMPV G protein contributes to neutrophil infiltration into the airways of infected mice. BALB/c mice were infected with rHMPV-ΔG or rHMPV and BAL collected at day 1 after infection. Total number of (a) neutrophils; (b) lymphocytes and (c) monocytes/macrophages was determined by cytopsin analysis and total number of cells enumerated by total BAL cell counts. \(n = 4–10\) mice/group. Mean ± SEM are shown. *** \(p < 0.005\).

### 4.3.3 HMPV G Regulates Lung Cytokine and Chemokine Profile in the Lungs of Infected Mice

To further elucidate the role of G protein in the production of neutrophil chemoattractants and proinflammatory cytokines, we sought to assess the production of lung cytokine and chemokine profile after 24 h of rHMPV-ΔG infection and compare it to that of rHMPV. Cell-free supernatants from BAL samples were analyzed by multiplex assay, using multi-Plex cytokine detection, as described in methods. As shown in Figure 4.3, the levels of TNF \((\downarrow 58\%)\), IL-17\((\downarrow 44\%)\), CXCL2\((\downarrow 40\%)\), VEGF \((\downarrow 46\%)\), CCL3\((\downarrow 54\%)\), and CCL4 \((\downarrow 68\%)\), were decreased
(as indicated) in mice infected with rHMPV-ΔG as compared with those infected with full-length rHMPV, suggesting that G protein contributes to the production of these chemokines in vivo. However, when compared between both groups of infected animals, no significant difference was observed in the production of IL-1α, IL-1β, IL-4, IL-5, IL-6, IL-9, IL 10, IL-12 p70, IL-13, IL-15, M-CSF, G-CSF, GM-CSF, IFN-γ, CXCL1, CXCL5, CXCL9, CXCL10, CCL2, CCL5, CCL11, and LIF. Other cytokines were not induced by the recombinant viruses in the infected mice (IL-2, IL-3, IL-7, and IL-12 p40).

Figure 4.3. HMPV G alters cytokine profile in infected mice. BALB/c mice were infected with rHMPV-ΔG or rHMPV and BAL collected next day. BAL samples from each group of mice were assessed for cytokine/chemokine production by a multi-Plex cytokine detection system. $n = 4–10$ mice/group. Mean ± SEM are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. 
4.4 Discussion

The regulation of the inflammatory immune response by infectious agents involves several factors from the host and the pathogen. Viral infections are known to alter the innate immune response [31,32]. In fact, several viral proteins are known to inhibit the IFN response in vitro [29,33,34] and in vivo [35]. In that regard, despite previous studies have demonstrated that the attachment glycoprotein (G) of HMPV inhibits the IFN response in vitro [28,29], this work demonstrates, for the first time, that the HMPV G protein inhibits the IFN response in vivo. Due to the restriction of high viral titers of the purified recombinant viruses, the final administered viral inoculum was $5 \times 10^4$ PFU/mouse. However, that amount of viral inoculum was enough to induce an IFN-α response that allowed us to define the inhibitory effect of the HMPV G protein in vivo. That inhibitory effect was validated by the fact that both viral inoculums (rHMPV and rHMPV-ΔG) were comparable as they were purified and titrated by the same methods, and no difference was observed in the viral gene expression when measured in the lung samples from the infected mice (Figure 4.1b).

There is evidence that interferons have pleiotropic immune functions in several models. In an experimental mouse model, type I IFN suppresses neutrophil recruitment by negatively regulating CXC chemokine expression in influenza [23,36], herpes simplex-1 [22], Listeria monocytogenes [24], and tumor-associated diseases [26]. Thus, the observed increased production of IFN-α by rHMPV-ΔG, due to its regulatory effect [22,37], may contribute to the suppression of the recruitment of neutrophils induced by HMPV. On this subject, several studies indicate that neutrophils are the predominant cell population recruited to the alveolar spaces in HMPV-infected mice during the early phase of the infection (Figure 4.2 and [18,19,20]). However, to the best of our knowledge, these findings represent the first evidence that the
HMPV attachment protein contributes to the recruitment of neutrophils into the lungs. Moreover, the UV treatment of rHMPV significantly reduced the number of neutrophils, indicating that the recruitment of these cells is dependent on viral replication and the de novo synthesis of the G protein. These observations are in line with studies with other respiratory viruses, including those with respiratory syncytial virus (RSV), an HMPV-close-related human paramyxovirus, in which it has been shown that the fusion (F) protein, of the RSV 2–20 strain, contributes to the infiltration of neutrophils into the lungs of infected mice [38]. Similarly, influenza A virus, an ssRNA ortomyxovirus, appears to regulate neutrophil infiltration into the alveolar spaces through the expression of the PB1-F2 protein [39]. This suggests that different surface viral proteins can specifically contribute to the infiltration of neutrophils to the respiratory tract.

