Alveolar Macrophages Contribute to the Pathogenesis of Human Metapneumovirus Infection while Protecting against Respiratory Syncytial Virus Infection

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Abstract

Human metapneumovirus (hMPV) and respiratory syncytial virus (RSV) are leading causes of upper and lower respiratory tract infections in young children and among elderly and immunocompromised patients. The pathogenesis of hMPV-induced lung disease is poorly understood. The lung macrophage population consists of alveolar macrophages (AMs) residing at the luminal surface of alveoli and interstitial macrophages present within the parenchymal lung interstitium. The involvement of AMs in innate immune responses to virus infections remains elusive. In this study, BALB/c mice depleted of AMs by intranasal instillation of dichloromethylene bisphosphonate (L-CL₂MBP) liposomes were examined for disease, lung inflammation, and viral replication after infection with hMPV or RSV. hMPV-infected mice lacking AMs exhibited improved disease in terms of body weight loss, lung inflammation, airway obstruction, and hyperresponsiveness compared with AM-competent mice. AM depletion was associated with significantly reduced hMPV titers in the lungs, suggesting that hMPV required AMs for early entry and replication in the lung. In contrast, AM depletion in the context of RSV infection was characterized by an increase in viral replication, worsened disease, and inflammation, with increased airway neutrophils and inflammatory dendritic cells. Overall, lack

of AMs resulted in a broad-spectrum disruption in type I IFN and certain inflammatory cytokine production, including TNF and IL-6, while causing a virus-specific alteration in the profile of several immunomodulatory cytokines, chemokines, and growth factors. Our study demonstrates that AMs have distinct roles in the context of human infections caused by members of the *Paramyxoviridae* family.

Keywords: human metapneumovirus; respiratory syncytial virus; viral infection; pathogenesis; alveolar macrophages

Clinical Relevance

In this study we tried to establish for the first time the role of alveolar macrophages (AMs) in the context of human metapneumovirus (hMPV) infection and to compare side-by-side the function of these cells in controlling antiviral responses, clinical disease, and airway inflammation in hMPV and respiratory syncytial virus infection. Our study demonstrates that AMs have distinct roles in the context of human infections caused by members of the *Paramyxoviridae* family.

Human metapneumovirus (hMPV), a paramyxovirus discovered in 2001, is an important cause of acute respiratory tract infections in infants and children (1, 2).

Recent prospective surveillance studies conducted in the United States over a period of three to six seasons have shown that the annual rate of hospitalization

associated with hMPV is the same as the rate of hospitalization associated with influenza virus (3). Although the clinical features of hMPV infection are similar to

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those caused by other respiratory viruses, these studies have reported that hospitalized infants with hMPV infection had more severe disease, requiring longer intensive care therapy (3, 4). The pathophysiology of hMPV infection is largely unknown. Few studies have examined in experimental animal models the contribution of adaptive immunity to the antiviral response and to the pathogenesis of hMPV-mediated disease, and there is almost no information on the role of innate immune response in this infection. Indeed, some of the scientific information has been extrapolated by the vast literature on human respiratory syncytial virus (RSV), a related member of the *Paramyxoviridae* family, taxonomically included in the *Pneumovirus* subfamily, which is the most common cause of bronchiolitis and pneumonia in young children (5).

Alveolar macrophages (AMs) are strategically situated as a first lung defense against respiratory pathogens and therefore play a central role in innate host defense and in the maintenance of immunological homeostasis (6). They are capable of sensing pathogen-associated molecular patterns and of initiating innate and adaptive immune responses against invading pathogens. AMs are also a primary source of inflammatory and immunomodulatory cytokines in lungs, and in general their depletion has been shown to result in an impaired host response against viral and bacterial pathogens (7, 8). For example, mice that are depleted of AMs or have constitutive deficiencies in macrophage function show enhanced RSV replication and viral-induced airway occlusion, respectively (7–9), suggesting a protective role of AMs in the context of human paramyxovirus infections.

The aim of this study was to establish for the first time the role of AMs in the context of hMPV infection and to compare side-by-side the function of these cells in controlling antiviral responses, clinical disease, and airway inflammation in hMPV and RSV infection. Dichloromethylene bisphosphonate (clodronate or L-CL₂MDP)-encapsulated liposomes administered via different routes to mice, including intranasal and intratracheal, are taken up by phagocytic macrophages, resulting in selective elimination of specific macrophage populations with minimal effects on nonphagocytic cells (7, 8, 10–17).

