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Role of Mucin 19 in the Respiratory Tract

Kaitlin McBride
Louisiana State University and Agricultural and Mechanical College, kaitlin.elise92@gmail.com

ROLE OF MUCIN 19 IN THE RESPIRATORY TRACT

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by
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To my late father,

I promised I would make you proud

ACKNOWLEDGMENTS

I would like to thank Dr. Guerrero-Plata for mentoring me these last two years. She has answered many questions, trained me on many lab techniques, and expanded my overall knowledge of immunology, as well as general science. Thank you for pushing me and helping me to exceed my own expectations for this master's study.

I would also like to thank my lab member, Reddy Cheemarla, for training me in lab techniques and protocols, as well as helping me and giving input on my project.

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ABSTRACT

Human metapneumovirus (HMPV) is a negative sense, single stranded RNA virus belonging to the *Pneumoviridae* family, and represents an important pathogen that causes severe respiratory disease worldwide. There is currently no vaccine against HMPV, so it is important to study the aspects of the immune response induced by HMPV. Because infiltration of mucus is a hallmark of HMPV infection, it is warranted to study the role of mucus in the disease process. Mucin proteins make up the major component of mucus and can be found within the airway and lungs. Previous work from our laboratory demonstrated a high upregulation of mucin 19 mRNA, both in human bronchial epithelial cells, as well as BALB/c mice that had been infected with HMPV. These findings led me to my hypothesis that mucin 19 plays a role in HMPV induced pathogenesis. Using a muc19$^{+/+}$ and muc19$^{-/-}$ mouse model of HMPV infection, I was able to show that muc19 is the predominant mucin expressed in the lungs after HMPV infection. In addition, I demonstrate that the lack of muc19 yields a significantly lower CD4+ T cell response in both the lungs and lymph nodes of infected mice. Further analysis revealed that the dendritic cell compartment was also altered by removing muc19. My work suggests that mucin 19 potentially regulates dendritic cell maturation, leading to the generation of tolerogenic dendritic cells. These novel findings yield relevant information regarding the contribution of the mucins, specifically muc19, on the immune response induced by respiratory viral infections in the lung.

CHAPTER 1
INTRODUCTION

Human metapneumovirus (HMPV) was discovered in 2001, although seroprevalence studies suggest it had been circulating for at least 50 years prior [46]. HMPV is a negative sense, single stranded RNA virus belonging to the newly formed *Pneumoviridae* family [1]. It infects people of all ages, but the populations most at risk for illness are children, elderly, and immunocompromised. Approximately 4-16% of patients with acute respiratory tract infections test positive for HMPV. Virtually all people have been infected with HMPV by the age of 5, however, children with underlying pulmonary, cardiac, or neural disorders, as well as those who were born prematurely stand a much higher risk of severe disease [35]. Symptoms of HMPV often include those common to other respiratory illnesses like rhinorrhea, cough, or fever, although other symptoms including conjunctivitis, vomiting, diarrhea, rash, and otitis media have been reported. Individuals who develop severe disease may exhibit bronchitis, bronchiolitis or even pneumonia, and may be hospitalized [44]. There is currently no vaccine against HMPV and treatment is supportive [35].

Because infiltration of mucus is a hallmark of HMPV infection, it is important to study the role of mucus in the disease process. Mucus has been shown to primarily act as a protective barrier to pathogens and toxins throughout the body, including in the eyes, respiratory tract, gut, and reproductive tract. [29, 38, 41, 43]. The primary component of mucus is mucin proteins, which are a family of complex O- linked glycosylated proteins, rich in serine and threonine, with various numbers of tandem repeats. There exists two types of mucins: membrane bound, and secreted. The membrane bound mucins, like MUC1 or MUC4, are often found at the apical surface of ciliated cells, while the secreted mucins are expressed by goblet cells, or mucus cells

within the submucosal glands [48]. Figure 1 is a diagram of mucus cells with secreted mucins (Fig. 1A), like MUC5AC or MUC19, and membrane bound mucins (Fig. 1B).

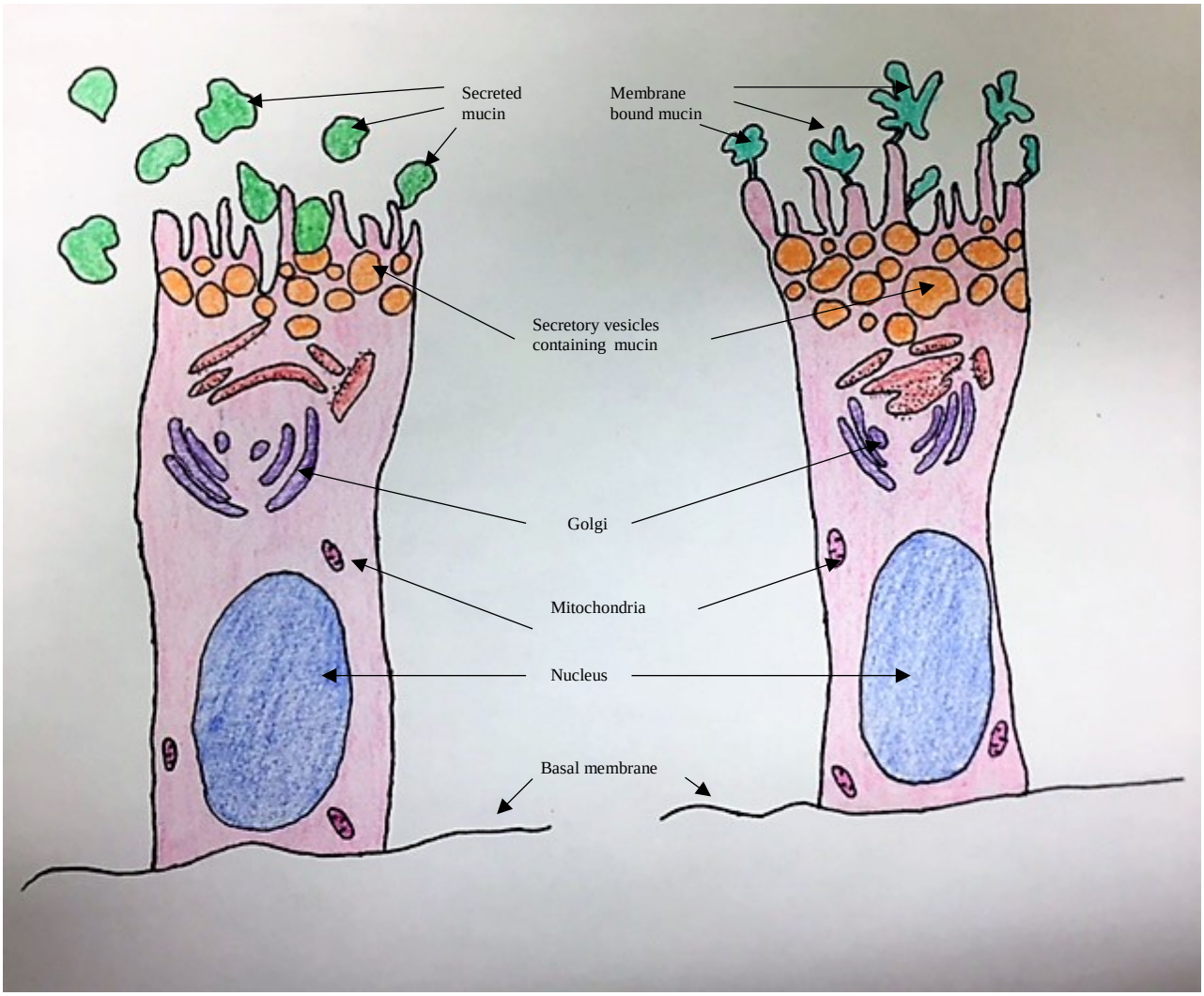

Figure 1. Diagram of mucus cells. A) Mucus cells can either secrete mucins, B) or have mucins which are bound to the membrane. (*McBride, K.E.)*

To date, there have been 22 mucins found in humans and 16 of those can be found within the airway and lungs [9, 22, 25, 30]. While the primary role of mucins is to act as a surface protectant, many have secondary functions within the disease process. Mucin 1 for example has

been shown to be upregulated in some tumor cells, and help exacerbate cancers. It is also known for mediating growth factors like connective tissue growth factor (CTGF), or platelet-derived growth factors (PDGF-A and B) which activate MAP kinase pathways, making it easier for proliferation and survival/ growth of tumor cells [33]. In human lungs specifically, mucins 5ac and 5b have been shown to be very important and genetic mutations within these genes have shown links to various lung diseases [47]. One study found that muc5b knockout mice had higher levels of bacteria and inflammation in the lungs as well as middle ear, due to a lack in mucocilliary clearance [42]. While there are several reports indicating the importance of mucins in respiratory viruses like influenza, rhinovirus, and respiratory syncytial virus, there is still much to learn about the role of mucins in other respiratory viruses with high morbidity and clinical relevance, such as HMPV [4, 6, 52].

