Murine Cytomegalovirus Influences Foxj1 Expression, Ciliogenesis, and Mucus Plugging in Mice with Allergic Airway Disease

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We have followed throughout time the development of allergic airway disease (AAD) in both uninfected mice and mice infected intranasally with murine cytomegalovirus (MCMV). Histological evaluation of lung tissue from uninfected mice with AAD demonstrated mucus plugging after 14 and 21 days of ovalbumin-aerosol challenge, with resolution of mucus plugging occurring by 42 days. In MCMV/AAD mice, mucus plugging was observed after 7 days of ovalbumin-aerosol challenge and remained present at 42 days. The level of interleukin-13 in bronchoalveolar lavage fluid from MCMV/AAD mice was decreased compared with AAD mice and was accompanied by increased levels of interferon- γ . Levels of Muc5A/C, Muc5B, or Muc2 mucin mRNA in the lungs of MCMV/AAD mice were not elevated compared with AAD mice. MCMV was able to infect the airway epithelium, resulting in decreased expression of Foxj1, a transcription factor critical for ciliogenesis, and a loss of ciliated epithelial cells. In addition, an increase in the number of epithelial cells staining positive for periodic acid-Schiff was observed in MCMV/AAD airways. Together, these findings suggest that MCMV infection of the airway epithelium enhances goblet cell metaplasia and diminishes efficient mucociliary clearance in mice with AAD, resulting in increased mucus plugging. (Am J Pathol 2008, 172:714–724; DOI: 10.2353/ajpath.2008.070462)

Asthma is an inflammatory disease of the lower airways, characterized by airflow obstruction, airway hyperreactivity, and airway inflammation. Mucus hypersecretion can contribute to airway obstruction in asthmatic patients, particularly during periods of exacerbation of the disease.^{1,2} In fact, several studies have reported that

airway obstruction is a key feature in fatal asthma with occlusions being comprised of mucus, inflammatory cells, and shed epithelial cells.^{3–7} The pattern of these elements within the plug suggests that they represent multiple or ongoing events rather than a single inflammatory response and emphasize the chronic nature of asthma.⁷ In humans, mucus is produced by the submucosal glands and goblet cells. Under healthy conditions, goblet cells comprise a small percentage of the airway epithelium, but in asthma can increase in number to become 20 to 25% of the epithelial population in both central and peripheral airways.⁸ In contrast, submucosal glands in the mouse are few, surrounding only the tracheae, and a limited number of goblet cells are present under normal conditions; however, this animal model has proven to be a useful tool in understanding certain mechanisms involved in mucus hypersecretion and goblet cell hyperplasia. In several mouse models of asthma, interleukin (IL)-13 has been shown to be the crucial driving factor for the production of mucus.^{9–12} These findings have been supported by *in vitro* studies in human epithelial cells, where IL-13 has been shown to enhance a mucus hypersecretory phenotype by increasing goblet cell density.¹³

Viral respiratory infections have been closely associated with asthma, potentially resulting in three separate outcomes: causative, exacerbating, or preventative. Being prevalent during infancy, respiratory syncytial virus (RSV) infection is associated with the first episodes of wheezing in the majority of infants, causing airway inflammation and airway obstruction.^{14–17} However, by the age of 2 nearly all children have been infected with RSV, but not all children develop asthma. Thus, it is unclear whether RSV infection can initiate or cause asthma, placing some infants at risk for the development of the disease, or whether this is merely an association. In contrast, it is generally accepted that viral respiratory infections can lead to the exacerbation of asthma in individuals who

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have been diagnosed with the disease. Epidemiological studies have shown that viral respiratory infections, particularly rhinovirus, coincide with asthma attacks in 80 to 85% of school-age children and 44% of adults, resulting in decreased lung function and increased symptom scores.^{18,19} Finally, the idea that viral pathogens may protect individuals from the development of asthma is controversial and is based on the observation that larger families that experience more infections have a lower risk of developing asthma.^{20–23}

The mechanism by which viral respiratory pathogens influence asthma is unknown. It is clear, however, that respiratory viruses share a common ability to infect the airway epithelium. In this manner viruses can directly damage the epithelial barrier, allowing the passage of more irritants and allergens into the lung, as well as increase epithelial cell shedding.²⁴ Repair and regeneration of damaged airway epithelium is complex and several transcription factors important in ciliogenesis and lung morphology have been shown to play a role.²⁵ Furthermore, viral respiratory infections activate the host's immune system, triggering a nonspecific proinflammatory response that is necessary to control the viral infection and a virus-specific CD8⁺ T-cell response, resulting in the production of interferon (IFN)- γ and clearance of the virus. It is conceivable that such changes in the local cytokine environment, triggered by viral infection, may play an important role in influencing asthma.