The observed contribution of the HMPV G protein to the recruitment of neutrophils in the infected mice, suggest that G protein may also regulate the expression of those cytokines and/or chemokines that control the recruitment of neutrophils to the alveolar spaces. Opposite to the effect of HMPV G protein on the IFN response, we observed some changes in CCL3, CCL4, VEGF, TNF, IL17, and CXCL2, which are all recognized mediators involved in the neutrophil recruitment to the sites of insult. In this work, we observed that the lack of G protein in HMPV resulted in the reduced expression of TNF and IL-17, which are known to promote the expression of neutrophil chemotactic cytokines. In fact, IL-17 together with TNF can synergistically induce the endothelial expression of neutrophilic chemokines including CXCL2 [40], which may explain also the reduced expression of CXCL2 observed in the rHMPV-ΔG-infected mice. In addition, we speculate that due to its suppressive effect on TNF [41], the IFN-α response in rHMPV-ΔG could be linked to the reduced production of TNF. In the same context, IFN-α has been reported to inhibit IL-17 production in PBMC’s from patients with chronic
active Hepatitis B infection (CAHB), suggesting the pleiotropic effect IFN-α has on proinflammatory cytokines associated with neutrophil activation and chemotaxis [42]. Moreover, type I IFN has also been shown to repress CXCL2 production in in vivo [23] and in vitro [36] settings, suggesting that the increased IFN-α response in rHMPV-ΔG could contribute to the observed diminished production of CXCL2. Furthermore, the absence of G protein led to a decrease in the expression of VEGF, which could also impact the neutrophil numbers to the site of infection since VEGF contributes to the recruitment of proangiogenic neutrophils from the circulation to the tissues [43]. These results are in line with data from melanoma studies in vitro, where IFN-α treatment significantly reduced the expression of VEGF suggesting a suppressive effect of Type I IFN on this cytokine [44,45]. Finally, the neutrophil recruitment after rHMPV-ΔG infection could have also been altered by the reduced expression of CCL3 and CCL4, which are neutrophil-active chemokines [46]. However, studies in vitro in an epithelial cell line (A549) indicate that the HMPV G protein rather inhibited the expression of CCL3 and some other cytokines [29]. This discrepancy might be due not only to the inherent differences of the experimental models, but also to the exerted effect of HMPV G protein on the complex microenvironment in vivo, where several cellular populations, including macrophages, lymphocytes, endothelial, and epithelial cells, mediate the overall cytokine/chemokine production. In fact, similar observations to the current work have been reported in a mouse model of pneumonia virus, where the nonstructural (NS) proteins NS1 and NS2 (rPVM ΔNS1ΔNS2; rPVM ΔNS2) antagonize IFN responses in vivo, but on the other hand, induced lower amounts of proinflammatory cytokines in the airways when compared to rPVM virus [35]. However, data on viral proteins regulating neutrophilic responses to the sites of inflammation is limited and further work is warranted. Overall, data from this work suggest that the attachment
protein of HMPV regulates neutrophil recruitment to the lungs by modulating the production of neutrophil chemoattractants.

In summary, the above results demonstrate a novel role for the attachment protein of HMPV as a contributing factor for neutrophil recruitment to the sites of infection. This effect appears to be influenced by the regulation of neutrophil chemoattractants and an exacerbated response of type I IFN production. We have recently demonstrated that neutrophils exert a protective effect to HMPV-induced pathogenesis in mice [20]. However, an exacerbated accumulation of neutrophils contribute to severe pulmonary inflammation [17]. Therefore, a controlled balance of neutrophil accumulation in the airways after HMPV infection would be helpful for the outcome of the infected individuals. In this regard, the attachment protein of HMPV represents an attractive target for future therapeutic applications to reduce an excessive accumulation of neutrophils in the airways.

4.5 References


CHAPTER 5
HOW DOES THE HUMAN METAPNEUMOVIRUS REGULATE NEUTROPHIL INFILTRATION INTO THE AIRWAYS?

5.1. Summary

Since its discovery in 2001, human metapneumovirus (HMPV) has been recognized as a respiratory pathogen of clinical significance that infects children, the elderly and immunocompromised patients [1,2]. Infection by this virus is characterized by acute lower respiratory tract infection and symptoms range from common cold-like syndrome to more severe manifestations such as bronchiolitis and pneumonia, which require hospitalization. Thus, HMPV can cause severe pulmonary inflammation. Recent clinical evidence indicates that neutrophil infiltration is induced within the airways of children with HMPV-induced bronchiolitis [3]. This phenomenon has been reproduced in experimental mouse models of infection by several groups [4–7]. However, limited information has been reported regarding the interaction between HMPV and neutrophils in the lungs of infected mice.