Our research group has previously reported that one mucin that has been found to be highly upregulated in an alveolar epithelial cell line during HMPV infection is MUC19 [3]. Mucin 19 is a secreted mucin that is found in numerous tissues around the body. In 2008, MUC19 was identified on the surface of the eye, and believed to help with homeostasis and lubrication of ocular tissue since patients with Sjögren's Syndrome, who symptomatically have dry eye, had reduced amounts of MUC19 [51]. MUC19 was also found in the inner ear, and showed upregulation when exposed to inflammatory cytokines, suggesting a role in ear infections [24]. In studies in the gut, polymorphisms within the mucin 19 gene have been linked to inflammatory diseases like Crohn's Disease [5]. Muc19 expressed in the salivary glands of mice has been shown to limit dental disease caused by *Streptococcus mutans* [10]. There has been one genome-wide association study linking MUC19 polymorphisms to asthma [23], but

otherwise there is a lack of knowledge about this particular mucin's presence and function within the lungs and airway. The lack of information on mucin 19 in the lungs warrants further research.

Previous work in our laboratory using normal human bronchial epithelial (NHBE) cells infected with HMPV indicate that MUC19 was predominantly expressed over any of the other mucins tested (*Banos-Lara MDR, Uche IK, and Guerrero-Plata A. Unpublished)*. This finding begged for questioning if the same thing happened *in vivo*. Thus, further studies using an experimental BALB/c mouse model of HMPV infection were performed. Interestingly, HMPV also induced a large amount of muc19 in the lungs of infected mice compared to the other mucins tested (*Banos-Lara MDR and Guerrero-Plata A. Unpublished*). These two key pieces of preliminary data are what shaped my current project. The idea that mucus had a secondary role in infection, something besides acting as a barrier, was quite intriguing. These data led me to embark upon the current project with the following hypothesis: *mucin 19 plays a role in HMPV induced pathogenesis.* Within this hypothesis there were two main objectives: 1) to determine the disease phenotype of my animal model, and 2) to define the role of mucin expression in HMPV immune response in an experimental mouse model.

CHAPTER 2
MATERIALS AND METHODS

Virus

HMPV (strain CAN97-83) was grown in LLC- MK2 cells (ATCC) in minimum essential medium containing 1μg trypsin/ml. Virus was allowed to propagate for 3-4 days before being filter purified [7-8]. Viral titer was determined by immunoassay [7, 8, 16]. Briefly, virus was serially diluted on LLC-MK2 monolayers in 24-well plates and rocked for 2 hours before applying a methylcellulose overlay. Five days later, monolayers were washed, then incubated with anti-HMPV antibody (1:1000) and stained with HRP labeled goat anti-mouse IgG antibody (1:500). Infected cells were detected using 3-amino 9-ethyl-carbazole, and counted under a microscope.

Mice and Infection Protocol

Mice used in this work were mucin 19 knockout mice (NFS/N.Cg-*Muc19*$^{tm2.1Culp}$/Mmucd), purchased from MMRRC at U.C. Davis. Due to the cross strain background, animals homozygous for both mucin 19 alleles (Muc19$^{+/+}$) were used as wildtype. Male and female animals aged 5 to 8 weeks old were used for these experiments. Mice were anesthetized with a mixture of ketamine and xylazine, and then infected intranasally with 1x10^7 PFU HMPV, while mock infected animals were inoculated with 50μl of sterile PBS [7,8].

Sample Collection

Mice were euthanized by combination of intraperitoneal injection of ketamine and xylazine and exsanguination via the femoral vessels. Bronchoalveolar lavage fluid (BAL) was collected by inserting a cannula into the trachea and flushing 1mL of PBS into the lungs twice.

Cell free supernatants were then kept at -75°C until further analysis. A small piece of lung tissue was flash frozen in liquid nitrogen until the time of RNA extraction using RNeasy kit (Qiagen). For lung cell analysis, lungs were perfused and kept on ice until their digestion using collagenase to obtain a single cell suspension, as previously described [8]. Spleens and lung draining lymph nodes were also collected, then pushed through 70μm mesh filters and washed with PBS/0.5% BSA to create a single cell suspension. For histologic analysis, lungs were perfused and fixed in 10% formalin phosphate and embedded in paraffin. To determine viral load, whole lungs were flash frozen in liquid nitrogen and stored at -75°C until analysis.

ELISAs

The mucin 19 (Cusabio), mucin 5ac, and mucin 5b (mybiosource) ELISAs were run by manufacturers' guidelines using the cell free supernatant from BAL diluted 1:2. OD was read at 450nm on a SpectraMax M2 microplate reader (Molecular Devices).

Lung Viral Replication

Whole lungs were homogenized and 2-fold serial dilutions were performed with the supernatant, which was then plated onto LLC- MK2 monolayers with a methylcellulose overlay. 6 days later plaques were visualized with HRP staining as described above.

Pathology

Lung tissue was sectioned from paraffin blocks, and stained with hematoxylin and eosin. A blind analysis and scoring was performed by a veterinary pathologist as previously described

[8], and lungs were assigned a pathology score percentage. Lungs were analyzed by enumerating the layers of inflammatory cells surrounding the vessels and bronchioles. To obtain the final percentage score, the number of abnormal perivascular and peribronchial spaces were divided by the total number of spaces.

Flow Cytometry

Single cells suspensions were incubated with anti-CD16/32 (clone 2.4G2) antibody at 4°C for 30 minutes before applying specific antibodies: anti-CD8a (clone 53-6.7), anti-CD3e (clone 145-2c11), anti-CD4 (clone RM4-5), anti-γδ T cell receptor (clone GL3), anti- MHC II (clone 2G9), anti-CD11b (clone M1/70), anti-CD80 (clone 16-10A1), anti-CD86 (clone GL1), and anti-CD40 (clone 3/23), anti- IL-4, anti- IFNγ, anti-CD49b (clone DX5), anti-LY6G (clone 1A8), and anti-IL 17a (clone TC11-18H10); all from BD Pharmingen, and anti-CD11c (clone N418), anti-F4/80 (clone BM8) from eBioscience. After application with specific antibodies, cells were incubated for an additional 30 minutes, at 4°C in the dark, before being washed with PBS/ 1%BSA and fixed with 2% paraformaldehyde [8]. Cells were analyzed using a FACScan flow cytometer (BD Biosciences) and FlowJo software (v 10.0.7, Tree Star, Inc.).

Intracellular Cytokine Staining

After a single cell suspension was obtained, cells were counted and resuspended at 10^6 cells per ml in RPMI medium, containing 10% FBS and 1% penicillin/streptomycin. 50ng/ml PMA, 500ng/ml ionomycin, and 10μg/ml Brefeldin A were added to each sample, and then incubated for 4-6 hours at 37°C. Cells were then harvested and washed with PBS/ 1% BSA. Cells were stained for extracellular markers, then fixed with 4% formaldehyde in hypertonic

PBS. Cells were then permeabilized and stained for intracellular cytokines by applying a 2x concentrated anti-cytokine antibody in saponin buffer (PBS/ 1%BSA/ 0.5% Saponin). After 30 minutes at room temperature, cells were washed in saponin buffer, and then resuspended in PBS/BSA and analyzed on a FACScan flow cytometer (BD Biosciences) and using FlowJo software (v 10.0.7, Tree Star, Inc.).

Statistics

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, unless otherwise noted. Significance between two groups was evaluated using a two tailed Student's t-test. All statistics listed are reported as mean ±SEM. Statistical analyses were performed using Graph Pad InStat 3 (GraphPad Software). $P< 0.05$ was considered statistically significant.