Although RSV and rhinovirus are the primary viral respiratory pathogens associated with changes in asthma, other viruses can also influence this disease. For example, human cytomegalovirus, a β -herpesvirus, has been associated with exacerbations of asthma in adults.²⁶ Therefore, we have examined the ability of murine cytomegalovirus (MCMV), which shares many biological properties with human cytomegalovirus, to influence the ovalbumin (OVA)-induced murine model of allergic airway disease (AAD). Our initial studies demonstrate that MCMV infection enhanced mucus plugging in small airways during acute AAD.²⁷ In the present studies we found that enhanced mucus plugging in MCMV/AAD mice was observed during both acute and chronic airway disease, but was not associated with increased mucin gene expression or increased IL-13 expression as compared to AAD mice. However, MCMV infection decreased Foxj1 expression, a transcription factor important in ciliogenesis, and resulted in changes in the ciliary phenotype of the airway epithelium that may hinder efficient mucociliary transport, thereby enhancing mucus plugging.

Materials and Methods

Animals and Viruses

Seven-week-old female C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were provided sterile food and water, and housed in microisolators under specific pathogen-free conditions. Their welfare was in accordance with institutional and

Office of Laboratory Animal Welfare guidelines. All animal handling and manipulations were preapproved by the Animal Care Committee at the University of Connecticut Health Center.

MCMV strain K181, originally obtained from Dr. M.C. Jordan (Oregon Health Sciences University, Portland, OR), was commercially screened for the presence of other pathogens and scored negative. Recombinant MCMV, expressing the enhanced green fluorescent protein under the control of the immediate early 2 promoter, was a gift from Dr. John Hamilton (Duke University, Durham, NC).²⁸

OVA Study Protocol

OVA sensitization and aerosol challenge of C57BL/6J mice has been previously described for our model of AAD.²⁷ Briefly, mice were given three weekly intraperitoneal injections of a suspension containing 25 μ g of OVA (grade V; Sigma, St. Louis, MO) and 2 mg of aluminum hydroxide [Al(OH)₃] in 0.5 ml of saline. One week after the last injection, mice were exposed to 0.2% aerosolized OVA generated by a BANG nebulizer (CH Technologies, Inc., Westwood, NJ) for 1 hour per day, delivered in a nose-only inhalation exposure chamber with space for exposing 48 mice simultaneously (In-Tox Production, Moriarty, NM). The concentration of OVA-aerosol used in these experiments differed from the 1% OVA solution used in our previous studies²⁷ and was chosen to maximize differences in parameters between AAD and MCMV/AAD mice. This procedure was repeated daily for 1, 3, 7, 10, 14, 21, or 42 days, as noted in the text, and mice were sacrificed 24 hours after the last OVA-aerosol challenge. Two control groups are routinely included in our studies: naïve mice that received no treatment and OVA-sensitized mice that received three weekly injections of OVA/Al(OH)₃, but are not exposed to OVA-aerosol. No differences in measured outcomes have been noted between these two controls.

MCMV/AAD mice were sensitized and exposed to OVA-aerosol as described above with one addition. Viral infection was performed intranasally 1 week before the start of OVA-aerosol challenge, using 2×10^4 PFU of MCMV. Two control groups were included in these studies: MCMV/OVA-sensitized mice that received three weekly injections of OVA/Al(OH)₃ and were intranasally infected with MCMV, but were not exposed to OVA-aerosol and MCMV infected mice that were infected with MCMV, but not sensitized to OVA/Al(OH)₃ or exposed to OVA-aerosol. No differences in measured outcomes were noted between these two controls. At the time of sacrifice, confirmation of MCMV infection was determined by the presence of virus in the salivary gland using a standard plaque assay.

Analysis of Bronchoalveolar Lavage Fluid (BALF)

Twenty-four hours after the final OVA-aerosol challenge, the lungs from each animal were lavaged *in situ* with five 1-ml aliquots of sterile saline. The total number of viable

leukocytes was determined by trypan blue exclusion using a hemocytometer. Leukocyte differentials were determined by May-Grunwald/Giemsa staining of cytocentrifuged BALF preparations.

Measurement of Cytokines in BALF and Serum

BALF, collected after 1, 3, 7, 10, 14, 21, or 42 days of OVA-aerosol challenge, was concentrated 10-fold using a Centriplus YM-10 filtration device (Amicon, Beverly, MA), aliquoted, and stored at -80°C until use. The presence of IFN- γ (Pierce-Endogen, Rockford, IL) and IL-13 (R&D Systems, Minneapolis, MN) were assayed by enzyme-linked immunosorbent assay, testing an undilute and a 1:2 dilution of concentrated BALF. The limit of detection was 10 and 1.5 pg/ml for IFN- γ and IL-13, respectively.

