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Rhinovirus colocalizes with CD68- and CD11b-positive macrophages following experimental infection in humans

J. Kelley Bentley, PhD[a], **Uma S. Sajjan, PhD**[a], **Marta B. Dzaman, MD**[b], **Nizar N. Jarjour, MD**[c], **Wai-Ming Lee, PhD**[d], **James E. Gern, MD**[c], and **Marc B. Hershenson, MD**[a,e]

Marc B. Hershenson: mhershen@umich.edu

[a]Department of Pediatrics and Communicable Diseases, University of Michigan Medical School, Ann Arbor, Mich

[b]Department of Neurosurgery, University of Michigan Medical School, Ann Arbor, Mich

[c]Department of Medicine, University of Wisconsin School of Medicine and Public Health, Madison, Wis

[d]Department of Biological Mimetics, Frederick, Md

[e]Department of Molecular and Integrative Physiology, University of Michigan Medical School, Ann Arbor, Mich

To the Editor

The precise mechanisms by which rhinovirus (RV) causes exacerbation of asthma remain unclear. The current explanation is that RV infection of airway epithelial cells induces release of chemokines, thereby attracting inflammatory cells and augmenting pre-existing allergic inflammation. Genes encoding chemokines are upregulated following experimental RV infection of the nasal epithelium,[1] and experimentally infected asthmatic subjects show increased airway neutrophils, lymphocytes, and eosinophils.[2] However, the notion that viral infection simply augments asthmatic airway inflammation may not explain how asthmatic subjects suffer manifestations of lower airways disease after colds while subjects without asthma do not. Could asthmatic subjects have a qualitatively different immune response to rhinovirus infection than do subjects without asthma?

To address this question, we developed a mouse model of RV-induced asthma exacerbation.[3] Mice were treated with intraperitoneal and intranasal ovalbumin and then infected with RV. Following ovalbumin and RV, airways inflammation and hyper-responsiveness increased significantly over that generated by either ovalbumin or RV alone. In addition, we found that CD68-positive mononuclear cells, rather than or in addition to epithelial cells, were a major source of CCL11, IL-4,[3] and CCL2[4] expression. While RV nonproductively infects bronchoalveolar macrophages *in vitro*,[5] the former data demonstrate that monocytes may contribute to RV-induced airway responses *in vivo*.

To examine the role of monocytes/macrophages in RV infection in humans, we performed a preliminary study examining lower airway biopsy specimens from control and allergic

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asthmatic subjects experimentally infected with RV16. Subjects were originally enrolled between 2002 and 2004.[6] All the subjects experienced cold symptoms. We studied samples taken during the acute cold phase (4 days after infection). To unmask antigens, tissue slides were heated under pressure for 90 secondsin 150 mM NaCl and 0.01 M citrate buffer (pH 6.0). To block unreacted formaldehyde groups, samples were incubated in 100 mM ammonium chloride in 70% ethanol or 0.02% sodium borohydride in PBS for 10 minutes. Tissues were blocked for 2 hours with 5% goat serum in Tris-buffered saline with 0.05% Tween-20 (pH 7.6). Sections were incubated with AlexaFluor 594 (Molecular Probes, Portland, Ore)-conjugated R16-7,[6] an antibody that binds to the RV16 VP2 capsid protein and, depending on the experiment, mouse antihuman CD68 (clone KP1; EBioscience, San Diego, Calif), CD11b, CD11c, and/or isotype controls IgG_1 κ, IgG_{2b}, or IgG_{2a} (all from Biolegend, San Diego, Calif). Antibodies were conjugated with AlexaFluor 488 or 633. Nuclei were stained with Hoescht 33258. Images were visualized by using an Axioplan microscope with Apotome attachment or LSM 510 confocal microscope (Carl Zeiss, Thornwood, NY).

Samples from 4 controls and 11 asthmatic subjects were obtained (for tabulation of staining results, see Table E1 in this article's Online Repository at www.jacionline.org). Because of prior use of the blocks and high autofluorescence, only a limited number of slides were available for study. Three of 4 controls and 8 of 11 asthmatic subjects showed a positive RV16 signal. Of the 3 control samples with RV, 2 demonstrated positive staining for RV but no CD68 staining, indicating successful epithelial infection with RV and an absence of macrophages (Fig 1, *A*-*E*). The remaining control sample demonstrated a few CD68-positive cells present in the epithelial layer, but no colocalization with RV. Of the 8 asthma samples with a positive RV signal, 7 were stained for CD68 (the eighth was not stained). Six showed colocalization of RV and CD68. Three samples contained an ample epithelial layer studded with RV-positive and RV-, CD68-double positive cells (Fig 1, *F*-*J*). Some CD68-positive cells displayed a different morphology than did surrounding cells. The remaining samples from asthma patients demonstrated epithelial damage (all 6 samples from asthmatic subjects with colocalization are shown in Fig E1 in the Online Repository at www.jacionline.org). In these cases, RV-, CD68-double positive cells were present in the airway lumen.