CHAPTER 3
RESULTS

Analysis of mucin 19 expression in an experimental mouse model of HMPV infection

In this work, an experimental mouse model based on the NFS/N.Cg-*Muc19*$^{tm2.1Culp}$/Mmucd strain was used because the muc19 deficient mice used in this work are of the same (NFS/N.Cg-*Muc19*$^{tm2.1Culp}$/Mmucd) background. Therefore, the protein expression of mucin 19 in the wild type animals in response to HMPV infection was first evaluated. For that purpose, Muc $19^{+/+}$ mice were infected with HMPV or PBS and then bronchoalveolar lavage (BAL) samples were collected at 12, 24, 48, and 72 hours post infection. BAL samples were analyzed for their muc19 protein content by ELISA assays. As shown in Figure 2A, protein levels showed a significant spike (741.4±150.4 ng/ml) of muc19 at 12 hours post infection, while levels slowly decreased out (53.0±11.2 ng/ml) to 72 hours post infection. In addition, a comparative analysis was performed of muc19 expression with other highly known respiratory mucins, mucin 5ac and mucin 5b, which have been called the major airway mucins, and are known for playing important roles in infection, chronic lung disease, as well as cancer [18, 41, 42, 47, 54]. In fact, muc5ac and muc5b are known to be induced by HMPV infection in the lungs of mice, as our group previously reported [7]. Thus, protein levels of muc5ac and muc5b were determined by specific ELISA assays, using the same BAL samples analyzed for muc19 quantification. Analysis of the data indicates that muc19 levels (741.4±150.4 ng/ml) were higher than those of both muc5ac (4.6±0.6 ng/ml) (Fig. 2B) and muc5b (5.6±0.8 ng/ml) (Fig. 2C).Comparative analysis (at the same scale) of the protein expression of muc19, muc5ac, and muc5b led me to determine the statistical differences among the 3 mucins, in which muc19 was

significantly higher at all time points measured (Fig. 2D). These novel data demonstrate that muc19 is the predominant mucin expressed in the lungs during infection with HMPV.

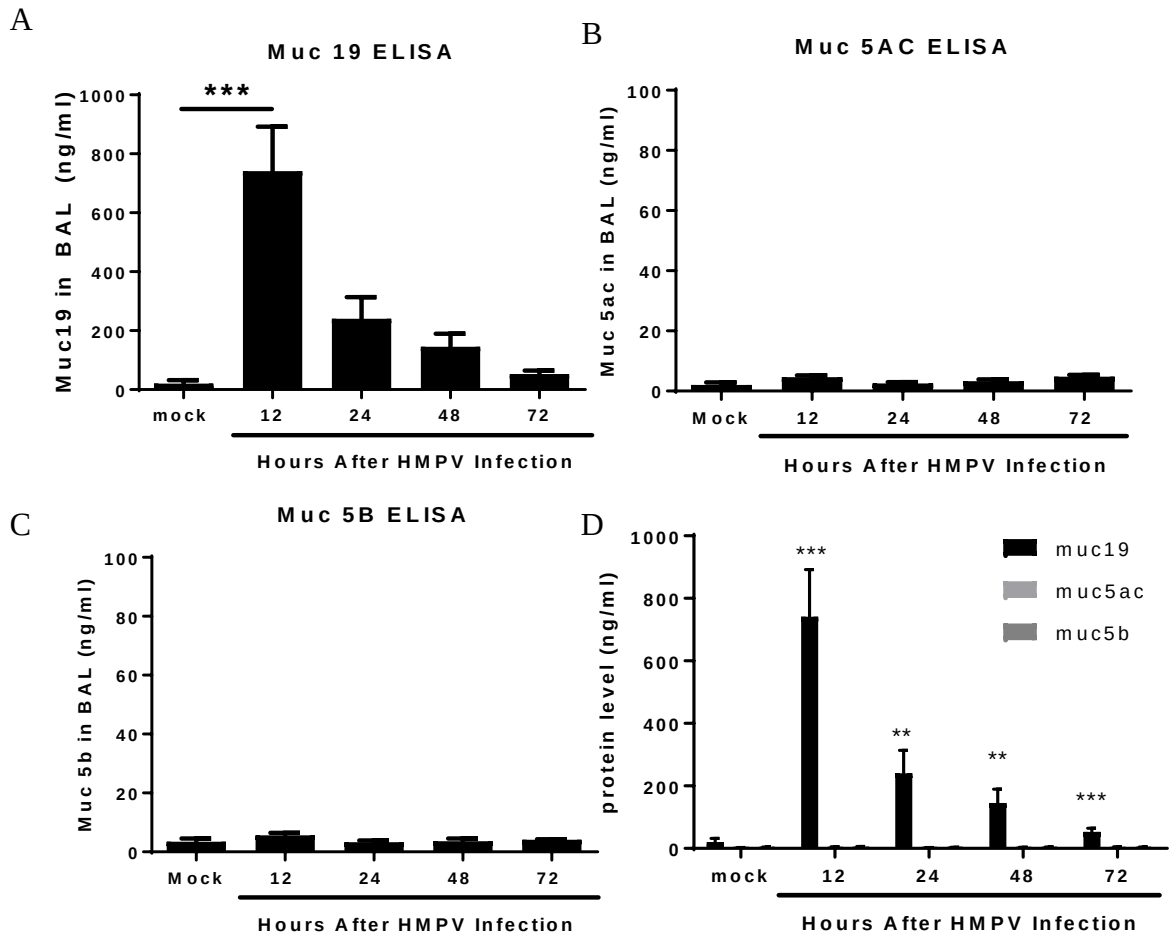

Figure 2. Muc19 is the predominant mucin expressed in the lungs during HMPV infection**.** Muc19$^{+/+}$mice were infected intranasally with HMPV. BAL samples were collected at 12, 24, 48, and 72h after infection. Expression of A) muc19; B) muc5ac; and C) muc5b was determined by ELISA assays. D) Comparative analysis of the expression of the 3 airway mucins. Data are representative of 2 separate experiments with similar results. (**P <0.01, *** P <0.001, ANOVA) (n= 7 mice)

Phenotypic characterization of HMPV infected muc19 deficient mice

Next, the role of mucin 19 was examined in some phenotypic disease parameters induced by HMPV infection, such as lung viral replication and inflammation. For lung viral titers, lung

tissue was collected at different time points. Infectious HMPV in the lung samples was determined by methylcellulose plaque assays. As shown in Figure 3A, the lack of muc19 led to a significantly lower viral titer at day 3 after infection when compared to wild type mice. To further determine the effect of muc19 in viral clearance, lungs were also analyzed 8 days after infection. However, by 8 days post infection, neither group had measurable plaques (Fig. 3B).

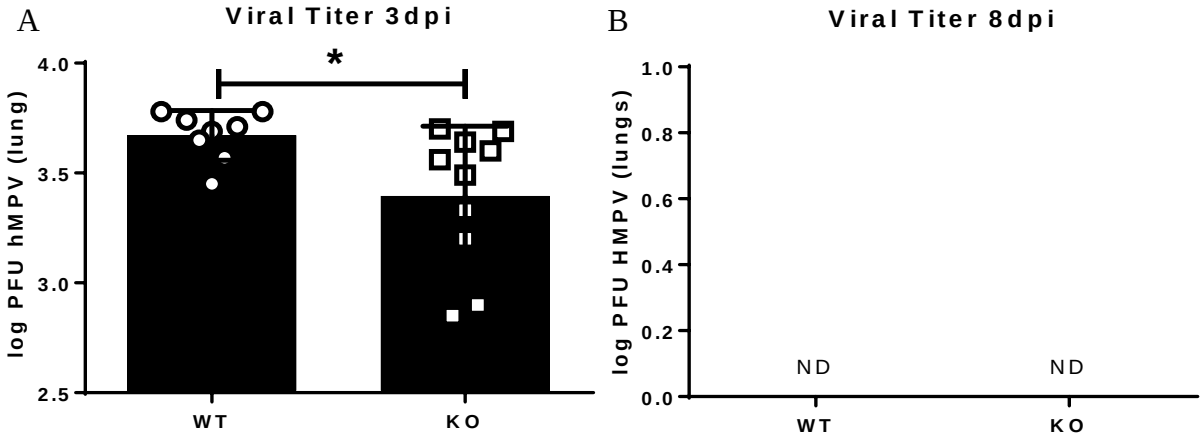

Figure 3. Lung viral replication. Lung tissue was collected at different time points to determine HMPV viral load within the lungs. A) At 3 days post infection, muc19$^{-/-}$ mice had a significantly lower viral titer, but B) by 8 days post infection, both mice had full clearance of virus. ND- not detected (*P<0.05) (n= 8-10 mice)

Although viral load is an objective assessment of infection, there have been several clinical reports saying that viral load of HMPV does not necessarily correlate with disease severity in human patients [36, 50]. Therefore, as an objective measure of lung disease, an assessment of lung inflammation at day 7 (when pulmonary inflammation peaks in mice) was determined [16]. Lungs were fixed with formalin, embedded in paraffin, stained with hematoxylin and eosin, and then evaluated by a veterinary pathologist. As shown in Figure 4,

there exists a significant amount of inflammation induced by HMPV infection when compared to the mock infected mice. However, the lack of mucin 19 does not appear to have an effect on lung pathology (Fig. 4).