In understanding that interaction, we have previously demonstrated that depletion of neutrophils led to an increased disease severity and lung inflammation, indicating a protective role of these cells during HMPV infection [4]. Furthermore, we have also explored the mechanisms by which HMPV infection contributes to the recruitment of neutrophils in the lung. In this context, we have investigated the role of HMPV attachment protein in contributing to the recruitment of neutrophils into the airways [8]. The attachment glycoprotein (G) of HMPV is one of the three putative membrane proteins expressed by the virus. Though, the attributed function for the attachment protein is to aid in the attachment of the virus to the target cells, several

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studies have demonstrated that cell attachment and fusion can happen in the absence of HMPV-G protein [9,10]. Apart from its roles in viral infection, the G protein is also known to modulate several aspects of the innate immune response [11–13]. However, the contribution of this protein in pulmonary inflammatory responses during HMPV infection remains poorly understood.

Therefore, we speculated on two previous findings in which it was indicated that interferon has a regulatory effect on neutrophil recruitment [14–17], and that G protein of HMPV suppresses interferon responses, as we and other groups have previously reported [11,13]. Based on those observations, we hypothesized that the viral G regulates neutrophil infiltration in HMPV-infected animals. In order to test that, we used a recombinant virus lacking the G protein (rHMPV- G), as described previously [11], and infected BALB/c mice intranasally with rHMPV-G or with the full-length recombinant HMPV (rHMPV), as a reference control. Recruitment of neutrophils into the airways was quantified by differential cell counts, and the production of interferon and additional cytokines was measured by ELISA and multiplex cytokine detection assays, respectively. Analysis of the interferon production by the rHMPV- G in BALB/c mice indicated that the absence of the attachment protein increased the levels of IFN-α in the lungs of the infected mice. Furthermore, those mice infected with the rHMPV- G showed decreased neutrophil infiltration into the airways compared with the ones infected with the rHMPV, but did not have an effect on macrophages or lymphocytes, indicating that the G protein was found to have a specific effect on neutrophils. Those findings led us to explore the effect of G protein on additional cytokines/chemokines. We observed that the absence of G protein also decreased the lung cytokine/chemokine profile such as TNF-α, VEGF, IL-17, CCL3, CCL4 and CXCL2, all known to have a chemotactic effect on neutrophils and to contribute to the recruitment of these cells to the site of infection. Overall, these findings demonstrate that the attachment protein of
HMPV contributes to the recruitment of neutrophils during the viral infection. This happens potentially through a mechanism that involves the inhibition of interferon responses by the HMPV-G protein, which in turn would allow the induction of high levels of neutrophil-chemotactic cytokines, and consequently higher numbers of neutrophils present in the airways of the infected animals. Whether other HMPV proteins could also play a role in the neutrophil recruitment is unknown. But it is likely that proteins that modulate the interferon response could have an effect on neutrophilic chemokines, based on the effect of interferon on neutrophil responses [14–17]. In conclusion, our study highlights the important role of viral proteins in neutrophil recruitment, which is not trivial, given the critical contribution neutrophils have in the inflammatory outcome of HMPV-infected individuals [3]. That is, excessive recruitment of these cells during the infection can have a negative outcome, leading to exacerbated inflammatory responses, so their recruitment during HMPV needs to be regulated. In this context, our work demonstrates the contribution of the viral attachment protein to the recruitment of these cells into the airways. On the other hand, we have also reported that the absence of neutrophils leads to an increased inflammatory response during HMPV infection, mediated by γδ T cells [4]. Thus, upon HMPV infection, a fine balance of neutrophil response is necessary for an optimal activation of the immune system and controlled inflammatory response. Hence, these findings help further our understanding of the complex interplay between the beneficial and detrimental roles of neutrophils in the inflammatory responses during HMPV infection.

5.2 References


CHAPTER 6
PRENATAL SMOKE EXPOSURE EXACERBATES LUNG INFLAMMATORY RESPONSE BY MODULATING NEUTROPHIL INFILTRATION TO THE LUNGS AFTER RSV INFECTION

6.1 Introduction

Tobacco smoke exposure is considered a worldwide problem and an important cause of morbidity and mortality in active as well as passive smokers. In particular, parental smoking during pregnancy has been associated with a range of adverse health effects in both smoking parents as well as in offspring. These adversities include but are not limited to COPD, asthma exacerbation, increased frequency of lower respiratory tract infections, cardiovascular diseases, cancer predisposition and otitis media [1-6]. In understanding the underlying mechanism behind these adversities, several constituents of tobacco smoke, known to have immunomodulatory effects have been shown to cross the placenta, thus affecting the development of immune system of the fetus in-utero [7]. Therefore, it is evident that in-utero exposure to second hand smoke (SHS) results in alteration of host immune responses in the offspring [8, 9].

Despite these finite evidences on how in-utero exposure predisposes offspring to altered immunity, there is limited to no evidence on the effect of SHS on host responses during lower respiratory tract infections, particularly respiratory viral infections. Epidemiological studies show that prenatal tobacco smoke exposure via active and passive maternal smoking leads to increased severity of bronchiolitis in infants [10]. One such leading cause of bronchiolitis in infants is infection with respiratory syncytial virus (RSV), a negative sense, single-stranded RNA pneumovirus causing acute lower respiratory tract infection. Findings from previous studies indicate that in RSV associated bronchiolitis in infants, neutrophils are the predominant cell population recruited to the airways after infection [15,16, 21]. Thus it is evident that neutrophils
play a crucial role in RSV induced inflammatory responses and contributing to bronchiolitis. Also, recent studies emphasize more on a potential link between cigarette smoke, neutrophils and associated inflammation [11-14]. Nevertheless, the mechanism behind SHS induced immune exacerbations during RSV infection has not been documented. In understanding this aspect, we sought to investigate the effect of in-utero SHS exposure on RSV induced inflammatory exacerbations in the neonatal murine lung.

