Rhinovirus-Induced Macrophage Cytokine Expression Does Not Require Endocytosis or Replication

Thomas G. Saba^{1*}, Yutein Chung^{1*}, Jun Young Hong², Uma S. Sajjan¹, J. Kelley Bentley¹, and Marc B. Hershenson^{1,2}

Departments of ¹Pediatrics and Communicable Diseases and ²Molecular and Integrative Physiology, University of Michigan Medical School, Ann Arbor, Michigan

Abstract

Rhinovirus (RV) is responsible for the majority of virus-induced asthma exacerbations. We showed previously that RV infection of ovalbumin-sensitized and -challenged BALB/c mice induces production of type 2 cytokines from M2-polarized macrophages. In the present study, we sought to determine the mechanism of RV-induced cytokine expression. We infected bone marrow–derived macrophages (BMMs) from BALB/c mice with RV serotype 1B, a minor group virus that infects mouse cells. Selected cultures were pretreated with IL-4, a type 2 cytokine increased in allergic asthma. RV infection of untreated cells increased messenger RNA and protein expression of the M1 cytokines TNF- α , CXCL1, and IL-6 but failed to induce expression of the M2 cytokines CCL22 and CCL24. Cells pretreated with IL-4 showed decreased expression of M1 cytokines but increased expression of Ym-1, Arg-1 (M2 markers), CCL22, and CCL24. Infection with

ultraviolet (UV)-irradiated, replication-deficient RV elicited similar cytokine responses, suggesting that the outcome is replication independent. Consistent with this, viral RNA copy number did not increase in RV-treated BMMs or bronchoalveolar macrophages. RV-induced cytokine expression was not affected when cells were pretreated with cytochalasin D, suggesting that viral endocytosis is not required for the response. Finally, RV-induced cytokine expression and viral attachment were abolished in BMMs from myeloid differentiation factor 88 and Toll-like receptor (TLR)2 KO mice, suggesting a specific requirement of TLR2. We conclude that RV elicits a proinflammatory cytokine response in BMMs through a cell-surface-mediated, TLR2-dependent mechanism that does not require viral endocytosis or replication.

Keywords: asthma; exacerbation; M2 polarization; Toll-like receptor 2; virus

Viral-induced exacerbations are a major cause of morbidity in asthma. Viral infections account for approximately 80% of asthma exacerbations in children and for nearly 50% in adults (1, 2). Rhinovirus (RV), a single-stranded RNA virus belonging to the *Picornavirus* family, is consistently the most frequent pathogen identified. However, the precise mechanisms underlying RV-induced asthma exacerbations are not known.

Until recently, the only cell type shown to be infected by RV was the airway epithelial cell. However, it is conceivable that RV directly infects airway inflammatory

cells. Recent studies indicate that inoculation of monocytes with RV induces cytokine expression *in vitro* (3–8), although the level of viral replication in these cells is small or negligible. Recently, we demonstrated that, in ovalbumin (OVA)-sensitized and -challenged mice, RV colocalizes with eotaxin- and IL-4–positive lung macrophages (9), suggesting that lung macrophages may play a role in the airway inflammatory response to RV *in vivo*. Furthermore, prior allergen treatment changed the activation of lung macrophages to an M2 polarized state, thereby influencing their repertoire of

cytokine responses. Finally, we recently reported colocalization of RV and the macrophage marker CD68 in biopsies of experimentally infected humans with asthma (10). It is therefore conceivable that the enhanced inflammatory response to RV in patients with allergic asthma is due to viral stimulation of recruited, activated macrophages that are normally not present in control individuals.

In the present study, we sought to determine the cellular mechanisms underlying RV-induced cytokine expression in macrophages. Specifically, we asked what events in the viral life cycle—binding to the

(Received in original form August 8, 2013; accepted in final form November 26, 2013)

*These authors contributed equally to this work.

This work was supported by National Institutes of Health grants R01 HL081420 (M.B.H.) and T32 HD007513 (T.G.S.).