While CD68 is an accepted marker for monocytes/macrophages, other markers exist, including CD14, CD11c (resident macrophages), and CD11b (recruited macrophages). Because of intense nonspecific staining, we were unable to reliably test for CD14. However, we were able to stain 4 samples from asthmatic subjects for CD11b and CD11c. We observed colocalization of CD11b and RV16 in all 4 samples studied (samples with colocalization are shown in Fig E2 in the Online Repository at www.jacionline.org). In 2 cases, we found colocalization of RV, CD68, and CD11b (Fig 2, *A*-*E*). Finally, all 4 slides tested demonstrated CD11c-positive cells, but in only 1 instance was there colocalization with RV16. In 1 subject, CD11c-positive cells took on the appearance of dendritic cells (Fig 2, *F* and *G*). Together, these data suggest that following experimental infection of patients with allergic asthma, RV colocalizes with CD68 and CD11b in airway monocyte/macrophages.

Macrophages play a critical role in innate immunity, including defense against pulmonary pathogens and the inflammatory response. Mice with allergic airways disease show increased alternatively activated, M2-polarized lung macrophages that produce type 2 cytokines in response to RV stimulation.[3,4] Recruited CD11b-positive macrophages promote eosinophilic airway inflammation in mouse asthma models.[4,7] Enhanced numbers of alternatively activated macrophages are found in the airways of subjects with asthma.[8] It is therefore conceivable that the enhanced inflammatory response to RV in patients with

allergic asthma is due to infection of recruited, activated macrophages that are normally not present in control individuals.

This study has limitations that should be considered in interpreting these findings, including the age of tissues, limited number of slides, and harsh unmasking protocols. We do not know whether the colocalization we observed represents replicative infection of airway macrophages, or simple endocytosis of the virus. Finally, under specific conditions, airway epithelial cells may take on features of phagocytic cells, including CD68 expression.[9] Nevertheless, based on costaining with CD11b and morphologic differences, RV-, CD68-double positive cells in the airway epithelium are likely to be monocytes.

We conclude that RV is ingested by recruited CD68-positive and CD11b-positive macrophages in asthmatic humans, providing direct evidence that tissue macrophages in the lower airways contribute to anti-RV responses *in vivo*. A prospective study is needed to definitively characterize the role of monocytic infection in RV-induced asthma exacerbations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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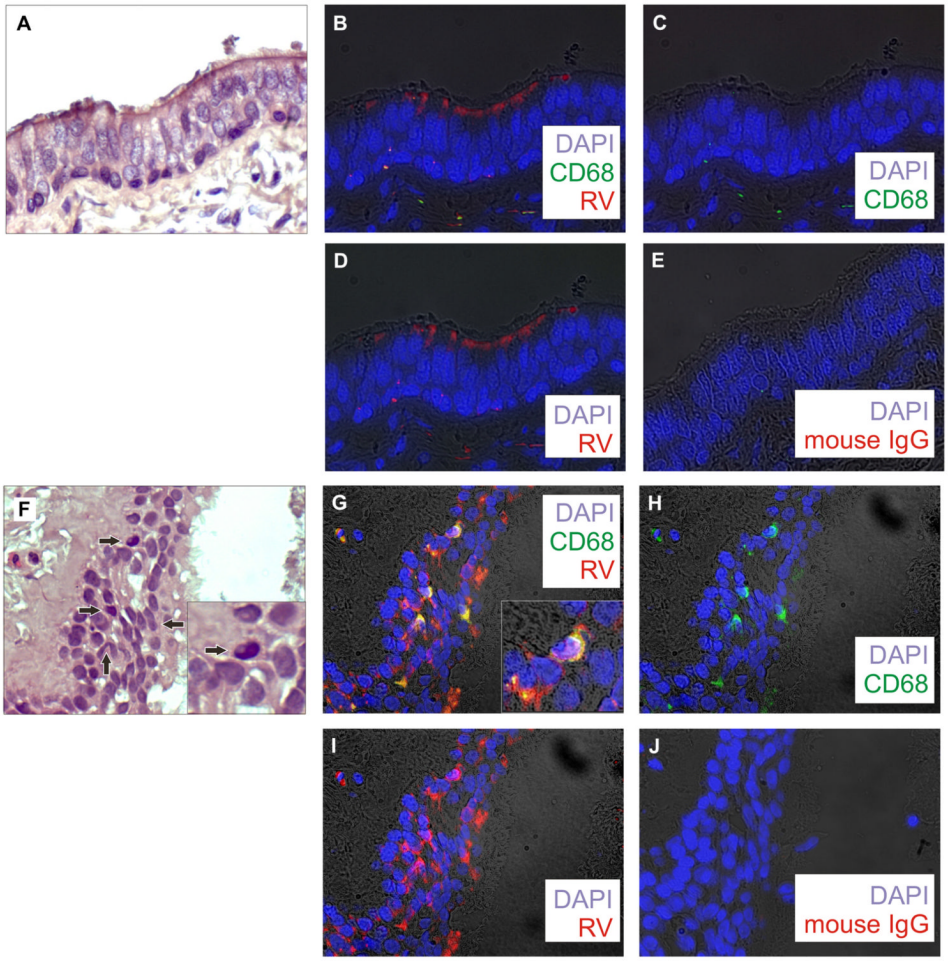

Fig 1.