In some studies, the depletion of macrophages from the lungs has been shown to be associated with an enhanced pulmonary immune response characterized by dendritic cell (DC) trafficking and increased cytotoxic T cell responses (15, 18–20). Thus, using this well-established method for the depletion of AMs from mouse lung (7–10, 12–17, 20), we dissected the function of such cells in hMPV-induced clinical illness, airway inflammation, and airway pathophysiology. Our results demonstrate that AMs are the major cellular source of IFN- α/β in the lung, after hMPV infection, but, in contrast to findings in other viral infections including RSV, AMs do not exert a protective anti-hMPV function; rather, they contribute to viral replication and airway disease, suggesting a distinct role of these cells in response to different viral pathogens, even if they belong to the same family.

Materials and Methods

Virus Preparations

The hMPV strain CAN97–83 was obtained from the Centers for Disease Control (Atlanta, GA), with permission from Dr. Guy Boivin at the Research Center in Infectious Diseases, Regional Virology Laboratory, Laval University, (Quebec City, PQ, Canada) and propagated as previously described (21). RSV (Long strain) was grown in HEP-2 cells (American Type Culture Collection, Manassas, VA) and purified as described elsewhere (22, 23). The virus titer was determined by a methylcellulose plaque assay (24).

Macrophage Depletion and Infection of Mice

Female, 8- to 10-week-old, BALB/c mice were purchased from Harlan (Houston, TX) and housed under pathogen-free conditions in the animal research facility of the University of Texas Medical Branch, Galveston, Texas, in accordance with the National Institutes of Health and University of Texas Medical Branch institutional guidelines for animal care. For depletion of macrophages, mice were inoculated intranasally with a single dose of 100 μ l of L-CL₂MBP (a kind gift of Roche Diagnostics, Mannheim, Germany) as described elsewhere (10). Equal volumes of empty liposomes or PBS served as controls. Forty-eight hours later, L-CL₂MBP and

control mice were infected intranasally with 10⁷ plaque-forming units of sucrose-purified hMPV or RSV diluted in PBS in a total volume of 50 μ l. An equal volume of sucrose in PBS served as mock infection. To study the role of macrophages in viral propagation, in some experiments, AM depletion was performed 48 hours after infection (14, 16). Details regarding collection of bronchoalveolar lavage, viral titration, clinical illness, measurement of airway hyperresponsiveness (AHR), cytokines and chemokine measurement, and cell recruitment in bronchoalveolar lavage (BAL) and lungs are provided in the online supplement.

AM Isolation and Infection

AMs were obtained from BALB/c mice by gentle BAL as previously described (25). Adherent AMs (in 48-well plates) were then infected with hMPV or RSV (multiplicity of infection [MOI] of 5) and cultured in 0.2 ml RPMI-1640 in a 5% CO₂ incubator. PBS alone was used for controls. At different times after infection, culture supernatant was collected, and cytokines and IFN were measured. For viral replication, adherent AMs were seeded in 48-well tissue culture plates at 5 $\times$ 10⁴ cells per well and infected with hMPV or RSV at an MOI of 0.1. At various time points after infection, cell supernatant and cells were sonicated and homogenized, and the homogenate supernatant was tested for viral replication as described for lung titration.

Cell Death Assay

Cell death was measured by photometric detection ELISA (Roche, Indianapolis, IN), which detects histone-associated DNA fragments (mono- and oligonucleosomes) generated by necrotic or apoptotic cells. Further details are provided in the online supplement.

Statistical Analysis

Statistical analyses were performed with the InStat 3.05 Biostatistics Package (GraphPad, San Diego, CA). To ascertain differences between two groups, Student's *t* test was used, and if more than two groups were compared, one way one-way ANOVA was performed. Values of *P* < 0.05 were considered statistically significant. Unless otherwise indicated, values for all measurements are expressed as the mean $\pm$ SEM in the figures.