Correspondence and requests for reprints should be addressed to Marc B. Hershenson, M.D., University of Michigan Medical School, Medical Sciences Research Building 2, 1150 W. Medical Center Dr., Ann Arbor, MI 48109-5688. E-mail: mhershen@umich.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 50, Iss 5, pp 974–984, May 2014

Copyright © 2014 by the American Thoracic Society

Originally Published in Press as DOI: 10.1165/rcmb.2013-0354OC on December 10, 2013

Internet address: www.atsjournals.org

host cell, endocytosis, or RNA replication—are required and sufficient for cytokine production. To accomplish this, we used bone marrow–derived macrophages (BMMs), a model system that allows the study of relatively large numbers of homogeneous cells. BMMs were studied in the presence or absence of IL-4 to stimulate the M2 polarization. We found that, whereas RV undergoes entry into host macrophages, cytokine responses were independent of viral endocytosis or replication and were dependent on Toll-like receptor (TLR)2 signaling, defining a potential mechanism by which RV could induce exacerbations of asthma.

Materials and Methods

Additional details on the methods may be found in the online supplement.

Animals

Animal usage followed guidelines set forth in the Principles of Laboratory Animal Care from the National Society for Medical Research. Female BALB/c (age 6–8 wk), B6.129P2(SJL)-*Myd88^{tm1.1Def}/J* myeloid differentiation factor (MyD)88 knockout (KO), B6N.129S1-*Tlr3^{tm1Flv}/J* TLR3 KO, and B6.129-*Tlr2^{tm1Kir}/J* TLR2 KO mice were studied (Jackson Laboratories, Bar Harbor, ME).

Generation of RV

RV1B (ATCC, Manassas, VA) was concentrated, purified, and titered as described previously (11–13). RV1B was ultraviolet (UV) irradiated using a CL-1000 crosslinker (UVP, Upland, CA) at 100 mJ/cm² for 15 minutes on ice.

Isolation and Infection of BMMs

Bone marrow was isolated and cultured using L929 supernatants as a source of macrophage colony-stimulating factor (14). After 7 days, cells were infected with RV or with replication deficient, UV-irradiated RV (UV-RV) at a multiplicity of infection of 5.0 for 1 hour at 33°C. After removal of viral inoculum, fresh medium was added, and cells were incubated for 8 hours and then lysed with Trizol (Sigma-Aldrich, St. Louis, MO). A MyD88/TRIF KO macrophage cell line was provided by Dr. Gabriel Nunez (University of Michigan) (15, 16).

Isolation and Infection of Mouse Bronchoalveolar Lavage Macrophages

Mice were injected intraperitoneally with 200 µl of a 0.5 mg/ml solution of alum and endotoxin-free OVA or PBS (Sigma-Aldrich) on Days 1 and 7 and were treated intranasally with 50 µl of a 20 mg/ml OVA or PBS on Days 14, 15, and 16. Mice were killed, and adherent bronchoalveolar lavage (BAL) cells (> 85% macrophages) were extracted and stimulated with RV or UV-RV *ex vivo*. After 8 hours, cells were lysed with Trizol. Selected mice were inoculated with RV1B on the day of the last OVA treatment as described previously (9).

Cell Treatments before Infection

BMMs were polarized to an M2 phenotype by treatment with 30 ng/ml IL-4 (Peprotech, Rocky Hill, NJ) overnight before infection. To assess the role of phagocytosis, BMMs were pretreated with 2 µM cytochalasin D (Sigma-Aldrich), 1 µM brefeldin A, 30 nM latrunculin A (Tocris, Minneapolis, MN), or DMSO for 60 minutes. TLR2 blocking was achieved by adding 0.5 µg/ml of a monoclonal TLR2 antibody (eBioscience, San Diego, CA) or an isotype control antibody for 10 minutes before infection with RV or UV-RV.

Measurement of Cytokine Expression

BMM and BAL macrophages were analyzed for cytokine messenger RNA (mRNA) expression by quantitative real-time PCR. Signals were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the comparative CT (2^{-ΔΔCT}) method and expressed as fold-change versus GAPDH. Cell supernatants analyzed for cytokine protein by multiplex immune assay (Bio-Rad, Hercules, CA).