In the work here, we demonstrate using a neonatal mouse model of in-utero smoke exposure, the effect of cigarette smoke on lung inflammation and neutrophil response after RSV infection. Our findings indicate that in-utero SHS exposure exacerbated lung inflammation upon RSV infection, which is mediated by increased infiltration of neutrophils into inflamed airways. Furthermore, neutrophils from SHS exposed and RSV infected neonates were highly activated, as indicated by increased CXCR2 expression. Overall, our work demonstrates that in-utero SHS exposure modulates lung inflammatory responses during RSV infection in neonatal mice.

6.2 Materials and Methods

6.2.1 Virus

RSV strain A2 (American Type Culture Collection, Manassas, VA) was grown and titrated in Hep2 cells (ATCC) as described previously [17].

6.2.2 Mice and smoke exposure

Eight- to 10-wk-old BALB/c mice were purchased from Envigo (Indianapolis, IN, USA). All mice were housed in specific pathogen-free conditions in accordance with the Louisiana State University Institutional Animal Care and Use Committee. For smoke exposure, BALB/c mice were acclimatized to tobacco smoke for 2 weeks prior to mating. The experimental study design
is presented in Fig. 6.1. Briefly, male and female mice were exposed to 100% mainstream smoke using Scireq inExpose system (Montreal, QC, CA). Mice were exposed to 4-3R4F filtered research cigarettes (University of Kentucky, Lexington, KY) with 30-minute gap between each cigarette for 5 days a week. Once pregnant, only female BALB/c mice were exposed to mainstream smoke until delivery. Pups from unexposed mice were considered as air exposed mice.

6.2.3 Infections

Neonatal mice (5 days old) were infected with 5 x 10^5 PFU of RSV in 10µl total volume via the i.n. route. Mock-infected animals received 10µl sterile PBS (herein, referred to as mock).

6.2.4 Histopathology analysis of lungs

The lungs of the mice were inflated with buffered formalin phosphate (10%) by intratracheal instillation. Wax embedded tissues were used to prepare 5-µm thick lung sections and stained with Hematoxylin & Eosin. Stained slides were visualized by bright-field microscopy using the highthroughput slide-scanner NanoZoomer and the NanoZoomer digital pathology view software (both from Hamamatsu Photonics, Hamamatsu City, Japan), as previously reported [18]. Additional lung sections were PAS stained to assess goblet cell hyperplasia and calculate mucus index. PAS stained slides were visualized same as H&E slides using NanoZoomer and the NanoZoomer digital pathology view software. Mucus index was calculated as the area of PAS positive airway epithelium/total area of the conducting airway epithelium.
6.2.5 Bronchoalveolar lavage cell and fluid collection

As previously described [18], following euthanasia, we lavaged the lungs of each mouse 2 times with 150 µL phosphate-buffered saline (PBS). The BAL was centrifuged at 14000 rpm for 1 min and supernatant was stored at −70 °C for subsequent analyses.

6.2.6 Detection of Cytokines and Chemokines

Cytokine and chemokine release in cell-free BAL supernatants was determined using the Milliplex MAP™ 32-Mouse-Plex cytokine detection system (Millipore, Billerica, MA, USA), according to the manufacturer’s instructions. The assay panel included the following cytokines and chemokines: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL 10, IL-12 p40, IL-12 p70, IL-13, IL-15, IL-17, M-CSF, G-CSF, GM-CSF, IFN-γ, TNF, CXCL1, CXCL2, CXCL5, CXCL9, CXCL10, CCL2, CCL3, CCL4, CCL5, CCL11, LIF, and VEGF. The range of sensitivity of the assay is 3.2 to 10,000 pg/ml.

6.2.7 Flow cytometry

For flow cytometry staining, lung single-cell suspensions were obtained as described previously [19]. Briefly, cells were incubated with anti-CD16/CD32 (Clone 2.G2) antibody at 4°C for 30 min. Cells were then washed with 1X PBS/1% BSA and stained with anti-Ly6G (Clone RB6-8C5), anti-CD11b (Clone M1/70) for 30 min at 4°C (BD Pharmingen, San Jose, CA, USA). After the incubation, cell were washed with PBS/1% BSA and fixed with 1% paraformaldehyde. Cells were run on FACSscan flow cytometer (BD Biosciences, San Jose, CA, USA) and data analyzed using FlowJo software (version 10; Tree Star, Ashland, OR, USA).
6.2.8 Statistical Analysis

Statistical significance was calculated by one-way ANOVA to ascertain the differences between 3 or more animal groups, followed by a Tukey-Kramer test to correct for multiple comparisons. For comparison of 2 groups, a student’s t-test was used (two-tailed, two-sample equal variance). All analyses were performed using Graph Pad InStat 3 (GraphPad Software, La Jolla, CA, USA). P ≤ 0.05 was considered statistically significant and unless otherwise indicated, results are expressed as mean ± SEM.