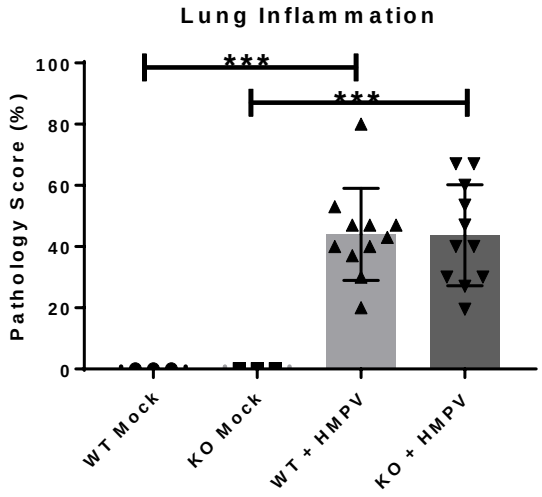

Figure 4. HMPV induced lung inflammation. H&E staining at 7 days post infection shows a significant amount of inflammation induced by HMPV infection compared to mock- infected mice, but muc19 does not affect inflammation. (***P <0.001, ANOVA) (mock= 3, n= 11 mice)

Lung cell infiltration analysis after 1 day of infection with HMPV

To further investigate the role of mucin 19 in HMPV induced immune response, the effect of muc19 on the innate cellular infiltrates of the lungs was analyzed. Based on the results observed in Figure 2 (that muc19 was expressed early in infection), lung samples were collected at day 1 after infection to look for differences in innate cells including neutrophils, macrophages, NK cells, and γδ T cells. Lung cell suspensions were stained with specific combinations of antibodies. Neutrophils were defined as Ly6G+/CD11b+; macrophages were identified as F4/80+/ CD11b+; NK cells were identified as DX5+; γδ T cells were identified as γδTCR+/ CD3e+. There was a significant increase in both neutrophils and macrophages between the mock

and infected animals (Fig. 5A-B). However, there was no outstanding differences in cellular content between the WT and KO animals infected with HMPV.

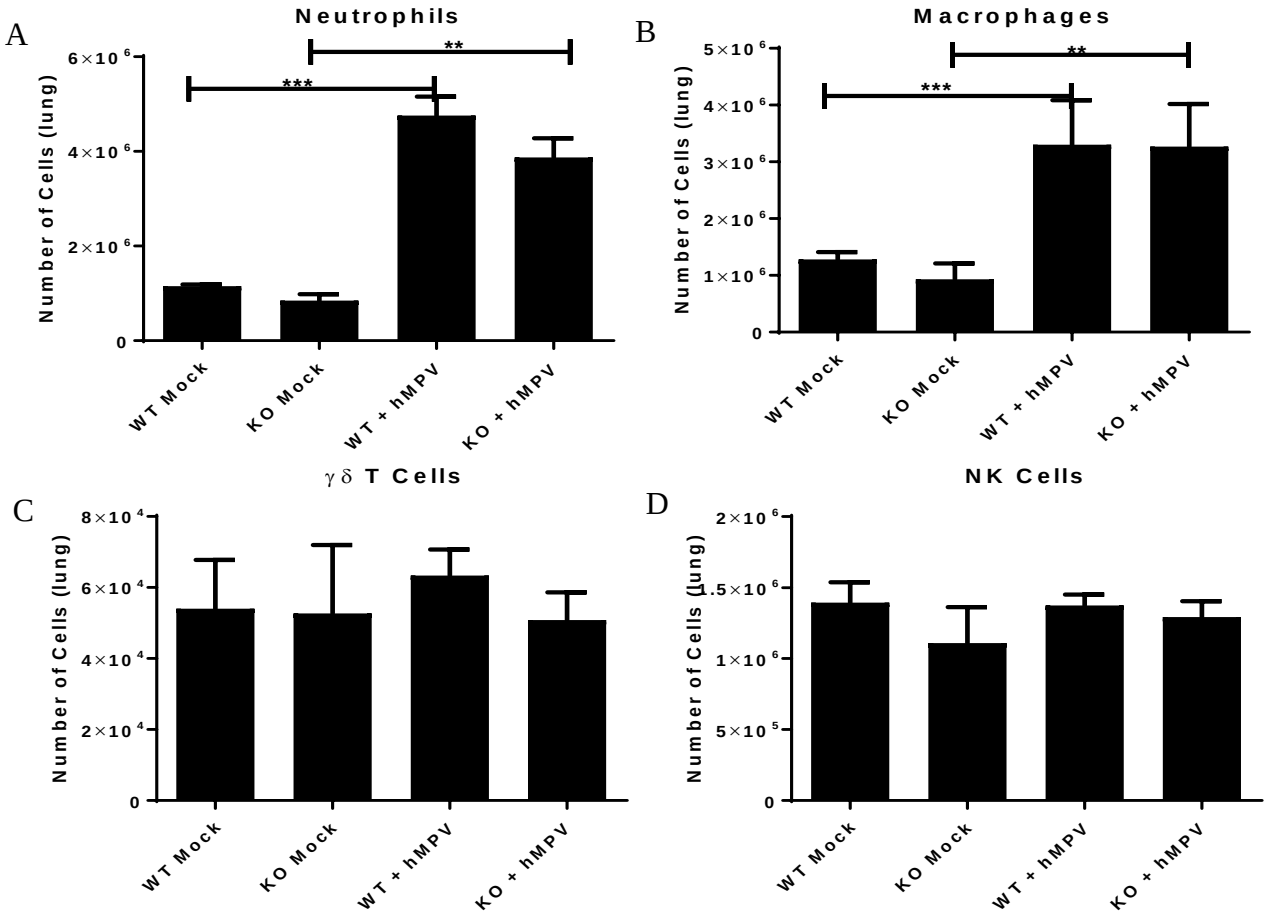

Figure 5. Day 1 cellular analysis of the lungs. Flow cytometry analysis of the lungs at day 1 show minimal differences between infected WT and KO mice. A) Neutrophils and B) macrophages are significantly upregulated by HMPV infection. However, there is very little increase in numbers between mock and HMPV infected animals for both C) γδ T cells and D) NK cells. (**P <0.01, ***P <0.001, ANOVA) (n= 9 mice)

Lack of mucin 19 alters the pulmonary CD4+ T cell response after HMPV infection

Although mucins are generally known for their role as protective barriers in innate immunity, there have been reports emerging about mucins' capabilities to regulate both the

number, as well as the type of T cell response [31, 34, 47]. Based on this notion, the T cell response was evaluated at day 7 after infection, when T cell recruitment is increased in the lungs of HMPV infected mice [27]. Lung cell suspensions were stained with specific combinations of antibodies. γδ T cells were identified as mentioned above; CD4+ and CD8+ T cells were identified as CD3e+/ CD4+ and CD3e+/ CD8a+, respectively. The results of these experiments indicate that there was a significant decrease in CD4+ T cells in the lungs of muc19 deficient mice after HMPV infection (Fig. 6A). However, that difference did not extend to either CD8+ T cells (Fig. 6B), or γδ T cells (Fig. 6C). These results suggest that muc19 alters specifically the CD4+ T cell response.