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Fresh lung tissue was homogenized in 1 ml of Ultraspec RNA solution (Biotecx Laboratories, Houston, TX) and stored at -80°C until use. Total RNA was isolated by chloroform extraction and ethanol precipitation, according to the manufacturer's specifications, and used in RT-PCR assays. Five μg of total RNA was treated with 5 U of DNase I (Invitrogen, Carlsbad, CA) for 15 minutes at room temperature, and was then used to generate cDNA templates for PCR with the SuperScript First-Strand (Invitrogen) and random hexamers in 50- μl total volume. Reactions lacking the reverse transcriptase served as a control for the presence of genomic DNA. To determine the levels of mucin mRNA by PCR, 5 μl of cDNA was incubated with the following primers: Muc5A/C, 5'-TGACCTCAGGTATTTACAC-3' (sense) and 5'-TAGTTCTTAGCCCTGCATTGC-3' (antisense); Muc5B, 5'-GCACCTGCTGTCAGGAGAGCA-3' (sense) and 5'-TGGTCAGTTGTGCAGTTCTG-3' (antisense); and Muc2, 5'-TTTGGGAACATGCAGAAGATCAAC-3' (sense) and 5'-CGTGAGAGCACTGGCGCGAGA-3' (antisense). GAPDH primers [5'-ATGTTCCAGTATGACTCCACT-3' (sense) and 5'-CCACAATGCCAAGTTGTCAT-3' (antisense)] were added to each PCR reaction as an internal control. The following PCR conditions were implemented: 94°C for 3 minutes, 35 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 2 minutes, followed by a final extension at 72°C for 7 minutes. The lungs from three individual mice were examined at each time point and the resulting PCR products were resolved on a 2% agarose gel. The intensity of the mucin bands and GAPDH was determined using IPLab Gel software (BD Biosciences, Rockville, MD).

Real-Time PCR for Mucin and Foxj1 mRNA

Total RNA from lung tissue was treated with DNase I as described above. Real-time PCR was performed in duplicate for Muc5A/C, Muc5B, Gob-5, foxj1, and β -actin in a final volume of 25 μl containing 12.5 ng of total RNA

and the iScript one-step RT-PCR kit with SYBR Green (Bio-Rad, Hercules, CA) using an iCycler iQ real-time PCR detection system (Bio-Rad). The primers were: Muc5A/C, 5'-AAAGACACCAGTAGTCACTCAGCAA-3' (sense) and 5'-CCACATGGGGTCACACTTC-3' (antisense)²⁹; Muc5B, 5'-CATGCTCACAAAAGCAGATG-3' (sense) and 5'-CCAGGGTTTATTGCAGCTTA-3' (antisense); Gob-5, 5'-TCAGGCTGTGGATAAGTCC-3' (sense) and 5'-TATCTTCAGCACGTGGATGC-3' (antisense)³⁰; Foxj1, 5'-GAGTGAGGGCAAGAGACTGG-3' (sense) and 5'-TCAAGTCAGGCTGGAAGGTT-3' (antisense)³¹; and β -actin, 5'-GATGCCACAGGATTCCATA-3' (sense) and 5'-AGAGGGAAATCGTGC GTGAC-3' (antisense) at concentrations of 325 nmol/L, 400 nmol/L, 300 nmol/L, 150 nmol/L, and 220 nmol/L, respectively. The PCR conditions were as follows: 50°C for 10 minutes for the RT step, 95°C for 3.5 minutes followed by 40 cycles of 95°C for 10 seconds, then 58°C for 20 seconds. The efficiency of all PCR reactions was greater than 90% and the relative fold-increase of mucin, Gob-5, or Foxj1 was calculated using the C_T (threshold cycle) method as compared to the housekeeping gene β -actin.

Lung Histology and Immunohistochemistry

Unmanipulated, noninflated lungs were removed, fixed with 10% buffered formalin, and embedded in paraffin by conventional methods. Tissue sections were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) or Alcian blue (data not shown). Histological examination of lung tissue infected with recombinant MCMV was processed in an identical manner. For analysis of Foxj1 expression, noninflated lungs were fixed with fresh 4% paraformaldehyde in phosphate-buffered saline for 24 to 48 hours at 4°C and embedded in paraffin. Five- μm tissue sections were deparaffinized in toluene, then rehydrated through graded alcohol to water. Exogenous peroxidase was quenched by incubating the slides in 3% H_2O_2 in water for 15 minutes at room temperature. Antigen retrieval was performed by incubating the slides in citrate buffer, pH 6.0, at 95°C for 20 minutes, followed by cooling at room temperature for 20 minutes. The samples were incubated with Foxj1 antibody (1:4000; Upstate, Lake Placid, NY) in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin for 1 hour at room temperature, then washed in PBS. The secondary antibody, biotinylated anti-mouse IgG from the M.O.M kit (Vector Laboratories, Burlingame, CA), was used according to the manufacturer's instructions, then tissues were counterstained for 30 seconds with hematoxylin. In addition, the total number of cells, as well as the percentage of PAS-positive or Foxj1-positive cells, was determined as follows. Sequential lung tissue sections from the same AAD and MCMV/AAD mice, used for examination of Foxj1 expression above, were stained with PAS, followed by hematoxylin or Foxj1, followed by hematoxylin. The number of positive cells in airways that demonstrated PAS staining ($<150\ \mu\text{m}$ in diameter) was determined, examining at least four airways for each group and counting a total of six to eight sections with 50 μm of basement membrane.

Statistical Analysis

A statistical comparison between two groups at a single time point was calculated using an unpaired *t*-test. Analysis of multiple time points or experiments with three or more groups was performed using one-way analysis of variance, followed by Fisher's PLSD. All tests were performed using the software StatView 4.5 (Abacus Concepts, Inc., Berkeley, CA). A probability of $P < 0.05$ was considered significant. Data are presented as a mean $\pm$ SEM.