Figure 6.1. In-utero cigarette smoke exposure model representation.

6.3 Results

6.3.1 RSV infection exacerbates lung inflammation in secondhand smoke exposed neonatal mice

To define the role of maternal smoke induced immune exacerbation in offspring, we infected secondhand smoke exposed or unexposed day 5 neonates with RSV or Control (PBS) for 7 days. At day 7 after infection, lungs were collected, and tissue sections were processed for lung pathological evaluation (Fig. 6.2A). Lung histopathological analysis revealed that mice that were unexposed or exposed to second hand smoke (SHS) show minimal to no airway inflammation. However, after RSV infection, unexposed mice showed an increased pathology score, and the lung inflammation for RSV-infected mice after SHS exposure was significantly higher when
compared to the RSV-infected unexposed group, suggesting an exacerbated airway inflammation by the effect of smoke in the RSV-infected animals (Fig 6.2B).

Figure 6.2. RSV infection after second hand smoke (SHS) exposure exacerbates lung inflammation. In-utero smoke exposed or unexposed neonatal mice were infected with \(5 \times 10^5\) PFU of RSV. At day 7 after infection, lungs were perfused and fixed in 10% buffered formalin and embedded in paraffin. Multiple 4µm-thick sections were stained with haemotoxylin & eosin (H&E) to assess lung inflammation (Magnification: 40X). \(n=4-10\) mice. Control mice were mock infected with PBS. Mean ± SEM are shown. **\(P<0.01\), ***\(P<0.005\).
6.3.2 RSV infection after in-utero smoke exposure alters lung cytokine/chemokine profile

In further delineating the exacerbated inflammatory responses observed in SHS exposed and RSV-infected neonates, the concentration of cytokines and chemokines were measured. For this, neonatal mice that were SHS exposed or unexposed were infected i.n. with RSV or PBS, and BAL samples were obtained from each group at d 7 after infection (when the inflammatory response was determined). The level of cytokines and chemokines in BAL were measured using a multiplex cytokine detection system. As shown in Figure 6.3, we found that SHS exposure led to an increased response of inflammatory and immunoregulatory cytokines after RSV infection. We observed a 2-fold increase in KC (CXCL1, a potent neutrophil chemokine), in SHS RSV-infected group compared to RSV infected alone mice. On the other hand, other cytokines and chemokines were altered with no significant differences between the different experimental groups.
In-utero smoke exposed or unexposed neonatal mice were infected with $5 \times 10^5$ PFU of RSV. BAL collected at day 7 after infection was used for cytokine/chemokine Mean ± SEM is shown. n= 5-12 mice. Control mice were mock infected with PBS and smoke or air exposed. Mean ± SEM are shown. **$P \leq 0.05$.

6.3.3 Secondhand smoke exposure increases neutrophil influx into the airways of RSV infected neonates.

Based on the cytokine and chemokine profile assayed in Figure. 6.2, we sought to characterize the cell subsets infiltrated in the lungs. We focused on the recruitment of neutrophils since CXCL1 recruits and activates these cells. For that, lungs were harvested at the peak of inflammation (d 7 p.i.). Lung single-cell suspensions were prepared from mock-infected, SHS exposed, RSV infected or SHS RSV infected mice. The infiltration of neutrophils was assessed
by flow cytometry analysis by staining the lung single-cell suspensions for CD 11b and Ly6G (Fig. 6.4A-B). Data shown in figure 6.4 indicates that SHS exposure followed by RSV infection led to an increased influx of neutrophils into the lungs during peak inflammation (d 7 p.i.). We found that SHS exposure significantly increased the recruitment of neutrophils after RSV infection, both percentage (Fig. 6.4A) and numbers (13± 2 x 10^5 cells) (Fig. 4B), compared with mice that were RSV infected alone (7.5± 1 x10^5 cells), mock-infected (6.5± 1 x10^5 cells), or SHS exposed (9± 0.5 x10^5 cells). These findings strongly suggest that neutrophils are key players in exacerbated lung inflammation in these mice.
Figure 6.4. RSV infection in SHS exposure induces neutrophil influx into the lungs. In-utero smoke exposed or unexposed neonatal mice were infected with $5 \times 10^5$ PFU of RSV. At day 7 after infection, lungs cells were stained with specific antibodies and analyzed using flow cytometry to measure neutrophil responses. A) Neutrophil gating strategy. B) Total neutrophil counts from FACS analysis. n= 4-10 mice. Control mice were mock infected with PBS. Mean ± SEM are shown. **$p \leq 0.05$, ***$p < 0.005$. 
6.3.4 *Neutrophils from SHS and SHS+RSV infected neonates are highly activated.*