Results

Effect of AM Depletion on Virus-Induced Disease, Lung Inflammation, and Airway Function

To dissect the role of AMs in hMPV infection, AMs were depleted from the lungs of BALB/c mice via intranasal administration of 100 μ l of clodronate liposomes (L-CL₂MBP). Because in preliminary experiments empty liposomes used as controls for L-CL₂MBP also induced some reduction in macrophage number (17), inoculation of PBS was used in all subsequent experiments as control for treatment. AM depletion was first confirmed by BAL cell staining. Consistent with previous reports, intranasal administration of L-CL₂MBP resulted in 65 to 80% depletion of AMs compared with PBS-treated control mice ($P < 0.001$) (see Figure E1A in the online supplement). AM depletion by L-CL₂MBP was confirmed by flow cytometry analysis of BAL cells using anti-CD11c and anti-F4/80 antibodies recognizing mouse AMs. A significant reduction in AMs was observed in BAL of mice treated with L-CL₂MBP compared with PBS-treated animals (Figure E1B). Depletion of AMs was evident as early as Day 1 and persisted for up to 5 days after treatment (Figure E1C). To assess the role of AMs in hMPV-induced disease, various parameters of disease were evaluated in AM-depleted and control mice, including body weight loss, clinical illness, AHR, and pathology score (as a measure of peribronchial and perivascular inflammation). All the experiments were performed and analyzed in four groups of mice (i.e., AM-depleted mice with or without infection and control mice not depleted of AMs with and without infection). For better clarity, the results are presented in the figures for the two infected groups with and without AM depletion (the uninfected groups of animals did not show signs of disease or change whether depleted of AMs or not). After hMPV infection, AM-depleted mice demonstrated significantly less body weight loss as compared with AM-undepleted mice (Figure 1A). Assessment of lung inflammation at Day 5 and 7 after infection by histopathology score showed significantly reduced cellular infiltration in AM-depleted mice compared with undepleted mice (Figure 1B). We have

previously shown that mice infected with hMPV demonstrate increased baseline Penh values, denoting airway obstruction, up to Day 12 after infection and a dose-dependent increase in AHR when challenged with methacholine at Day 14 after infection (26). In agreement with our findings of body weight loss and pathology score, AM-depleted mice infected with hMPV showed a significant improvement in parameters of lung function, as shown by a reduction in airway obstruction (i.e., basal Penh values), starting at Day 5 after infection and continuing to Day 14 after infection (Figure 1C), as well as significantly less AHR at Day 21 (Figure 1D), compared with undepleted mice.

To determine whether the contribution of AMs to the pathogenesis of disease is common to other paramyxovirus infections, we performed experiments in AM-depleted mice infected with RSV. In contrast with our findings in hMPV infection, but in overall agreement with previous reports in the RSV model, AM depletion was associated with significantly greater lung inflammation (Figure 1B) and AHR at Day 5 (Figure 1C) and Day 14 (Figure 1D) in RSV-infected mice compared with undepleted RSV-infected mice.

Effect of AM Depletion on Inflammatory Cell Recruitment after Infection

BAL and lung differential cell count were performed to ascertain the kinetics of macrophage depletion and inflammatory cell recruitment after infection. As expected, in hMPV and RSV infection the macrophage number in BAL and lung tissue was significantly lower in macrophage-depleted mice at early time points (1–3 d after infection), compared with undepleted mice, with no difference between the two groups with both infections at later time points after infection (data not shown). Whereas in hMPV-infected mice AM depletion resulted in significantly decreased lymphocyte recruitment in the BAL compared with undepleted mice, in RSV-infected mice increased BAL lymphocytes were observed after AM depletion, in particular at Day 8 after infection (Figure 2A). These results were consistent with the pathology scores (Figure 1B). BAL differential staining was also corroborated by flow cytometric

staining of lymphocytes (CD4⁺ and CD8⁺ T cells) isolated from the lungs. Compared with undepleted mice, AM-depleted mice had significantly lower numbers of CD4⁺ T cells at Day 8 after hMPV infection, whereas there was increased lymphocyte recruitment (CD4⁺ and CD8⁺ T cells) in response to RSV infection (Figure 2B). Moreover, compared with undepleted mice, a significant increase of inflammatory DCs (CD11c^{hi}MHCII^{hi}CD11b⁺) and neutrophils (CD11b^{hi}Gr1^{hi}) was observed in the lungs of AM-depleted mice infected with RSV but not in mice infected with hMPV (Figure 2C). No significant differences were observed in other cell types, including interstitial macrophages, plasmacytoid DCs, and NK cells, in AM-depleted mice after infection with hMPV or RSV (data not shown).