Presence of Viral RNA

Positive-strand viral RNA was measured by quantitative one-step, real-time PCR (17).

Confocal Fluorescence Microscopy

BMMs on collagen-coated glass slides were incubated with rat anti-mouse CD68 (AbD Serotec, Raleigh, NC), anti-TLR2 clone T2.5 (eBioscience), and partially purified antiserum against RV1B (ATTC) as described previously (18).

Flow Cytometry

Twenty-four hours after stimulation with RV, BMMs were harvested and stained with

fluorescent antibodies against CD11b, CD11c, CD86, CD80, and the mouse MHCII analog IA/IE (BioLegend, San Diego, CA). The fluorescence levels of fixed cells were analyzed using FACS CANTO (BD Biosciences, San Jose, CA).

Data Analysis

Data are represented as mean ± SEM. Statistical significance was assessed by one-way ANOVA and Student-Newman-Keuls multiple range test.

Results

IL-4 Induces M2 Polarization of BMMs and Enables RV-Induced Type 2 Cytokine Expression

BMMs from BALB/c mice were cultured overnight in the presence or absence of IL-4 and stimulated with RV or replication-deficient UV-RV for 8 hours. Expression of Ym-1 and Arg1 were significantly up-regulated in BMMs pretreated with IL-4 (Figures 1A and 1B), suggesting that IL-4 is sufficient for polarization to an alternatively activated M2 phenotype. In untreated BMMs, stimulation with RV or UV-RV significantly increased transcript levels of the M1 cytokines TNF-α, CXCL1, IL-6, and IL-12 (Figures 1C–1F). Replication-deficient, UV-irradiated RV has been previously shown to be sufficient for macrophage/monocyte and epithelial cell cytokine production, indicating that viral replication is not required for this process (5).

BMMs pretreated with IL-4 before RV infection showed significantly lower levels of M1 cytokines compared with control cells. On the other hand, unlike control cells after RV infection, BMMs pretreated with IL-4 showed significant up-regulation of IL-4, IL-13, CCL17, CCL22, and CCL24 after stimulation with RV or UV-RV (Figures 1G–1K). There was no effect of IL-4 or RV on TGF-β (Figure 1L), and cells did not express significant levels of CCL26 (not shown). CCL17, CCL22, CCL24, CCL26, and TGF-β expression has been associated with the alternatively activated M2a phenotype (19, 20). CCL17 (thymus- and activation-regulated chemokine), CCL22 (macrophage-derived chemokine), and CCL24 (eotaxin 2) are macrophage-derived chemokines that have been previously associated with human eosinophilic asthma (21–24).

Figure 1. Bone marrow–derived macrophages (BMMs) polarize to an M2 phenotype when exposed to IL-4. BMMs were extracted from bone marrow of healthy mice and expanded in L9 media for 7 days. Adherent BMMs were pretreated with PBS or 30 ng/ml IL-4 overnight and stimulated with live rhinovirus (RV)1B or ultraviolet (UV)-irradiated replication-deficient RV (UV-RV) at a multiplicity of infection of 5.0 for 8 hours. Supernatants and cell lysates were collected for quantitative PCR and ELISA. Signals were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the comparative CT ($2^{-\Delta\Delta CT}$) method and expressed as fold-change versus GAPDH. (A, B) IL-4 treatment increased expression of the M2 markers Ym-1 and Arg1. (C–F) Quantitative PCR results for prototypical M1 cytokines. (G–L) Quantitative PCR for M2 cytokines. (M–O) Protein levels from supernatants measured using ELISA ($n = 3$). *Different from PBS ($P < 0.05$). †Different from media ($P < 0.05$; one way ANOVA).

Protein levels of cytokines were measured 24 hours after stimulation with RV or UV-RV (Figures 1M–1O). Consistent with our transcript data, RV significantly increased TNF-α and CXCL1 levels in untreated BMMs, whereas RV increased the level of CCL22 in BMMs pretreated with IL-4.