Results

MCMV Infects the Bronchial Epithelium

Although it is known that MCMV can infect the lungs of mice, infection of the airway epithelium has not been demonstrated. Thus, C57BL/6 mice were infected intranasally with a recombinant MCMV that expresses the enhanced green fluorescent protein. The mice were sacrificed after 7 days, when peak viral loads of MCMV were present in the lung.²⁷ Unmanipulated lung tissue was fixed in formalin and sequential sections stained with H&E (Figure 1A) or examined by phase (Figure 1B) and fluorescent (Figure 1C) microscopy. Fluorescent green staining was evident in the bronchial epithelium of airways less than 200 μm in diameter. No staining of the surrounding lung tissue, including the endothelium of blood vessel, was noted. Although MCMV is a lytic virus, no gross structural damage was evident on examination. This histological analysis demonstrated that MCMV can infect the bronchial epithelium.

Concomitant MCMV Infection Enhanced Mucus Production

We have previously shown that concomitant MCMV infection enhanced mucus plugging during the acute stage of AAD (after 7 days of OVA-aerosol challenge).²⁷ To determine the ability of MCMV to influence mucus plugging during the acute and chronic stages of disease, lung sections from uninflated, nonmanipulated lungs were isolated from AAD and MCMV/AAD mice after 1, 3, 7, 10, 14, 21, and 42 days of OVA-aerosol challenge and stained with PAS. As controls, lung sections from MCMV-infected and -uninfected naïve mice were also examined. Little to no PAS staining was observed in lung tissue from either AAD or MCMV/AAD mice on day 1 (not shown). Although the majority of airways from AAD and MCMV/AAD mice on day 3 did not stain with PAS, a limited number of airways displayed PAS-positive cells in both groups (Figure 2, A and B, respectively). By day 7, the number of PAS-stained airways increased in both AAD and MCMV/AAD mice (Figure 2, D and E, respectively), but mucus plugging of airways was evident only in MCMV/AAD mice. Extensive PAS staining of the airway epithelium, as well as mucus plugging, was present in both AAD and MCMV/AAD mice after 14 days (Figure 2, G and H) and 21 days (Figure 2, J and K) of OVA-aerosol challenge. By 42 days of OVA-aerosol challenge, only a few PAS-positive staining cells were observed in AAD mice and no mucus plugs were noted (Figure 2M). In contrast, mucus plugging was still evident in MCMV/AAD mice (Figure 2N). PAS staining was not present in lung tissue from naïve (Figure 2L) or MCMV mice corresponding to 3 (Figure 2C), 7 (Figure 2F), and 14 (Figure 2I) days of OVA-aerosol challenge (Figure 2, C, F, and I). Thus, concomitant MCMV infection in mice with AAD resulted in

Figure 1. MCMV can infect the bronchial epithelium. C57BL/6 mice were intranasally infected with a recombinant MCMV that expresses enhanced green fluorescent protein under the control of the immediate early 2 promoter. Seven days after infection, the mice were sacrificed and unmanipulated lungs were removed and fixed in formalin in preparation for histological evaluation. Images shown are from sequential lung tissue sections that were either stained with H&E (A) or not stained. The sections were observed under bright field (A), phase (B), or fluorescent (C) optics. The same tissue section is shown in B and C. Scale bar = 80 μm .

Figure 2. Histological evidence of mucus plugging in airways from AAD and MCMV/AAD mice. After 3 (**A, B**), 7 (**D, E**), 14 (**G, H**), 21 (**J, K**), or 42 (**M, N**) days of OVA-aerosol challenge, mice were sacrificed and unmanipulated lung tissue was processed for histological evaluation. Lung tissue from MCMV-infected mice corresponding to 3 (**C**), 7 (**F**), and 14 days (**I**), along with lung tissue from naïve mice (**L**), served as controls. PAS-stained lung sections from AAD, MCMV/AAD, MCMV, and naïve mice are shown here, with mucus plugs first observed in MCMV/AAD and AAD mice after 7 and 14 days of OVA-aerosol, respectively. Similar results were obtained with Alcian blue staining. Scale bar = 100 μ m.

Figure 3. A delay in airway inflammation in MCMV/AAD mice correlates with changes in Th1/Th2 cytokines. BALF was collected from AAD (**A**) and MCMV/AAD (**B**) mice after 1, 3, 7, 10, 14, 21, and 42 days of OVA-aerosol challenge by lavaging the lungs of each animal *in situ* with five 1-ml aliquots of sterile saline. The total number of viable leukocytes present in BALF was determined and differential leukocyte counts were performed on cytocentrifuged slide preparations stained with May-Grunwald/Giemsa. BALF was concentrated and assayed for the presence of IL-13 or IFN- γ by enzyme-linked immunosorbent assay. The results are expressed as the mean $\pm$ SE with a $n = 6$ for each time point of each group.

an earlier onset of mucus plugging (7 days of OVA-aerosol challenge) and prolonged mucus plugging (42 days of OVA-aerosol challenge).