Effect of AM Depletion on Cytokines, Chemokines, and Type I IFN Secretion

Infections caused by paramyxoviruses, including hMPV and RSV, induce production of proinflammatory cytokines and chemokines, some of which have been implicated in the pathogenesis and clinical manifestations of disease. The cellular source of such lung cytokines remains elusive, although AMs have been shown to be a major source of some of these cytokines. Thus, cytokines, chemokines, and growth factors were measured in the BAL of AM-depleted mice after infection with hMPV or RSV using Bio-plex arrays and ELISA. Significantly lower levels of IL-1 α , IL-1 β , TNF- α , and IL-6 were detected at all time points tested in hMPV-infected, AM-depleted mice compared with undepleted mice (Figure 3A). This finding was particularly dramatic for IL-6 and TNF- α , suggesting that AMs are the major cellular source of such cytokines in the lung. Similar results regarding TNF- α and IL-6 were observed in the context of RSV infection but not for IL-1 α and IL-1 β , both of which were significantly higher in the BAL fluid of AM-depleted mice compared with undepleted mice (Figure 3A). In addition, we found that, compared with undepleted mice, AM-depleted mice had greater concentrations of IL-12p40 and lower concentrations of granulocyte-macrophage colony-stimulating factor in response to hMPV or RSV infection (Figure 3B). On the other hand, whereas the neutrophil growth factor granulocyte colony-stimulating factor (G-CSF) was

Figure 1. Effect of alveolar macrophage (AM) depletion on human metapneumovirus (hMPV)- and respiratory syncytial virus (RSV)-induced disease. (A) Dichloromethylene bisphosphonate (L-CL₂MBP)-treated and untreated mice were infected with hMPV or RSV, and change in body weight was measured over a period of 14 days after infection. (B) Lungs were collected at Days 5 and 7 after infection, and pathology score was assessed by hematoxylin and eosin staining. Airway obstruction represented by baseline Penh for hMPV infection and airway hyperresponsiveness after methacholine challenge at Day 5 after infection after RSV infection (C) and airway hyperresponsiveness after methacholine challenge at Day 21 after hMPV infection and Day 14 after RSV infection (D) were determined by unrestrained plethysmography. Data are expressed as mean $\pm$ SEM ($n = 4$ mice/group) and are representative of three independent experiments. * $P < 0.05$ and *** $P < 0.001$ when comparing L-CL₂MBP-treated versus untreated infected mice.

Figure 2. (See figure legend on following page)

significantly reduced in AM-depleted mice infected with hMPV, levels were greater in AM-depleted mice at most time points after RSV infection (Figure 3B).

Regarding inducible chemokines, AM-depleted mice had higher amounts of CCL3 and CCL5 in response to hMPV infection (Figure 4A). In experiments with RSV infection, we found a significant decrease in the levels of CCL4 and CCL5, but levels of CCL3, with the exception of an early time point (12 h), were significantly greater in AM-depleted mice compared with undepleted mice (Figure 4A). Levels of IFN- γ , IL-4, IL-13, IL-17A, IL-17F (Bio-plex), and TGF- β (ELISA) were also measured. Similar levels of IL-13 and IFN- γ in BAL (Figure E2) and lung homogenates (data not shown) were observed in AM-depleted and undepleted mice after infection with hMPV or RSV. IL-4 was not detectable in the BAL in all groups of mice (data not shown). AM depletion greatly enhanced the levels of IL-17A (Figure E3) and IL-17F (data not shown) in AM-depleted mice infected with RSV. IL-17A and IL-17F were undetectable or were at the lowest level of assay detection in all groups of hMPV-infected mice. TGF- β was also detected in BAL of hMPV- or RSV-infected mice, but although AM depletion did not change the concentrations measured in hMPV infection, it significantly increased concentrations at all time points after RSV infection (Figure E3).

To better understand the contribution of AMs to type I IFN production in the lung, IFN- α and IFN- β levels were measured in BAL at various times points after infection. Our results show that AM depletion greatly reduced IFN- α and IFN- β production by hMPV infection (Figure 4B) and virtually abolished it in RSV-infected mice (Figure 4B). These results indicate that AMs are the major cellular source of inflammatory cytokines and type I IFN and that AMs play a major role in modulating the production of inducible chemokines and immunomodulatory cytokines in the airways by virus-specific mechanisms.