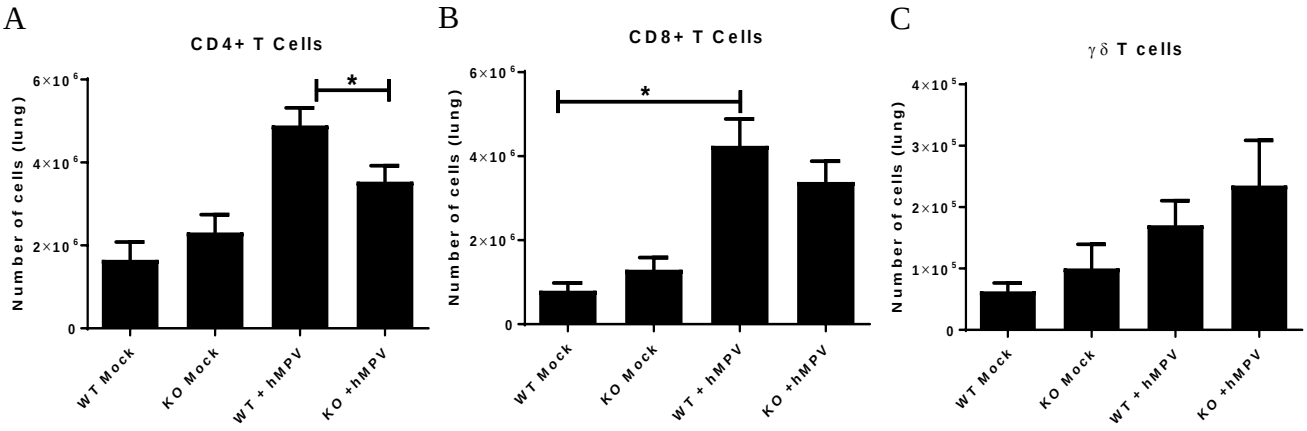

Figure 6. Muc19 alters CD4+ T cell response in lung of HMPV infected mice**.** 7 days after infection, lung suspensions were made to analyze the T cell compartment. A) HMPV infected KO mice show a significant decrease in the number of CD4+ T cells in the lungs. However, there is no difference between infected WT and KO mice in either B) CD8+ T cells or C) γδ T cells. (*P <0.05 ANOVA) (n=9-14 mice)

Phenotypic characterization of CD4+ T cell responses

Based on the observed altered T cell response, the CD4+ T cells induced during HMPV infection were further characterized. In order to evaluate that, the induction of Th1 (IFN-γ), Th2

(IL-4), and Th17 (IL-17) CD4+ T cell subsets in the lungs of infected mice were assessed. Lung tissue was collected at 7 days post infection. Once a lung single cell suspension was made, cells were stimulated with PMA, ionomycin, and Brefeldin A before staining with anti-CD3 and anti-CD4 antibodies. Cells were then fixed and permeabilized before staining with anti-IFN-γ for Th1, anti-IL-4 for Th2, and anti-IL-17a for Th17. Based on the experimental observations, no Th2 cells were induced during HMPV infection (data not shown). This finding is in line with previous observations that natural HMPV infection does not induce a Th2 response. Furthermore, although I consistently observed a significant decrease in the number of CD4+ T cells in the lungs of muc19$^{-/-}$mice (Fig. 6A), there was no difference in the Th1 subset (Fig. 7A) or Th17 subset (Fig. 7B) when comparing muc19$^{-/-}$ and muc19$^{+/+}$mice.

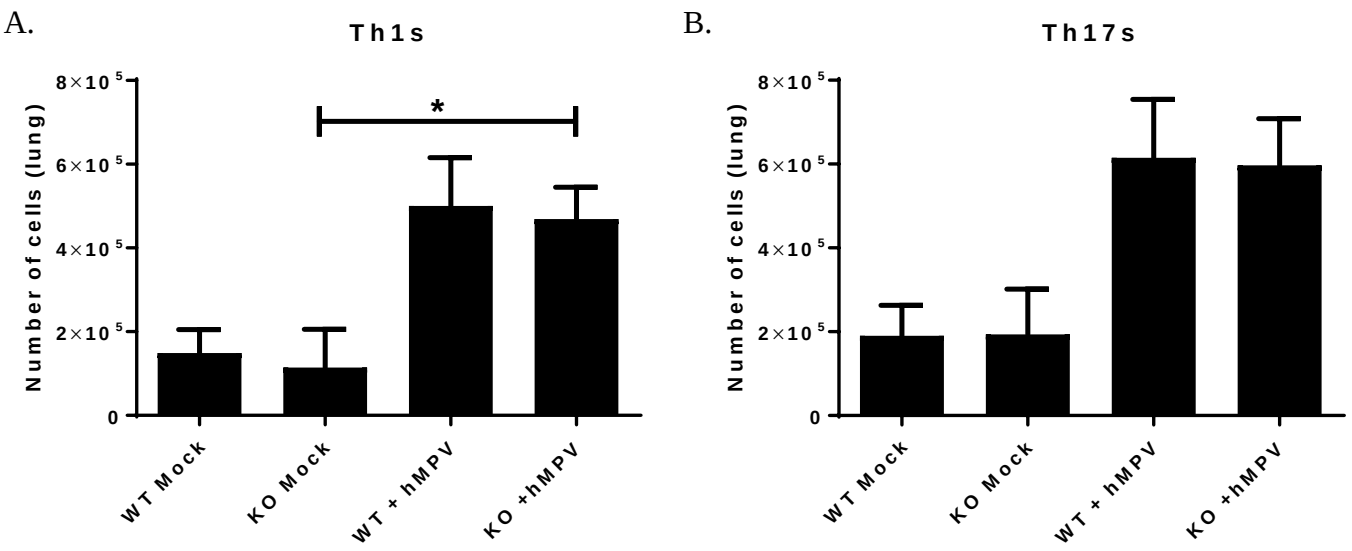

Figure 7. Intracellular cytokine staining to determine T cell subset. Lung suspensions from infected mice were stained with anti-IFN-γ or anti-IL17a antibody to determine T cell subset. At 7 days post infection there was no difference in A) Th1, or B) Th17 subsets in HMPV infected animals. (*P <0.05, ANOVA) (n= 9 mice)

Lack of mucin 19 decreases the LNs CD4+T cell response after HMPV infection

Based on the results found in lung tissue where CD4+ T cell numbers were decreased in muc19 deficient mice, additional studies on the T cell responses were done in draining lymph nodes (LN). Lung draining LNs were collected at day 7 after HMPV infection. Cell suspensions were stained as indicated in the methods to identify CD4+ and CD8+ T cells. Data shown in Figure 8 demonstrates that the lack of muc19 also altered the CD4+ T cell response in the LNs because WT infected mice had $2.22x10^6$ CD4+ T cells, while KO infected mice had only $1.06x10^6$ CD4+ T cells (Fig. 8A). This finding suggests that muc19 has a general effect on CD4+ T cell responses during HMPV infection. On the other hand, no differences were observed in the CD8+ T cells in the LNs (Fig. 8B), confirming the specific role of muc19 on CD4+ T cells, as observed in the lung (Fig. 6A).

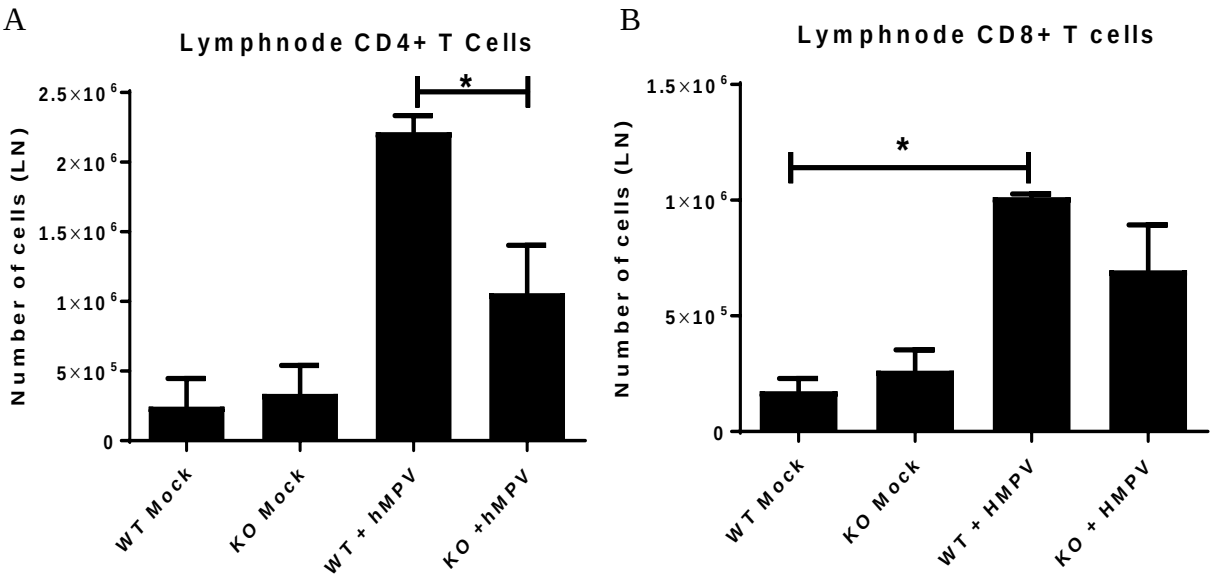

Figure 8. Muc19 decreases LN CD4+ T cell response. Lung draining lymph nodes were collected 7 days after infection to evaluate T cell response. A) Muc19 deficient mice infected with HMPV show significantly lowered CD4+T cell response, but not B) CD8+ T cell response in comparison to infected wild type animals. (*P <0.05, ANOVA) (n= 6-8 mice)

Lack of Mucin 19 increases pulmonary dendritic cell infiltration in HMPV infected mice

Because there was a consistent decrease in the numbers of CD4+ T cells induced by HMPV in muc19 KO mice in both lungs and LNs, it was speculated whether there might be some insufficiency in the antigen presenting cell compartment, leading to the lowered CD4+ T cell response. In order to test that, animals were infected intranasally with HMPV, then lungs were collected after 7 days of infection. Lung cell suspensions were stained with specific combinations of antibodies to analyze antigen presenting cells. Dendritic cells were defined as being $CD11c^{hi}$/MHC II^{hi}. Strikingly, a significant increase in dendritic cells were observed in the infected KO animals (Fig. 9).