IL-13 Production Was Decreased in MCMV/AAD Mice

IL-13 is a pleiotropic cytokine, produced by activated CD4⁺ Th2 cells, that has been associated with many features of asthma, including increased mucus production.^{9,10} To determine whether differences in IL-13 were associated with enhanced mucus plugging in MCMV/AAD mice, BALF was collected from AAD and MCMV/AAD mice from the same experiment and examined after 1, 3, 7, 10, 14, 21, and 42 days of OVA-aerosol challenge. As shown in Figure 3A, elevated levels of IL-13 were observed in BALF from AAD mice after 3 and 7 days of OVA-aerosol challenge. In contrast, the levels of IL-13 were reduced in BALF from MCMV/AAD mice after 3 days of OVA-aerosol challenge (Figure 3B, $P = 0.024$). As expected, increased levels of the Th1 cytokine IFN- γ were observed in MCMV/AAD mice after 1, 3, and 7 days of OVA-aerosol challenge. This increase in IFN- γ was in response to MCMV infection because IFN- γ levels in BALF from MCMV control mice peaked on day 0 (835.6 ± 231.6 pg/ml) and decreased thereafter (259.0 ± 25.6 pg/ml on day 3). IFN- γ was not detected in BALF from uninfected AAD mice (Figure 3A). Thus, concomitant MCMV infection altered the balance of Th1/Th2 cytokines in MCMV/AAD mice, dampening IL-13 production.

Examination of leukocytes collected from BALF from mice in the same experiment demonstrated that AAD mice developed airway inflammation and eosinophilia. As shown in Figure 3A, airway inflammation peaked in AAD mice after 7 days of OVA-aerosol challenge ($22.3 \pm 5.7 \times 10^5$ cells) and declined thereafter. The majority of these cells were eosinophils ($19.9 \pm 0.2 \times 10^5$ cells). In contrast, as shown in Figure 3B, peak airway inflammation and eosinophilia in MCMV/AAD mice was observed after 14 days of OVA-aerosol challenge ($27.6 \pm 7.6 \times 10^5$ leukocytes and $19.9 \pm 7.1 \times 10^5$ eosinophils). This delay in airway inflammation in MCMV/AAD mice may be attributable to changes in Th1/Th2 cytokine profiles noted above.

Expression of Mucin mRNA in Lung Tissue from AAD and MCMV/AAD Mice

Next, we determined if changes in mucin gene expression between AAD and MCMV/AAD mice could account for enhanced mucus plugging in MCMV/AAD airways. Using RT-PCR analysis, we screened for changes in mRNA levels of Muc5AC (Figure 4A), Muc5B (Figure 4B), and Muc2 (Figure 4C), the three lung mucin genes. Lung tissue from three individual mice were collected at each time point from AAD and MCMV/AAD mice after 1, 3, 7, 10, 14, 21, and 42 days of OVA-aerosol challenge. Lung tissue from naïve and MCMV-infected mice served as controls. The PCR products were resolved on an agarose gel and, to aid in the interpretation of the data, the intensity of each mucin band relative to GAPDH was determined (Figure 4, D–F). Elevated levels of Muc5AC mRNA were observed in lungs from both AAD and MCMV/AAD mice; however, expression of Muc5AC mRNA was detected earlier in AAD mice, after 7 days of OVA-aerosol challenge (Figure 4, A and D). Expression of Muc5B mRNA was consistent throughout time in AAD mice, whereas the levels of Muc5B mRNA in MCMV/AAD mice were more variable (Figure 4, B and E). Finally, no changes were observed in Muc2 mRNA between AAD and MCMV/AAD mice throughout the time course (Figure 4, C and F).

To confirm these results, real-time PCR analysis for Muc5AC and Muc5B were performed on AAD and MCMV/AAD mice after 3 and 7 days of OVA-aerosol challenge. These time points were chosen because mucus plugging was observed in MCMV/AAD mice, but not AAD mice, after 7 days of OVA-aerosol challenge. As shown in Figure 5A, after 3 days of OVA-aerosol challenge, elevated levels of Muc5AC mRNA were observed in the lungs of AAD mice as compared to naïve mice ($P < 0.05$). After 7 days, a marked increase in Muc5AC mRNA expression (>600 -fold) was observed in AAD mice as compared to naïve controls ($P < 0.0001$). Gob-5, a member of the calcium-activated chloride channel family, has been shown to be associated with the induction of Muc5AC expression.³² In our model, increased expression of Muc5AC in AAD mice was accompanied by a significant increase in Gob5 mRNA after both 3 and 7

days of OVA-aerosol challenge (Figure 5B; $P < 0.0001$ compared to naïve mice). A similar pattern of Muc5AC (>60 -fold) and Gob5 (~ 200 -fold) mRNA expression was also noted in lung tissue from MCMV/AAD mice, although the magnitude of the response even after 7 days of OVA-aerosol challenge was not as striking. Finally, no increase in the expression of Muc5B was observed in AAD or MCMV/AAD mice as compared to controls after 7 days of OVA-aerosol challenge (Figure 5C). Thus, the differences in mucus plugging observed in the lungs from MCMV/AAD mice as compared to AAD mice cannot be explained by increased Muc5AC, Muc5B, or Muc2 mRNA expression.