Effect of AM Depletion on Viral Replication in the Lung

To establish whether AMs play a role in antiviral responses, we measured viral titers at Days 3, 5, 7, and 9 after infection in lung tissue of AM-depleted and undepleted mice after infection with hMPV or RSV. A significant reduction in viral titers at Day 3 ($P < 0.05$) and Day 5 ($P < 0.001$) after infection was observed in hMPV-infected, AM-depleted mice compared with undepleted mice (Figure 5A), suggesting that AMs are necessary for optimal infection by hMPV. To further determine whether AMs play a role in facilitating the initiation and/or subsequent spread of infection, we depleted AMs 48 hours after viral inoculation and measured peak viral replication. No significant difference was observed in peak viral titers in hMPV infection when mice were depleted of AMs 48 hours after viral inoculation (Figure 5B), suggesting that AMs are required at the earliest time of hMPV infection for optimal viral replication. On the other hand, in agreement with previous reports, we observed a slight increase in peak viral titer in RSV-infected mice that were depleted of AMs before or 48 hours after viral inoculation (Figures 5A and 5B), suggesting that, in contrast to hMPV infection, AMs contribute to the overall antiviral activity of the lung against RSV. Complete viral clearance was noted by Day 7 after both infections.

To determine whether AMs are indeed susceptible to hMPV and RSV infection, AMs isolated from the BAL of BALB/c mice were exposed side-by-side to hMPV or RSV (MOI of 5). Western blot analysis of hMPV and RSV viral proteins showed that AMs can be similarly infected with a high inoculum of hMPV or RSV (data not shown). However, although RSV-infected AMs produced higher amounts of cytokines and most of the chemokines that were tested (IL-1, IL-6, TNF- α , IL-12p40, G-CSF, granulocyte-macrophage colony-stimulating factor, CCL3, and CCL4), hMPV-infected AMs produced significantly higher amounts of type I IFNs and CCL5

(Figure 6A). To further determine whether hMPV and RSV could result in a productive infection, AMs were inoculated at a low MOI (0.1), and viral titer was determined at subsequent days after infection. hMPV replication was detected at Day 1 and at lower titer up to Day 4 after infection, whereas a modest replication of RSV was detected only at 24 hours after infection but not at later time points (Figure 6B). These data suggest that hMPV, but not RSV, can establish a productive infection of mouse AMs.

Differential Fate of AMs after hMPV and RSV Infection

Based on the distinct characteristics of viral replication observed *ex vivo*, we asked whether the initial events that occur in the lung after inoculation with hMPV or RSV affect the fate of AMs in a virus-specific manner (i.e., AMs may affect the early phase of hMPV but not RSV replication). To determine the fate of AMs after infection with hMPV or RSV, BAL was collected from BALB/c mice from 2 to 24 hours after infection. AMs were identified based on CD11c expression, autofluorescence, and the side scatter. In uninfected mice, AMs represented greater than 90% of all BAL cells (Figure 7A). After inoculation with RSV, we observed a significant reduction in the number of AMs in BAL starting at 2 hours after infection and reaching a drop close to 80 to 90% by 6 hours after infection. On the other hand, only a modest decrease in the number of AMs was detected in mice that were inoculated with hMPV at 6 hours after infection. By 24 hours after infection, no further effect of infection on macrophages was noted in response to either virus (data not shown).

To assess whether AMs undergo cell death after exposure to RSV or hMPV, possibly explaining the disappearance of cells from the BAL (as in the case of RSV infection), we measured cell death using a cell death detection ELISA Kit (Roche). AMs were isolated from BAL and infected *ex vivo* with hMPV or RSV. At different

Figure 2. Effect of AM depletion on bronchoalveolar lavage (BAL) and lung cell recruitment after infection. L-CL₂MBP-treated and untreated mice were infected with hMPV or RSV, and BAL and lungs were collected at different time points after infection to determine differential cell counts by hematoxylin and eosin staining in BAL (A) and CD4 and CD8 T lymphocyte recruitment to the lung (B) as well as neutrophils (CD11b⁺Gr1⁺) and dendritic cells (CD11c^{hi}CD11b⁺) (C) by flow cytometry analysis after staining with specific antibodies. Data are expressed as mean $\pm$ SEM ($n = 5$ mice/group) and are representative of three independent experiments. * $P < 0.001$ when comparing L-CL₂MBP-treated versus untreated infected mice.