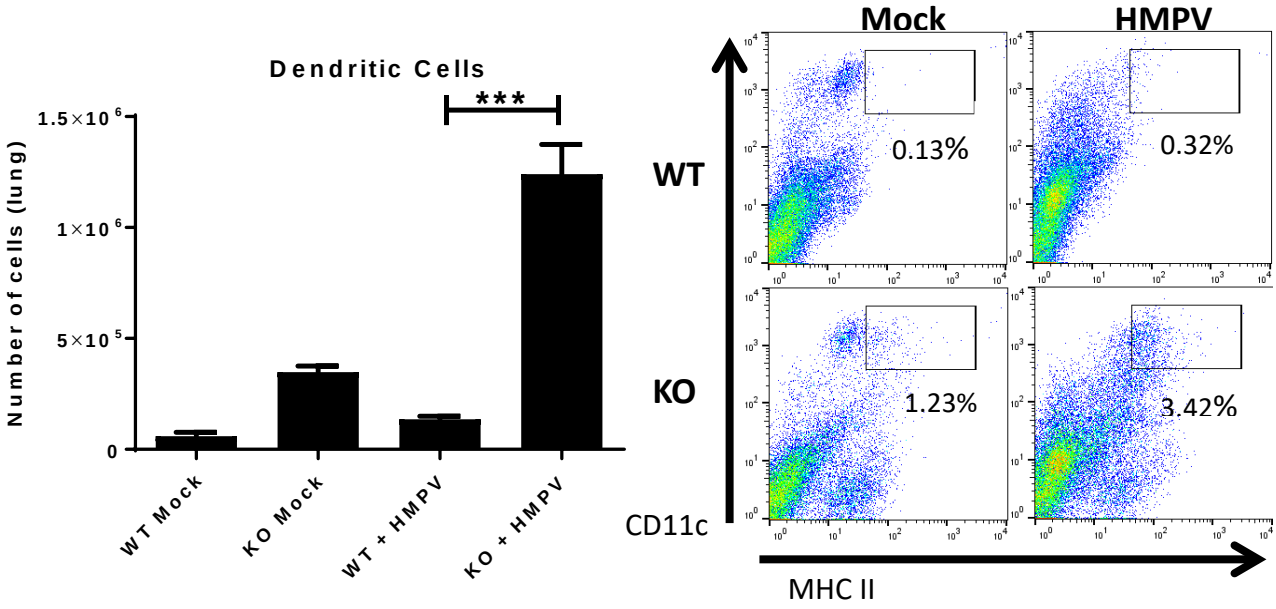

Figure 9. Lack of muc19 increases dendritic cell infiltration. Lungs were taken from HMPV infected $Muc19^{+/+}$ and Muc $19^{-/-}$ mice at 7 days post infection to look at antigen presenting cells. It was found that a lack of muc19 significantly upregulates dendritic cells. (***P <0.001, ANOVA) (n= 9 mice)

Macrophage infiltration analysis in the lungs of HMPV-infected mice

Because the increased numbers of dendritic cells in muc19 KO mice were so striking, the effect of muc19 on other antigen presenting cells, such as macrophages (F4/80+/ CDllb+) was also investigated. However, the difference in the number of macrophages between muc19$^{+/+}$and muc19$^{-/-}$ was unremarkable (Fig. 10). These results suggest that muc19 preferentially alters dendritic cells.

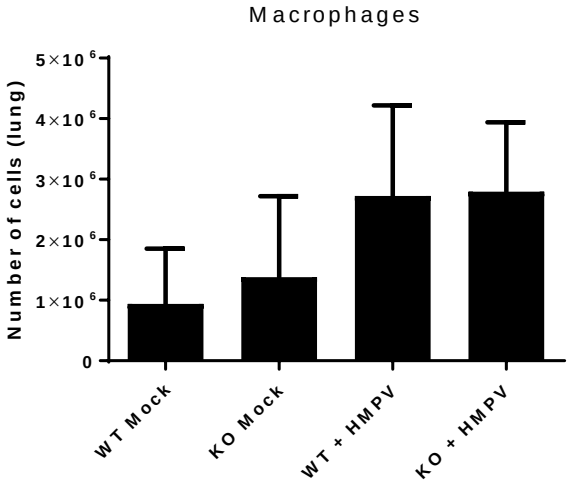

Figure 10. Muc19 has no effect on macrophages. Lungs taken from muc19 WT and KO mice 7 days after infection demonstrate that muc19 does not alter macrophages. ($P > 0.05$, ANOVA) (n=9 mice)

Impaired maturation in dendritic cells from muc19 KO mice infected with HMPV

While interesting, the dendritic cell finding was unexpected because in normal cases an upregulation in antigen presenting cells would lead to more T cell proliferation and activation, not less. This finding warranted a better characterization of the dendritic cell population in order to investigate their potential role in the observed diminished CD4+ T cell response found in muc19 deficient mice. To determine the activation and maturation of the dendritic cells, lungs

were collected at 7 days post infection. Lung cell suspensions were stained for dendritic cells as before. Additionally, cells were also stained with anti-CD80 or anti-CD86. In order to determine maturation state, dendritic cells were again gated with CD11c and MHC II, and then the mean fluorescent intensity (MFI) of the costimulatory molecules (CD80/86) was determined. The results showed that the MFI of CD80 was reduced by over 60% in the KO animals infected with HMPV (Fig. 11A). There was also a decrease in CD86 (Fig. 11B), as well as MHC II (Fig. 11C).