MCMV Infection Inhibits Foxj1 Expression and Alters the Ciliated Epithelial Cell Phenotype

Respiratory viral infections have been shown to influence mucociliary clearance of bacteria particles by decreasing Foxj1 expression, a transcription factor necessary for ciliogenesis.²⁵ Therefore, we investigated the influence of MCMV infection on Foxj1 expression in the airway epithelium. As shown in Figure 6, the levels of Foxj1 mRNA in lung tissue from AAD mice after 3 days of OVA-aerosol challenge were not significantly different as compared to naïve mice ($P = 0.27$). In contrast, both MCMV and MCMV/AAD mice displayed a significant reduction in Foxj1 mRNA ($P = 0.02$). To determine whether the decrease in Foxj1 mRNA expression, associated with MCMV infection, correlated with a decrease in Foxj1 protein, lung tissue from AAD and MCMV/AAD mice was examined by immunohistochemical staining for Foxj1 expression. As shown in Figure 7, Foxj1 expression (brown nuclear stain) was present in the airway epithelium of AAD mice, with staining evident in ~ 40 to 50% of the cells (Figure 7A). As evident on higher magnification, Foxj1-positive cells from both AAD and MCMV/AAD displayed a ciliated phenotype (Figure 7B). In comparison, Foxj1 expression appeared to be reduced in the airways of MCMV/AAD mice because the majority of epithelial cells stained positive with hematoxylin, not for Foxj1 (Figure 7C).

To confirm these findings, quantification of the percentage of Foxj1-positive cells in the airways of the AAD and MCMV/AAD mice, examined in Figure 7, was performed (Figure 8). We previously observed that after 3 days of OVA-aerosol challenge, histological evidence of disease (ie, inflammation or PAS staining) appeared limited in both groups of mice (Figure 2, A and B). Therefore, we focused on PAS-stained airways as an indication of

Figure 4. Expression of Muc5AC, Muc5B, and Muc2 mRNAs in the lungs of AAD and MCMV/AAD mice throughout time. Total RNA was isolated from the lungs of AAD and MCMV/AAD mice after 1, 3, 7, 10, 14, 21, and 42 days of OVA-aerosol challenge. RNA from the lungs of naïve and MCMV-infected mice, corresponding to day 0 and day 7, served as controls. RT-PCR analysis of Muc5AC (A), Muc5B (B), and Muc2 (C), along with GAPDH was performed for three mice at each time point, with a representative profile being shown. The intensity of the mucin and GAPDH bands was determined and expressed as a ratio of Muc5AC/GAPDH (D), Muc5B/GAPDH (E), and Muc2/GAPDH (F), with AAD and MCMV/AAD represented as solid and open circles, respectively.

Figure 5. Muc5AC and Gob5 mRNA expression was significantly elevated in AAD as compared to MCMV/AAD mice. Total RNA was isolated from lung tissue from AAD and MCMV/AAD mice after 3 (gray bars) and 7 (black bars) days of OVA-aerosol challenge, with lung tissue from naïve and MCMV-infected mice serving as controls. Quantitative analysis of Muc5AC, Gob5, and Muc5B mRNA expression was determined and normalized to GAPDH mRNA using real-time PCR. The Muc5AC, Gob5, and Muc5B gene expressions in AAD, MCMV/AAD, and MCMV-infected mice were expressed as fold increase compared with untreated naïve mice. Each bar represents the mean $\pm$ SE, with $n = 5$ for each group. * $P < 0.0001$ as compared to all other groups. † $P < 0.05$ as compared to naïve. N.D., not done.

disease, staining sequential lung tissue sections for Foxj1 expression or PAS. No changes in the total number of epithelial cells in airways from AAD or MCMV/AAD mice were observed, with 11.9 ± 0.4 and 12.7 ± 0.5 cells per $50 \mu\text{m}$ of basement membrane, respectively. In contrast, the percentage of Foxj1-positive cells in these airways decreased significantly from 57.4% in AAD mice to 46.2% in MCMV/AAD mice (Figure 8, $P = 0.005$). Furthermore, the percentage of PAS-positive cells increased from 31.4% in AAD mice to 45.2% in MCMV/AAD mice (Figure 8). This increase in PAS-positive cells in MCMV/AAD mice was not attributable to viral infection alone because little to no PAS staining was observed in airways from MCMV infected mice in the absence of AAD (Figure 2, C, F, and I). These results indicate that concomitant MCMV infection resulted in an altered epithelial cell phenotype, characterized by a decrease in Foxj1 expression and a loss of ciliated cells in airways demonstrating PAS staining. In addition, concomitant MCMV infection resulted in an increase in the number of goblet cells in these airways. The loss of ciliated cells, along with the increase in goblet cells, may contribute to the enhanced mucus plugging observed in MCMV/AAD mice.