Figure 3. Effect of AM depletion on cytokines and growth factors after infection. L-CL₂MBP-treated and untreated mice were infected with hMPV or RSV, and BAL was collected at different time points after infection to measure cytokines and growth factors by Luminex-based assay. (A) IL-1α, IL-1β, TNF-α, and IL-6. (B) IL-12 p(40), granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF). Data are expressed as mean ± SEM (*n* = 5 mice/group) and are representative of three independent experiments. *P < 0.001 when comparing L-CL₂MBP-treated versus untreated infected mice.

times after infection, cell supernatants and cell lysates were harvested. A rapid increase in the release of mono- and oligonucleosomes (i.e., histone complexed DNA fragments) was observed in the cell

supernatant of RSV-exposed AMs, an indication of a necrotic process (Figure 7B). On the other hand, a modest necrosis was observed in hMPV-exposed cells. We did not observe significant differences in the

histone-linked DNA fragments in cell lysates of RSV- or hMPV-infected cells compared with uninfected controls, suggesting that apoptosis may not represent a major pathway of cell death in AMs

Figure 3. (Continued).

exposed to these paramyxoviruses (data not shown).

Discussion

Using a BALB/c mouse model of experimental infection and a direct side-by-side comparison between pathogens, we show for the first time that resident AMs enhanced hMPV replication in the lung and significantly contributed to clinical disease, airway obstruction, and inflammation after infection. These findings were consistently underscored by the reduction in viral lung titer, lack of body weight loss, improvement in airway obstruction and AHR, and reduction in pathology of lung tissue in mice that were depleted of AMs before hMPV inoculation. The relevance of these data is

further supported by the observation that, in contrast to hMPV depletion of AMs before RSV infection resulted in an increase in viral replication and overall worsening of disease parameters and lung pathology. Our results in the RSV model are in agreement with reports by others (7–9) and are consistent in general with studies of other respiratory viral pathogens, including influenza virus, suggesting that AMs are indeed necessary for viral clearance and control of disease (16). The precise mechanism(s) by which AMs control viral infections is unclear but may include production of type I IFN, nitric oxide, and the phagocytosis of virus-infected cells by AMs (27). The potential mechanisms by which AMs, as in our study, contribute to rather than protect from hMPV infection are more puzzling. The reduction in BAL and lung

lymphocyte recruitment, in particular CD4⁺ cells that we observed after hMPV infection of AM-depleted mice, is likely to be a contributing factor to the overall milder airway disease observed in these mice compared with AM-competent controls. Indeed, we have previously shown that mice depleted of CD4 T cells have significantly reduced body weight loss and clinical disease after hMPV infection yet have comparable levels of viral replication in the lung (26). In addition, herein we found a significant reduction in neutrophil recruitment to the lung in AM-depleted, hMPV-infected mice compared with AM-competent animals, a finding that was in sharp contrast with the increased recruitment of such cells and inflammatory DCs in AM-depleted mice that were infected with RSV (Figure 2C). This increase in inflammatory cells may explain

Figure 4. (See figure legend on following page)

Figure 5. Effect of AM depletion on RSV/hMPV replication in the lung. (A) L-CL₂MBP-treated and untreated mice were infected with hMPV and harvested at Days 3, 5, and 7 after infection to determine viral titers by TCID₅₀ assay. (B) Mice were first infected with hMPV and were treated with L-CL₂MBP 48 hours later. At Day 5 after infection, lungs were collected, and viral titer was determined by TCID₅₀ assay. (C) L-CL₂MBP-treated and untreated mice were infected with RSV and harvested at Days 3, 5, and 7 after infection to determine viral titers by plaque assay. Mice were first infected with RSV and were treated with L-CL₂MBP 48 hours later. (D) At Day 5 after infection lungs were collected, and viral titer was determined by plaque assay. Data are expressed as mean $\pm$ SEM ($n = 5$ animals/group) and are representative of three independent experiments. nd = not detected. * $P < 0.05$ and ** $P < 0.01$ when comparing L-CL₂MBP-treated versus untreated infected mice.

the increase in lung inflammation and worsened lung function seen in AM-depleted, RSV-infected mice.

On the basis of our results and previous studies by others (7), it appears that AMs significantly contribute to the production of key innate and inflammatory cytokines, including IL-6 and TNF- α , both of which were dramatically reduced in BAL fluid of hMPV- or RSV-infected mice that were depleted of AMs. On the other hand, whereas AM-depleted mice that were infected with hMPV had a significant reduction in IL-1 α and IL-1 β in the BAL compared with undepleted mice, AM-depleted mice that were infected with RSV

showed a dramatic increase in the levels of both these pyrogenic cytokines as early as 12 hours after infection. Levels of IL-1Ra, an endogenous inhibitor of IL-1, were not affected by AM depletion in either model of infection (data not shown). Because IL-1 has been identified as a potent mediator of local inflammation in the lung, our findings suggest a key role of the AM-IL-1 axis in the context of respiratory viral infection pathogenesis. However, it is unclear why AM depletion resulted in opposite effects on IL-1 production in hMPV versus RSV infection. AMs may be the main source of IL-1 β in hMPV infection (explaining the reduced levels of this cytokine after AM