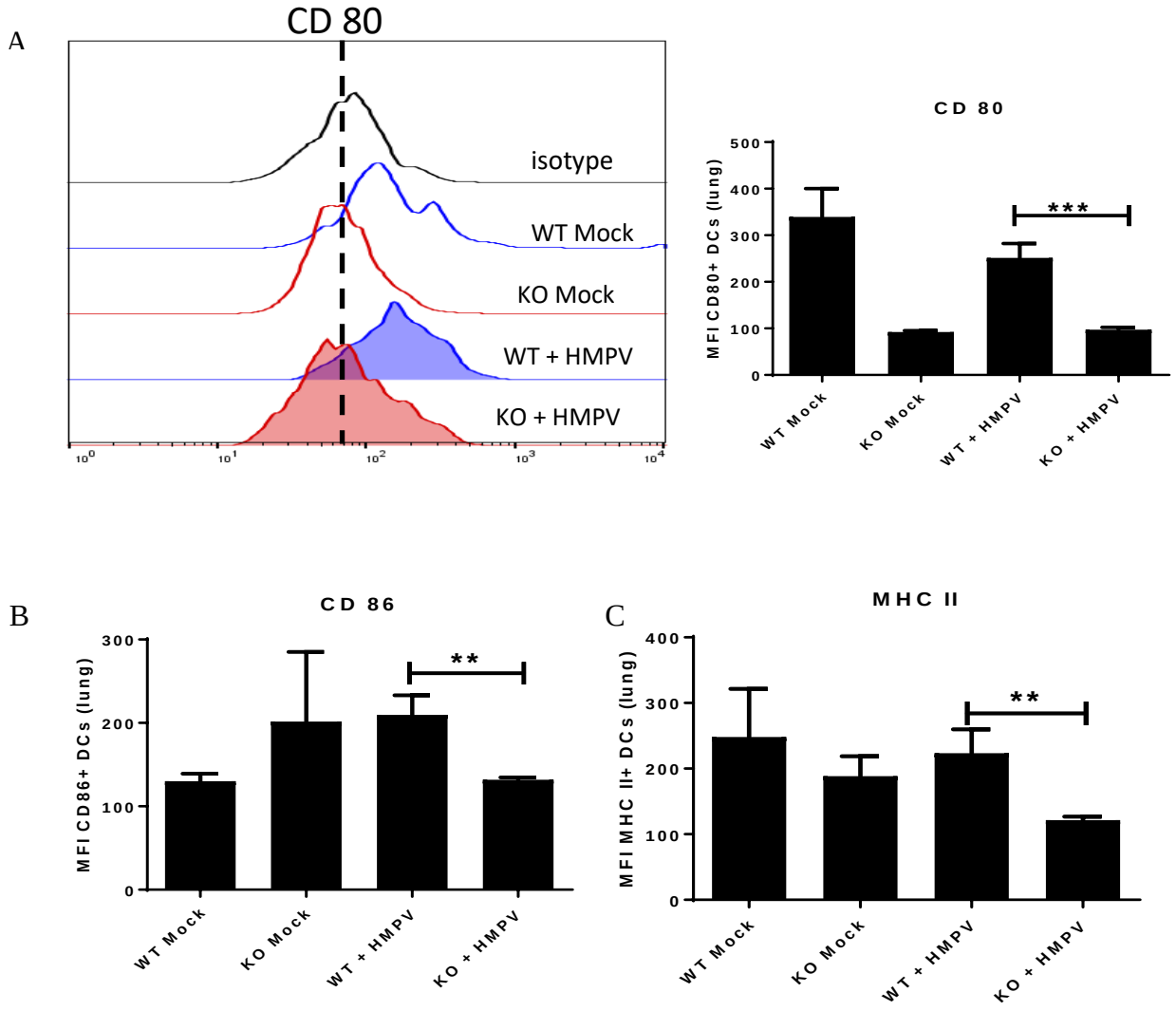

Figure 11. Dendritic cell characterization. Dendritic cells were analyzed 7 days after infection. DCs from Muc $19^{-/-}$ mice had less co-stimulatory molecules: A) histogram and mean fluorescent intensity (MFI) analysis for CD80 and B) MFI analysis for CD 86, C) as well as MFI analysis for MHC II expressed on their surface. (**P <0.01, ***P <0.001, student's t-test) (n= 6 mice)

These results suggest that the lung dendritic cells in the muc19 KO animals remain in an immature state. This finding led me to question whether the dendritic cells in the peripheral lymphoid organs also remained in an immature state. In order to test that, spleens were taken as a surrogate for secondary lymphoid organs and made into single cell suspensions. A characterization of the dendritic cells in the spleen was performed as it was in the lungs. This experiment yielded similar results to what was observed in the lungs, suggesting that splenic dendritic cells also contained less CD80 and MHC II (Fig. 12A-B). These data propose that the muc19 KO mice have dendritic cells that remain in an immature state.

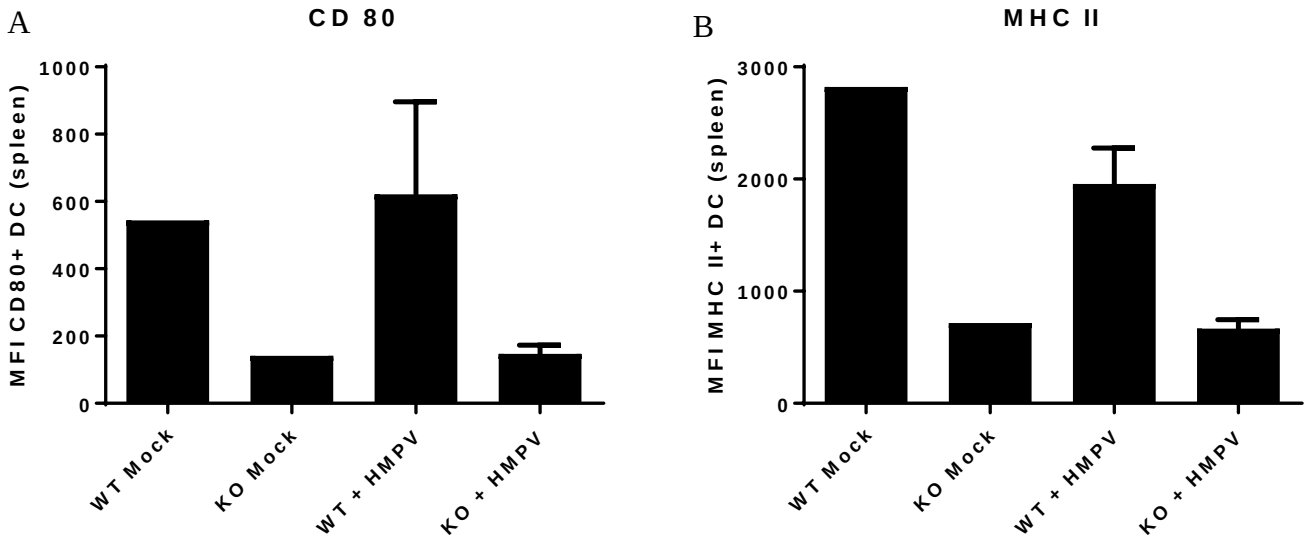

Figure 12. Splenic dendritic cell analysis. Spleens were harvested from HMPV infected mice after 7 days. It was found that splenic dendritic cells from the muc19 deficient mice had lower amounts of both A) CD80 and B) MHC II expressed on their surface. (Statistical analysis was not run due to small sample size) **(**mock=1, n=3 mice)

CHAPTER 4
DISCUSSION

Human Metapneumovirus (HMPV) remains one of the most common viral causes of acute respiratory tract infections, especially in children, elderly, and immunocompromised populations. Clinical symptoms can range from mild respiratory disease to severe bronchiolitis and pneumonia, some of which require hospitalization [19-20]. Reinfection in infants is common and thought to be partially due to their immunologic immaturity, however older children as well as adults can be reinfected [13]. Recent studies have shown that the immune system can build a robust response to clear the virus, although there is almost no lasting immunity or protective memory response [15]. Ongoing basic immunology research of HMPV is required in order for us to make effective treatments against it.

Because the lungs are one of the largest mucosal surfaces in the human body that contain mucus with a vast variety of mucin proteins, it is imperative that we study the role of these various mucins, both in normal functioning lungs, as well as injured and infected lungs. This work demonstrates that not only is mucin 19 expressed in the lungs, but it is also expressed at over 100 times that of other mucins (muc5ac and muc5b) with previously known importance in the lungs (Fig. 2). These findings led us to speculate that muc19 plays a role in HMPV induced pathogenesis. To test this idea, a muc19 deficient mouse model of infection was used, comparing $muc19^{+/+}$ (WT) and $muc19^{-/-}$ (KO) mice.

One of the novel findings in this work is the reduced CD4+ T cell response, both in the lungs and the LNs of muc19 KO mice. Mucus has been classically known as a protective layer and lubricant in the respiratory, gastrointestinal, and urogenital tracts, and other than causing difficulty for those with chronic lung infections, like asthma and cystic fibrosis, mucus had no

other known roles [21]. More recently though it has been shown that mucins can actually regulate and cause changes within the adaptive immune system. A report from 2005 showed that mucin 1 (MUC1) was actually expressed on the surface of human CD3+ T cells and inhibited their proliferation [2]. Another report from 2011 stated that muc1 was expressed on T-regulatory cells and could provide co-stimulatory or co-inhibitory signals based on the cell ratios present [28]. More in line with our findings, a study of colitis with a muc2 deficient mouse shows that a particular subset of DCs induces a robust Th17 response, which leads to disease severity in those mice lacking mucin 2 [49]. Overall, these scientific evidences demonstrate that mucins can regulate the adaptive immune response.

Because the idea of a largely uncharacterized mucin, such as muc19, altering the adaptive immune response is such a novel idea, we decided to look further into the regulatory mechanism of mucin 19 and that was where we found one of the most exciting results of this work. At 7 days post infection, it was found that the muc19 KO mice had a striking increase in pulmonary dendritic cells (DCs) (Fig. 9). Upon initial finding, these results were rather intriguing because under normal circumstances more antigen presenting cells (DCs) should yield a larger CD4+ T cell response. Since these findings were contrary to the expected ones, a series of characterization experiments on the dendritic cells to determine their level of activation and maturation state were performed. Upon infection, immature DCs are activated and release several cytokines (IL-1, IL-6, IL-12), and overexpress co-stimulatory molecules (CD80, CD86) as well as major histocompatibility complex (MHC) molecules class I and II, in a process known as maturation. That process allows the mature DCs to migrate to the peripheral lymphoid organs and present antigen to T cells [17]. T cell activation occurs at an immunological synapse. Proper formation of an immunological synapse between DCs and CD4+ T cells requires MHC II on the

antigen presenting cell to interact with the T cell receptor (TCR), as well as co-stimulatory molecules to bind with their specific ligands. While there are a variety of other molecules, such as integrins, that stabilize the synapse positionally, without MHC molecules, TCRs, or co-stimulatory molecules there would be no activation of T cells [39]. Interestingly, these results demonstrate that the muc19 KO mice had more DCs in the lung, but those cells expressed lower co-stimulatory molecules (CD80, and CD86), as well as MHC II molecules (Fig. 11). So while the muc19 KO animals have a much larger number of DCs, those cells appear less suitable for antigen presentation, suggesting an immature state of the DCs found in the muc19$^{-/-}$ mice.