Given that neutrophils are recruited by increased CXCL1, we then wanted to see if CXCR2 is the target receptor on neutrophils for CXCL1 mediated activation. Recent studies have also shown that CXCL1 mediated neutrophil trafficking in the lungs is via CXCR2 activation. For confirming this, we characterized the neutrophils in SHS + RSV mice lungs and screened them for CXCR2 up-regulation (Fig. 6.5A). Flow staining for neutrophils revealed that CXCR2 was indeed up-regulated in lung neutrophils from neonatal mice that were SHS exposed and SHS+RSV infected compared to the mock infected or RSV infection alone (Fig. 6.5B).

Interestingly, when we measured CXCR2 activation on lung neutrophils in mock-infected, SHS exposed, RSV infected or SHS exposed with RSV infection, we found that both SHS exposure alone (mean-21.48) and SHS exposed with RSV infection (mean-20.54) showed a robust increase in CXCR2 activation compared to RSV infected alone (mean-10.97) neutrophils suggesting the role of SHS in inducing neutrophil activation (Fig. 6.5B).
Figure 6.5. **SHS exposure highly activates infiltrated neutrophils.** In-utero smoke exposed or unexposed neonatal mice were infected with 5x10^5 PFU of RSV. At day 7 post infection, lungs cells were stained with specific antibodies and analyzed using flow cytometry to measure CXCR2 expression on neutrophils. A) CXCR2 expression analysis. B) Mean fluorescent intensity of neutrophil CXCR2 expression from FACS analysis. n= 4-10 mice. Control mice were mock infected with PBS. Mean ± SEM are shown. **P≤0.05.

### 6.4 Discussion

Based on reported evidences, it is evident that cigarette smoke alters host responses in active as well as passive smokers [8,9]. Furthermore, emerging data also support that maternal smoking during pregnancy also has an impact on developing immune system in the offspring. In fact, several constituents of tobacco smoke are known to cross the placenta and modulate the developing immune system in the fetus [7]. These alterations have an impact on immune outcomes during external insults. Nevertheless, knowledge on how secondhand smoke exposure alters immune outcomes during an external stimulus is not clear. One such insult known to be a
leading cause of morbidity and mortality in infants worldwide is respiratory syncytial virus infection, causing acute lower respiratory tract infection and associated bronchiolitis.

Therefore, our studies were designed to address the effect of in-utero smoke exposure on inflammatory responses in the airways during RSV infection. The key aspect we wanted to investigate is whether SHS exposure alters lung inflammatory response during RSV infection. If so, we wanted to identify the mechanism leading to this inflammatory exacerbation. RSV infection in mice leads to increased lung inflammation on day 7 after infection as indicated by high pathology score and release of associated mediators into the airways. So, to determine if SHS exposure and subsequent RSV infection have any effect on lung inflammation, we assessed lung pathology at day 7 after infection in both SHS as well as unexposed mice infected with RSV or mock infected. As shown in Figure 6.2, RSV infection in SHS exposed neonatal mice led to increased lung inflammation as indicated by pathology score compared to RSV infection alone or SHS alone. These findings demonstrated that in-utero smoke exposure alters lung immune responses and thus leads to exacerbated inflammation during RSV infection. Hence we further investigated the mechanism behind exacerbated lung inflammation observed in SHS neonates infected with RSV.

In further understanding the potential mechanism of the recruitment of neutrophils in RSV SHS-exposed mice, we assessed lung cytokine and chemokine profile, which would give us a sense of the immune cells recruited causing lung inflammation. We observed that, RSV infection in SHS exposed neonatal mice significantly increased the production of CXCL1 in the bronchoalveolar spaces. CXCL1 is a known potential neutrophil chemo-attractant produced mainly by epithelial cells, macrophages, γδ T cells and neutrophils and it aids in the infiltration of neutrophils from circulation to sites of insult. Therefore, this finding led us to speculate that neutrophils are the
one causing exacerbated lung inflammation in SHS neonates infected with RSV. As we speculated, SHS mice infected with RSV had a significant increase in the influx of neutrophils into the airways compared to RSV infection alone or SHS exposure alone. From our previous findings, we have shown that neutrophils when depleted during HMPV infection, a virus belonging to the same family as RSV, leads to altered immunity showing the beneficial role of neutrophils [18]. However, excessive recruitment of these cells to the site of infection has been shown to cause tissue damage and inflammation. Thus, a balance of these cells is necessary to impart beneficial immune response. Furthermore, we also observed that the neutrophils from SHS or SHS mice infected with RSV were highly activated as indicated by increased expression of CXCR2, a receptor expressed by neutrophils and binds ligands CXCL1/2. This effect was earlier demonstrated in adult mice exposed to mainstream cigarette smoke, wherein smoke exposure induced CXCL1 production and CXCR2 expression on neutrophils. Treatment with CXCR2 antagonists resulted in reduced infiltrating neutrophils and thus perivascular inflammation [20].