Discussion

Excessive mucus accumulation in the conducting airways has been reported to be a major contributing factor

in fatal asthma. Upon autopsy, mucus plugs partially or totally occlude both small and medium airways, with mucins being the primary component of these plugs.^{33,34} In the present studies, we have shown that concomitant MCMV infection enhanced mucus plugging during the early stages of AAD and prolonged mucus plugging throughout the course of disease. Enhanced mucus plugging was associated with an increase in goblet cell metaplasia, as well as a decrease in Foxj1 expression and the number of ciliated cells in the airway epithelium of MCMV/AAD mice. Because MCMV can infect the airway epithelium, these findings suggest a means by which this opportunistic respiratory pathogen can enhance mucus plugging in asthma by causing phenotypic changes in the airway epithelium that can enhance mucus production and decrease mucus clearance.

Ciliated epithelial cells that line the airway provide an important first defense against invading respiratory pathogens, allowing for their rapid removal through efficient mucociliary clearance. Several laboratories have demonstrated a critical role for Foxj1 (also called hepatocyte nuclear factor/forkhead homologue 4) in epithelial

Figure 6. MCMV infection reduced Foxj1 mRNA expression in the airway epithelium. Total RNA was isolated from lung tissue from AAD and MCMV/AAD mice after 3 days of OVA-aerosol challenge. Total RNA from lung tissue from naïve and MCMV-infected mice served as controls. Quantitative analysis of Foxj1 mRNA was determined by real-time PCR analysis and normalized to GAPDH mRNA expression. * $P < 0.02$ compared to naïve mice.

Figure 7. Fewer Foxj1-positive, ciliated epithelial cells were present in the airways of MCMV/AAD mice. Lung tissue from AAD mice (A) and MCMV/AAD mice (C) were fixed in 4% paraformaldehyde and routinely processed for histological evaluation. After 3 days of OVA-aerosol challenge, sequential lung sections were stained with anti-Foxj1 monoclonal antibody, followed by biotinylated anti-mouse IgG (brown nuclear stain) and counterstained with hematoxylin. A higher magnification of airway epithelium (B) demonstrates that Foxj1-positive cells were ciliated, regardless of whether they were from AAD or MCMV/AAD mice. Scale bars = $20 \mu\text{m}$.

Figure 8. Quantitation of Foxj1 expression in AAD and MCMV/AAD airways. Lung tissue from the same AAD and MCMV/AAD mice, as shown in Figure 7, were processed for histological evaluation after 3 days of OVA-aerosol challenge. Sequential lung sections were stained with PAS or anti-Foxj1 antibody, then counterstained with hematoxylin. The percentage of PAS-positive cells and Foxj1-positive cells, as well as the total number of cells, was determined. Each bar represents the mean $\pm$ SE, with $n = 6$ to 8 airway sections. * $P = 0.03$; $^{\dagger}P = 0.005$.

cell ciliogenesis, although its exact function remains uncertain. Expression of Foxj1 is restricted to the lung, choroids plexus, oviduct, testis, and brain, with expression being abundant in the nuclei of cells.^{35–38} Mice deficient in Foxj1 display changes in left-right asymmetry during development and an absence of ciliated airway cells in the adult.^{38,39} A similar outcome has been observed in individuals who suffer from the autosomal recessive disorder Kartagener's syndrome, including situs inversus and immobile cilia in the airway epithelium, placing these individuals at risk for chronic respiratory infections. Recent studies have demonstrated the importance of Foxj1 in the assembly of apical membrane protein complexes necessary for the organization of functional ciliated epithelial cells as well as a potential role for Foxj1 in the regulation of inflammation.^{40,41}

Many respiratory viruses target ciliated epithelial cells for infection because ciliary movement can play an important role in the spread of the virus.^{42,43} Intranasal infection of mice with Sendai virus (SdV) has been shown to result in airway injury, characterized by a transient decrease in ciliated epithelial cells and a corresponding decrease in Foxj1 expression.⁴⁴ The absence of ciliated epithelial cells in mice during SdV infection diminished their ability to clear *Haemophilus influenzae* from the lungs, suggesting a reduction in mucociliary function. Upon clearance of SdV from the lung, repair of the airway epithelium ensued, leading to the restoration of a ciliated phenotype and re-establishment of mucociliary function.⁴⁴ Like SdV, MCMV can infect airway epithelial cells, resulting in a decrease in Foxj1 expression and a reduction in ciliated epithelial cells. In MCMV/AAD, but not MCMV-infected mice (in the absence of AAD), prolonged mucus plugging was evident after 42 days of OVA-aerosol challenge, a time when resolution of disease has occurred in uninfected AAD mice. The reason for prolonged mucus plugging is unknown. MCMV, a β -herpesvirus, is not cleared from the host, but persists at low levels in the lung before becoming latent in this tis-

sue.^{45,46} The continued presence of virus within the lung, may contribute to mucus plugging in these mice. In addition, airway inflammation in patients with asthma has also been associated with a decrease in mucociliary clearance.⁴⁷ MCMV infection delayed airway inflammation and eosinophilia in mice with AAD; yet, peak inflammation reached similar levels as compared to uninfected AAD mice. Thus, delayed inflammation may also influence mucociliary clearance and enhanced mucus plugging during the chronic stages of disease in these mice.