depletion), whereas other inflammatory cells, such as DCs, may be the main source in RSV infection. If this is the case in RSV infection, AMs would exert an inhibitory function on other cells, for example regarding production of pyrogenic cytokines to maintain a homeostatic circuit and to prevent inflammatory injury to the lung. Indeed, AMs have been shown to suppress DC and T and B cell function and to crosstalk with airway epithelial cells through the production of nitric oxide, prostaglandin E₂, IL-10, and TGF- β (28–30).

Although with a different kinetics of production, we found that in hMPV and

Figure 4. Effect of AM depletion on chemokines and type I IFNs after infection. L-CL₂MBP-treated and untreated mice were infected with hMPV or RSV, and BAL was collected at different time points after infection to measure chemokines and type I IFN by Luminex-based assay and ELISA, respectively. (A) CCL3, CCL4, and CCL5. (B) IFN- α and IFN- β . Data are expressed as mean $\pm$ SEM ($n = 5$ mice/group) and are representative of three independent experiments. * $P < 0.001$ when comparing L-CL₂MBP-treated versus untreated infected mice.

Figure 6. Cytokine production and viral replication by murine AMs. AMs were isolated from BALB/c mice and infected *ex vivo* with hMPV or RSV (multiplicity of infection [MOI] of 5). (A) At 24 hours after infection, cell supernatant was collected, and cytokines, chemokines, and IFN- α and - β levels were measured. (B) AMs were infected with hMPV or RSV (MOI of 0.1), and the viral titer was measured at different days after infection by immunostaining for hMPV and plaque assay for RSV. Data are expressed as mean $\pm$ SEM and are representative of two to three independent experiments. * $P < 0.001$ when comparing with hMPV infected cells. nd, not determined.

RSV infections, depletion of AMs was associated with significantly higher levels of IL-12p40 in BAL. IL-12p40 is the subunit of the heterodimeric cytokines IL-12 (with the p35 subunit) and IL-23 (with the subunit p19) (31). IL-12p40 can also form the homodimer known as IL-12p80, which has high affinity for the IL-12R β 1 chain and as such has been shown to function as an efficient IL-12 antagonist (32). It has been shown that airway epithelial cells are a major source of IL-12p40 in the lung (33), and therefore it is reasonable to speculate that removal of an AM-driven negative signal/mediator may result in the increased production seen in our studies. Studies have shown that mice deficient in IL-12p40 have more severe airway inflammation and greater AHR after infection with RSV (34) or hMPV (35), and overexpression of IL-12p80 in mouse lung epithelium primes the host for a protective response against a lethal respiratory infection with parainfluenza virus (36). Therefore, the increase in the number of IL-12p80 homodimers (which represents a sizable percent of the total measurable IL-12p40) in our model could explain in part the milder disease, inflammation, and AHR in

AM-depleted mice that were infected with hMPV. On the other hand, the fact that we did not observe a similar reduction but rather observed an increase in disease severity in AM-depleted mice that were infected with RSV despite a robust production of IL-12p40 may be related to the fact that IL-12-dependent events have a variable role depending on the type of viral infection, as shown in studies using Sendai virus in which overexpression of IL-12p40 contributed to increased animal morbidity (33).

Overall, our findings suggest that AM depletion in the context of RSV infection results in a proinflammatory mucosal environment characterized by increased levels of G-CSF, IL-17, TGF- β , and CCL3 in addition to IL-1. These cytokines have been shown to be involved in the various aspects of pathogenesis of respiratory diseases and in human natural and experimental RSV infections by regulating lung inflammation, mucus production, oxidative response, airway neutrophilia, AHR, T cell responses, and viral replication (25, 37–41).