In summary, the current data demonstrates that in-utero smoke exposure modulates host inflammatory responses to RSV infection. Our results indicate that RSV infection in SHS exposed neonatal mice leads to increased production of CXCL1 in the airspaces and also increases the expression of CXCR2 on neutrophils. This causes CXCL1 to bind CXCR2 on these neutrophils and infiltrate them to lungs after RSV infection. Thus, increased influx of neutrophils leads to exacerbated lung pathology. These findings are summarized in a proposed model shown in Figure 6.6.
Figure 6.6. Simplified illustration of the effect of in-utero cigarette smoke exposure on RSV induced lung inflammation in neonatal mice. RSV infection in second hand smoke (SHS) exposed neonatal mice induces CXCL1 production and also up regulates CXCR2 expression on neutrophils. CXCL1 binds to its receptor CXCR2 and increased neutrophil infiltration to the site of infection (lungs), leading to exacerbated pulmonary inflammation.

6.5 References


CHAPTER 7
SUMMARY AND CONCLUDING REMARKS

7.1 Introduction

The experimental studies presented in this dissertation used two experimental mouse models of human pneumovirus infection to delineate the role of neutrophil in host immunity. We particularly looked at the role of these cells during human metapneumovirus (HMPV) and respiratory syncytial virus (RSV) infections, both belonging to the pneumovirus family and known to be the leading causes of lower respiratory tract infections in infants, elderly and immunosuppressed individuals worldwide. Chapter 1 provides an introduction on the immune responses induced by HMPV in the mouse model used in this dissertation (published in the peer reviewed journal- Pathogens). Chapter 2 describes the role of neutrophils in an experimental mouse model of HMPV infection (published in the peer reviewed journal- Journal of Leukocyte Biology). Chapter 3 describes the role played by γδ T cells in HMPV infection (manuscript under preparation). Chapter 4 and 5 emphasize on the role of viral attachment protein G in contributing to neutrophil recruitment to the airways during HMPV infection [both chapters have been published in peer reviewed journals: chapter 4 (published in the peer reviewed journal-Viruses), chapter 5 (published in the peer reviewed journal-Future Virology as an invited editorial)].

Chapter 6 explores the effect of neutrophils in the exacerbated pulmonary inflammation generated by in-utero smoke exposure followed by RSV infection (manuscript to be submitted).

Summarized below are the results of each chapter and discussion of their implications in understanding the immune responses to the two clinically important respiratory pneumovirus infections.
7.2 Summary of results

In chapter 2, we demonstrated the role of neutrophils in HMPV infection using an experimental mouse model of infection. This was achieved by effective depletion of neutrophils \textit{in vivo} using a monoclonal antibody, which binds specifically to and depletes neutrophils but not any other cell population. Neutrophil depleted mice infected with HMPV had increased disease severity (as indicated by enhanced body weight loss and high clinical score) and lung inflammation (as demonstrated by high lung pathology score) compared to neutrophil competent mice infected with HMPV. In further understanding the inflammation induced by lack of neutrophils, we observed increased production of IL-17 and CXCL1 in the airways and also increased number of $\gamma\delta$ T cells infiltrating the lung. This observation led us to suspect that $\gamma\delta$ T cells might be playing a role in inducing lung inflammation in the absence of neutrophils. To confirm this effect, we used a knock-out mouse model which lack the $\delta$ chain of the $\gamma\delta$ TCR and infected them with HMPV. Compared to wild-type, $\delta$TCR KO mice had significantly reduced lung inflammation at day 7 after HMPV infection (time point when HMPV induced lung inflammation peaks in a mouse model of infection). These results confirm two things: a) that neutrophils regulate lung inflammatory responses by suppressing $\gamma\delta$ T cell recruitment to the lungs meaning neutrophils have a beneficial role during HMPV infection and b) $\gamma\delta$ T cells during HMPV infection induce exacerbated lung inflammation as indicated by increased lung pathology. I further looked into the role of $\gamma\delta$ T cells during HMPV infection in chapter 3, as nothing has been characterized with these cells so far in HMPV induced host responses. In this work, I observed that $\gamma\delta$ T cells during HMPV infection regulate IL-6 production, and also regulate neutrophil recruitment to the airways. However, $\gamma\delta$ T cells were not involved in lung viral clearance after HMPV infection.
Chapters 4 and 5 describe the contribution of HMPV attachment protein (G) in neutrophil recruitment to the airways of infected mice. Previous studies from our lab and other groups reported the role of HMPV-G protein in regulating interferon responses in the lung [1, 2]. Furthermore, several studies have demonstrated that interferon has a suppressive effect on neutrophil infiltration to the sites of insult [3, 4, 5, 6, 7]. Based on these two observations, we investigated whether HMPV-G protein regulates neutrophil recruitment to the lungs after HMPV infection. We observed that infection of mice with rHMPV-ΔG (mutant virus which lacks the G protein) led to increased IFN-α production and decreased neutrophil influx into the airways compared to mice infected with rHMPV (recombinant full-length virus). However, this effect was only specific to neutrophil influx, as we did not see any change in lymphocyte or monocyte populations after infection with rHMPV-ΔG. Also, with increased IFN-α in rHMPV-ΔG mice, we also observed a decrease in several cytokines in the lung, which are known to have a chemotactic effect on neutrophils. These results confirm that HMPV-G protein regulates neutrophil recruitment to the airways after HMPV infection potentially through increased interferon responses.