IL-13 is a key regulator of asthma, resulting in the enhancement of airway inflammation and mucus production.^{9,10} Although IL-13 can directly induce mucus production in airway epithelial cells *in vivo*, *in vitro* studies have demonstrated that the influence of IL-13 is critically dependent on concentration.^{48,49} Exposure of human bronchial epithelial cells in culture to 1 ng/ml of IL-13 increased goblet cell metaplasia 5- to 10-fold, whereas exposure to 10 ng/ml markedly inhibited this phenotype.⁴⁹ Thus, it is possible that the lower levels of IL-13 present in BALF from MCMV/AAD mice were sufficient to initiate and enhance goblet cell metaplasia. It has also been suggested that IL-13 signaling plays a role in the transformation of ciliated epithelial cells into goblet cells.⁵⁰ Electron microscopy of tracheal epithelial cell cultures, treated with IL-13, provided evidence of a small subpopulation of ciliated cells that contained mucus granules. Similarly, treatment of differentiated human epithelial cells with IL-13 resulted in cells with a mucociliary phenotype, being positive for both Foxj1 and Muc5A/C.⁵¹ In our studies, co-staining with PAS and Foxj1 revealed that the majority of Foxj1-positive cells were negative for PAS and Foxj1-PAS double-positive cells were rare (data not shown). Furthermore, in MCMV-infected mice, in the absence of AAD, no increase in PAS-positive cells was observed, although a decrease in Foxj1 expression was noted. Thus, it is unlikely that the loss of ciliated cells per se directly leads to an increase in goblet cell numbers or an increase in mucus plugging.

Besides decreased mucociliary clearance and increased goblet cell metaplasia, mucus plugging can also result from increased mucin production. Changes in mucin expression can alter the composition of mucus, thereby affecting its viscoelastic properties and efficiency of clearance. Bacterial and viral infections, as well as other sources of inflammation, have been reported to enhance the expression of Muc2, Muc5AC, and Muc5B, three gel-forming lung mucins that contribute to the viscoelastic and adhesive properties of mucus.^{52–60} However, enhanced mucin mRNA synthesis was not observed in MCMV/AAD mice; Muc5AC and Gob5 mRNA expression, although elevated as compared to naïve mice, did not reach the levels of expression observed in AAD mice. These findings were surprising, because Muc5AC synthesis has been reported to be a good marker for goblet cell metaplasia.¹³ It is possible that increases in Muc5AC mRNA may not reflect increases in protein synthesis because posttranscriptional or post-translational regulatory mechanisms may be in place. The ability to uncouple these two events may allow further

insight into the mechanisms controlling mucin synthesis and goblet cell metaplasia.

In AAD mice, increased expression of Muc5AC mRNA, but not Muc5B or Muc2, was observed. This pattern of mucin expression is consistent with that found in lung biopsies from patients with asthma, in which the levels of Muc5AC are elevated as compared to Muc5B or Muc2.^{61,62} A role for Gob-5, a putative member of the calcium-activated chloride channel family, in the regulation of Muc5AC expression has been suggested. Overexpression of Gob-5 enhances epithelial cell metaplasia and mucus production in an OVA-induced model of asthma, whereas a deficiency in Gob-5 decreased mucus production.^{32,63} In contrast, studies by Robichaud and colleagues⁶⁴ examined mucus production in Gob-5-deficient mice in an OVA-induced model of asthma, but attenuation was not observed. In our studies elevated levels of Muc5AC mRNA were associated with increased levels of Gob-5 mRNA in lung tissue from AAD mice. This correlation between Gob-5 and Muc5AC mRNA expression was also observed in MCMV/AAD mice, albeit at lower levels. Intranasal infection with RSV in an OVA-induced model of inflammation was able to enhance both Gob-5 and Muc5AC expression as compared with uninfected OVA-sensitized and -challenged mice.⁵⁵ This increase in mucus production was associated with elevated levels of IL-17 in the lung. IL-17 has been shown to stimulate Muc5AC and Muc5B mRNA expression in human tracheo-bronchial epithelial cells.⁶⁵ We could not detect IL-17 in BALF collected from AAD or MCMV/AAD mice after 3 or 7 days of OVA-aerosol challenge (data not shown). This disparity may be attributed to differences in samples tested (lung tissue versus BALF) or be specific to the virus.

In this study we have detailed the progression of AAD in uninfected and MCMV-infected mice throughout time, monitoring several key parameters of allergic inflammation and mucus production. Concomitant MCMV infection shifted the balance of Th1/Th2 cytokines and delayed the peak of airway inflammation. More prominently, MCMV infection enhanced mucus plugging in the lower airways of mice with AAD via increased goblet cell metaplasia and the loss of ciliated epithelial cells. Although the underlying mechanism(s) by which MCMV infection can modify mucus production is not clear, the loss of ciliated cells was driven by a decrease in Foxj1 expression. Our results indicate that the consequence of concomitant MCMV infection includes exacerbation of pulmonary disease, most likely by altering the airway epithelial cell phenotype and decreasing mucociliary clearance.

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