One of the DC subsets described in the literature is tolerogenic DCs (tol-DCs), which are characterized for expressing an immature phenotype. Tol-DCs are essential for immune system regulation. Conventional dendritic cells are known for their abilities to establish a highly inflammatory state by activating lymphocytes, as well as secretion of pro-inflammatory cytokines. Alternatively, these tol-DCs are known for being in an immature state, with low levels of co-stimulatory molecules such as CD80 and CD86 [53]. Tol-DCs are important for maintenance of self-tolerance and regulate against autoimmunity. However, while the autoimmunity front is bounding with new information in this field, there is almost no knowledge of tol-DCs and their roles in infection. That makes the current observations on muc19 and tol-DCs highly relevant, because they identify tol-DCs as potential players in a pathogen-induced immunity. Over the years there have been new and better ways to generate tolerogenic DCs, which allowed for better understanding of these cells [32]. In the last decade, the field has grown so much that there are now clinical trials exploring the effectiveness of tolerogenic dendritic cell treatments for type 1 diabetes, multiple sclerosis, rheumatoid arthritis, Crohn’s disease, some cancers and even organ/ graft transplant rejection [37]. The original theory was that tolerance

was only induced because these cells possessed low levels of MHC and co-stimulatory factors, thus rendering them unable to present antigen. Although, it has been determined that microenvironment, as well as cytokines and growth factors all play a role in the induction of tolerance [12, 26, 45]. The main way tol-DCs induce immune tolerance is through altering T cell activation, though this can be done in a number of ways. The characteristic traits of having low MHC and co-stimulatory molecules can prompt T cell anergy, which might explain the current findings in my muc19 model. Another mechanism involves the upregulation of inhibitory factors, like programmed cell death ligand-1 (PDL-1), which bind to their receptors on T cells, and cause cell death. However, probably the most studied mechanism is the ability of tol-DCs to secrete regulatory cytokines IL-10 and TGFβ, which induce activation of T regulatory cells (Tregs) that can suppress primed T cells and skew the response toward a less active, non-inflammatory one [12,14]. Tregs that are induced by tol-DCs are known to be especially good at impairing inflammatory responses by reducing effector T cell responses, controlling neutrophil migration, and secreting suppressive cytokines (IL-10, TGFβ, IL-35, IL-27) [32].

A potential mechanism for the phenomenon found in this study is that in normal situations HMPV infection upregulates production of mucin 19, which in turns regulates dendritic cell maturation. Once the dendritic cells have matured, they migrate to the lymphoid tissues, where they present antigen, thus activating T cells. However, lack of mucin 19 likely induces tol-DCs, which have downregulated co-stimulatory molecules (Fig. 13). These immature dendritic cells would not efficiently migrate to lymphoid organs and would not activate T cells, and cause an immune tolerance, rather than a highly inflammatory state.

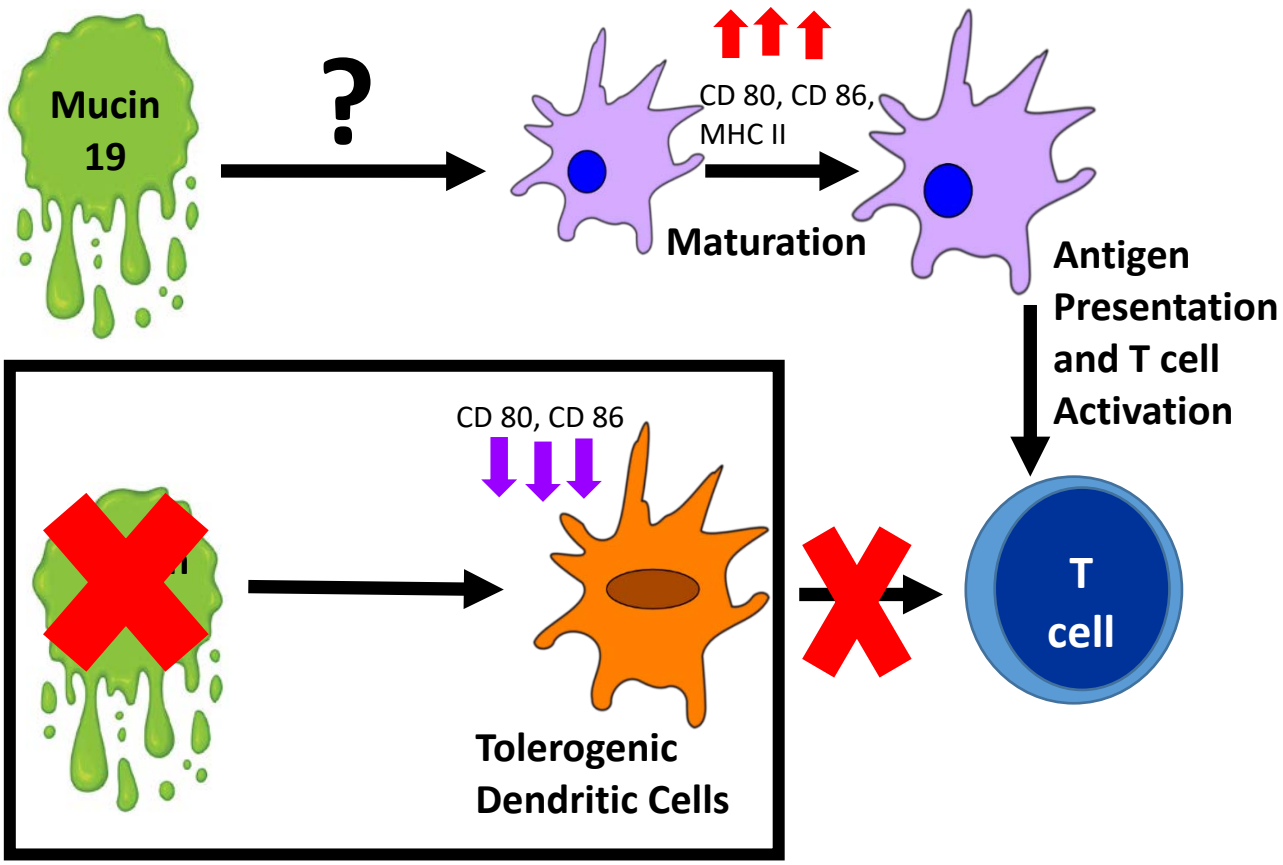

Figure 13. Potential model for observed decrease in CD4+ T cells. Mucin 19 regulates dendritic cell maturation through an undetermined mechanism, which allows them to present antigen and activate T cells. Lack of mucin 19 induces tolerogenic dendritic cells.

In conclusion, this thesis project studying the role of mucin 19 in an HMPV model of infection yields three novel findings: 1) It demonstrates that mucin 19 is a predominant mucin within the lung and does have secondary functions, 2) It reveals a positive role of mucin 19 on the CD4+T cell response, and 3) It shows that a lack of mucin 19 induces a population of tol-DCs, which could possibly play a role in reinfection or other subsequent respiratory infections. These highly relevant data open up new avenues of study to help better understand the intricacies of the immune response within the respiratory tract, not only for HMPV, but also for other significant respiratory pathogens.

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VITA

Kaitlin Elise McBride was born in Irving, Texas in 1992. Soon after her birth, her family moved back home to southeast Texas, where she could be surrounded by her large extended family. She graduated *Magna Cum Laude,* with a distinguished diploma from Port Neches-Groves High School in 2010. After graduation, she moved to Waco, TX to attend Baylor University. She enjoyed her four years at Baylor, and earned a Bachelor's of Science degree, majoring in Biochemistry, and minoring in Biology. After graduation, she moved back home and worked at a veterinary clinic to gain experience for her longtime goal of becoming a veterinarian. In the fall of 2016 she started her master's program at Louisiana State University in the Department of Pathobiological Sciences, where she is studying the role of mucin 19 in the respiratory tract. After completion of her master's work, she will begin her Doctor of Veterinary Medicine degree at Auburn University's College of Veterinary Medicine in Auburn, AL.