For chapter 6, we analyzed the findings described in chapter 2, where we confirm a protective and beneficial role of neutrophils during HMPV infection, wherein they regulate the infiltration of γδ T cells into the lungs, which induce pathology. However, neutrophils are considered "double-edged sword". In fact, several studies in different infectious disease models have confirmed that excess recruitment of these cells can also play a detrimental role in enhancing inflammation [8, 9, 10]. RSV infection in infants is associated with neutrophil induced bronchiolitis and studies have linked cigarette smoke to enhanced recruitment of neutrophils causing aggravated lung inflammation [11, 12, 13, 14, 15]. So, we explored the aspect of
neutrophil induced lung inflammation in in-utero smoke exposed (termed secondhand smoke exposed) neonatal mice infected with RSV. In order to explore this aspect, we used an in-utero cigarette smoke exposed mouse model of RSV infection. We observed that SHS exposed neonatal mice infected with RSV had increased lung inflammation (as indicated by lung pathology score) compared to unexposed neonatal mice infected with RSV. In further understanding the mechanism behind this increased lung inflammation, we observed increased production of CXCL1 in the lung, a potential neutrophil chemo-attractant. In accordance with this observation, SHS exposed neonatal mice infected with RSV had high influx of neutrophils into the airways compared to unexposed neonatal mice infected with RSV. Also, the neutrophils from SHS or SHS+RSV neonatal mice were highly activated as indicated by increased CXCR2 expression (a receptor for CXCL1). Altogether, data from this chapter indicate that SHS exposure and subsequent RSV infection induce exacerbated lung inflammation, which is mediated by neutrophils infiltrating the airways. Therefore, excess recruitment of neutrophils can play a detrimental role leading to exacerbated lung inflammation.

7.3 Conclusions and future direction

The data presented in this dissertation work has solid contributions in understanding the role of neutrophils in human respiratory viral infections, as their role has remained controversial due to the contradicting findings published so far. Based on the experimental evidences presented here, that neutrophils in host responses can be both beneficial as well as detrimental (Summarized in Figure 7.1 below). Thus, a fine balance in the recruitment of these cells is necessary to impart favorable clinical outcome.
Future studies are aimed at further understanding the mechanisms leading to neutrophil recruitment to the sites of infection, wherein blocking those cells lead to balanced infiltration and thus by less tissue damage. Also, exploring the mechanisms leading to the infiltration of γδ T cells in the absence of neutrophils during HMPV infection and the pathogenesis induced by them. Future research branching out from this work will greatly contribute to our understanding of the innate inflammatory response to viral infections and potentially help the design of effective therapeutic strategies for human respiratory pneumovirus infections and other respiratory infections that induce a robust response of neutrophils.
Figure 7.1. Summarized illustration on the role of neutrophils in respiratory pneumovirus infection. Neutrophils during HMPV infection regulate the recruitment of γδ T cell infiltration that in turn induce pathogenic responses in the lungs. Thus, neutrophils during HMPV infection play a beneficial role. However, RSV infection in neonatal mice exposed to cigarette smoke in-utero leads to exacerbated lung inflammation correlating with excessive recruitment of neutrophils. Therefore, neutrophils during RSV infection in neonatal mice in an exacerbated model of inflammation (in-utero cigarette smoke exposure) lead to exacerbated lung inflammation demonstrating a detrimental role of neutrophils. Based on these findings, we demonstrate that neutrophils can be both beneficial or detrimental during respiratory pneumovirus infections, therefore a fine balance in the recruitment of these cells is critical for a favorable outcome.

7.4 References


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Nagarjuna R. Cheemarla (Reddy) was born in Hyderabad, Telangana, India and got his Bachelor of Technology degree in Biotechnology from the Jawaharlal Nehru Technological University, India in 2010. Following graduation, Reddy moved to the United States in 2011 to pursue his Master of Science in Microbiology & Immunology from Wright State University, Dayton, Ohio. Reddy’s love for immunology and research interest led him to start his graduate education in 2013 in the Department of Pathobiological Sciences in the School of Veterinary Medicine. Reddy joined the laboratory of Dr. Antonieta Guerrero-Plata with research interest in viral immunology, which led him to investigate the role of innate immune cells in respiratory viral infections; the focus of this dissertation. Reddy plans to graduate in August 2018 and pursue his research career with an emphasis on infectious diseases and immunology.