Images of bronchial epithelium from control (**A-E**) and asthmatic (**F-J**) subjects experimentally infected with RV16. **A, F,** Hematoxylin and eosin. *Arrows* in *F* mark corresponding RV16-, CD68-positive cells. **B, G,** Merged immunofluorescence image showing staining for RV16 capsid protein *(red)*, CD68 *(green)*, and DAPI, a nuclear stain *(blue)*. Colocalization is yellow. **C, H,** CD68 staining. **D, I,** RV16 staining. **E, J,** Isotype control IgGs. (Original magnification, ×640). *DAPI*, 4′-6-Diamidino-2-phenylindole, dihydrochloride.

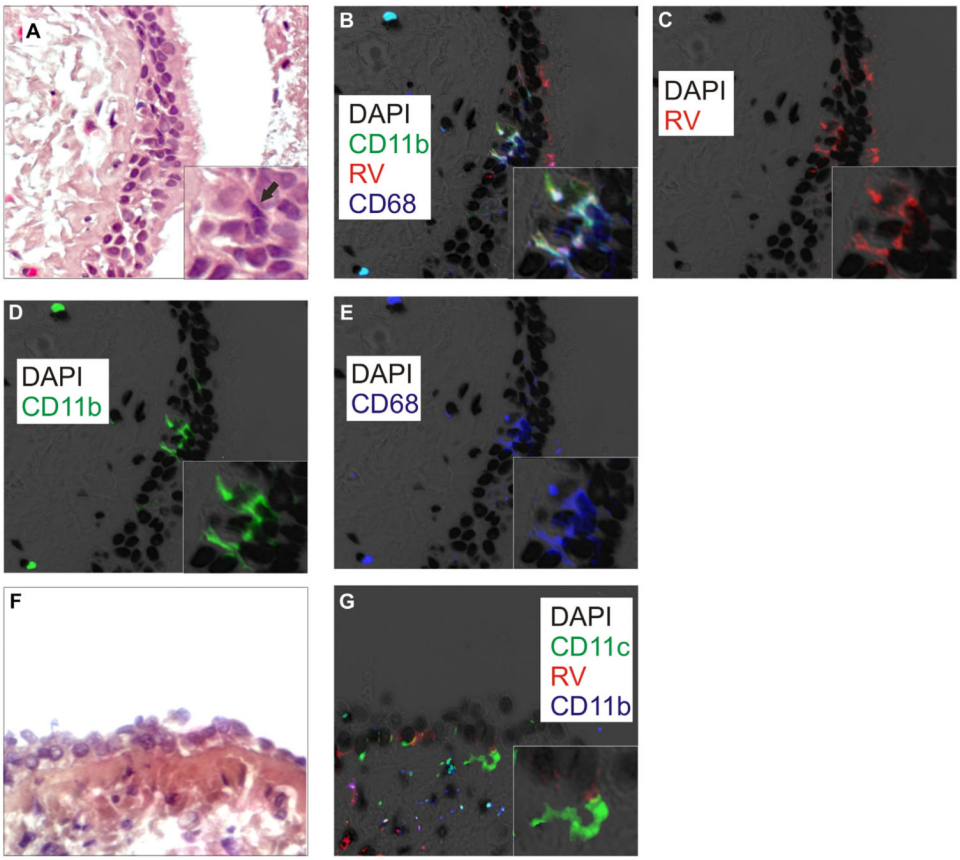

Fig 2.

Asthmatic tissue stained with DAPI *(black)*, R16-7 *(red)*, anti-CD11b *(green)*, and anti-CD68 *(blue)*. **A, F,** Hematoxylin and eosin. The *arrow* in A marks a corresponding RV16-, CD11b-, CD68-positive cell. **B,** Colocalization of CD11b and CD68 is *cyan*; colocalization of 3 markers is *white*. **C,** RV16. **D,** CD11b. **E,** CD68. (Original magnification, ×640; insets show group of costained cells.) **G,** Image showing RV *(red)*, CD11c *(green)*, and CD11b *(blue)*. CD11c-positive stellate cell underlying infected epithelial cell *(inset)*. *DAPI*, 4′-6-Diamidino-2-phenylindole, dihydrochloride.