AMs in our studies appear to be a major cell source of IFN- α / β in the lung after hMPV and RSV infection, similar to what

has been shown for other experimental infections in mice with RNA viruses (42). However, other studies using cell depletion approaches have shown that plasmacytoid DCs (43) and epithelial cells (44) are cell sources of type I IFNs after experimental infection of mice with RSV. In both of these studies and in our study, different strains of RSV were used. In our studies with RSV, AM depletion virtually abolished IFN- β production, but small amounts of IFN- α in BAL fluids were still detectable after AM depletion, and, in the case of hMPV infection, sizable concentrations of IFN- α and IFN- β were still measurable after AM depletion (Figure 4). Our experiments of *ex vivo* infection of AMs also demonstrate that these cells produce a significant amount of type I IFNs in response to hMPV and RSV infection. Altogether, these data support our findings that AMs are the major cells responsible for the production of type I IFNs and that they regulate the production from other cell types, such as epithelial cells. Irrespective of the cellular source, the deficiency of type I IFN may be sufficient to explain the moderate increase in RSV peak lung titer observed in our AM-depleted mice and in previous similar

Figure 7. Fate of AMs after infection with hMPV and RSV *in vivo* and *ex vivo*. Mice were infected with hMPV or RSV or were mock inoculated. BAL was collected at different times after infection, and cells were stained with anti-CD11c antibody and analyzed by flow cytometry. AMs were identified on the basis of the side scatter (SSC) and expression of CD11c. (A) The percentage of AMs is indicated in the gates. Data are representative of two independent experiments. (B) AMs isolated from BALB/c mice were infected *ex vivo* with hMPV or RSV (MOI of 5). At different times after infection, cell supernatant was harvested to measure cell death using a cell death detection ELISA kit. Data are expressed as mean $\pm$ SEM ($n = 6$ animals/group) and are representative of two independent experiments. * $P < 0.05$.

studies (7). Indeed, STAT-1 knock-out mice or mice treated with IFN- α show an approximately half log increase or decrease in RSV replication, respectively

(45, 46). However, despite the fact that hMPV-infected mice are more susceptible than RSV to the antiviral activity of type I IFN (46), we observed

lower viral titers in the AM-depleted mice, suggesting that the reduction of IFN- α/β in AM-depleted mice had no effect on hMPV replication.

The nature of hMPV and RSV interactions with AMs in the early phase of infection appears to be a critical event to understanding the distinct effect of AM depletion that we observed in these two infections. It was reasonable to postulate that AMs may represent the first cell target of hMPV infection in the lung and are therefore critical in the early phase of viral entry and replication. This is supported by our findings that, when treatment with L-CL₂MBP was performed 48 hours after mice had been inoculated with hMPV (rather than before inoculation), depletion of AMs did not alter lung viral replication. In support of a role of AMs as an early hMPV cell target, we show that hMPV can replicate in AMs *ex vivo* (isolated from mouse BAL), although the infection appears to be restricted at later time points and to be less efficient than in airway epithelial cells, the major target of this infection. On the contrary, RSV appears to be poorly capable of establishing a productive infection in murine AMs yet capable of triggering cytokine production, as previously reported (47).

To determine the fate of AMs after infection of mice with hMPV or RSV, we tracked AMs in BAL by flow cytometry and

showed that, whereas the total number/percent of AMs rapidly dropped within 6 hours of RSV inoculation, the number/percent of AMs was only slightly decreased at the same time points of hMPV infection. On the basis of the results of *ex vivo* DNA fragmentation assays, comparing AMs exposed to hMPV or RSV, the most likely explanation for the much more rapid disappearance of AMs from the airways of RSV-infected mice compared with hMPV-infected mice appears to be the much faster necrotic process that is triggered by RSV, similar to what has been demonstrated for influenza (48). Thus, it is possible that hMPV would establish an initial cycle of entry and replication in AMs, without killing the cells, and this initial “Trojan horse” process would be critical for subsequent progression of the infection and spreading to the airway epithelium, similar to what has been reported recently for measles virus, another member of the *Paramyxoviridae* family (49). Therefore, when AMs are depleted, as in our experimental mouse model, hMPV is severely impaired in its capacity to progress to a full-blown infection, resulting in lower viral replication in the lung and blunted disease.

On the other hand, RSV is not dependent on an initial infection of AMs for an optimal spreading to the airway mucosa; rather, it rapidly activates these cells for production of NF- κ B-dependent inflammatory cytokines (10), followed by killing of the cells via an inflammatory necrotic process (Figure 7).

In conclusion, in a mouse model of experimental hMPV infection, AMs appear to function as regulatory cells that enhance viral replication, clinical disease, airway obstruction, and lung pathology. These unexpected findings are in contrast to what has been observed by us and others in similar mouse models of RSV infection, in which AMs contribute to the antiviral innate immune response. These results underscore the distinct aspects of antiviral immunity in the context of paramyxovirus infections and the need to further understand the function of innate immunity in human infections caused by hMPV. ■

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