Effects of RV on Cytokine Expression of BAL Cells from OVA-Sensitized and OVA-Challenged Mice

To determine whether data from RV-stimulated BMMs reflect macrophage function *in vivo*, BAL cells were harvested from mice that were sensitized and

challenged with OVA or treated with PBS as a control. Macrophages were selected by plastic adherence. Adherent BAL macrophages were subsequently stimulated with UV-RV or RV *ex vivo* for 8 hours. The M2 markers Ym-1 and Arg1 were highly expressed in BAL cells from

OVA-sensitized mice but not from PBS-treated mice (Figures 2A and 2B). Similar to the BMM data from untreated cells, expression of the M1 cytokines TNF- α and CXCL1 was up-regulated in BAL macrophages from PBS-treated mice stimulated with UV-RV or RV (Figures 2C and 2D). In contrast, BAL macrophages from OVA-sensitized mice showed decreased M1 cytokine responses and increased M2 cytokine responses after RV infection (Figures 2E–2H), similar to the

pattern seen in IL-4 treated BMMs. Protein levels of secreted M1 and M2 cytokines from BAL cells 24 hours after RV stimulation were mostly consistent with corresponding mRNA expression (Figures 2I and 2J). Overall, these results confirm our previous data characterizing the response of BAL macrophages to RV infection (9) and demonstrate that IL-4-treated BMMs may be used as a model to study the interaction of RV with alveolar macrophages.

Effects of IL-4 on BMM Surface Markers and Viral Replication

Because macrophages are professional antigen-presenting cells, we examined whether costimulatory molecules on macrophages are up-regulated in response to RV. Harvested BMMs were pretreated with IL-4, stimulated with RV, and stained with fluorescent antibodies against the antigen-presenting cell surface markers CD11c, CD11b, the mouse MHCII analog IA/IE, and CD86. Detection of surface markers was performed using flow cytometry. Control BMMs were high in CD11b and low in CD11c, IA/IE, and CD86 (Figures 3A–3D). In the presence of IL-4, CD11c, and IA/IE were up-regulated, whereas CD11b and CD86 were unaltered. RV infection had no significant effect on the surface markers tested. We confirmed this result with BAL macrophages from OVA-treated mice. Our results indicate that RV does not induce a phenotypic change in macrophages via surface costimulatory molecule up-regulation.

We next examined whether the proinflammatory responses elicited by RV are dependent on viral replication. Although it has been demonstrated that macrophages allow RV entry (3, 4, 7, 25), the ability of RV to replicate in macrophages remains controversial. BMMs were infected with RV or replication-deficient UV-RV. HeLa cells (a cell line permissive to RV1B) were used as a positive control for RV replication. At various time points up to 24 hours after RV infection, cell lysates and supernatants were harvested for RV-positive strand RNA and viral titer assays, respectively. UV irradiation prevented RV replication (Figure 3E). Infected HeLa cells showed a logarithmic increase in positive-strand viral RNA (Figure 3F). (As expected, there was no increase in vRNA expression in cells inoculated with UV-irradiated replication-deficient RV.) In contrast to HeLa cells, viral RNA levels in BMM infected with RV failed to increase. There was no effect of IL-4 pretreatment. Similar results were found in mouse BAL cells from PBS and OVA-treated mice (Figure 3H). To further characterize viral replication, a plaque assay was performed to identify the titer of live RV released into the supernatant. An increase in viral titer beginning at 12 hours after infection was observed in RV-infected HeLa supernatants, but not in the supernatants of BMMs (Figure 3G).

Figure 2. Similar to IL-4-exposed BMMs, lung macrophages from ovalbumin (OVA)-sensitized and -challenged mice show M2 polarization. Bronchoalveolar lavage (BAL) macrophages were collected from either PBS or OVA-sensitized and -challenged mice and allowed to adhere to plastic for 3 hours *ex vivo*. BAL macrophages were then stimulated with RV1B and UV-irradiated RV1B as described in Figure 1. Messenger RNA (mRNA) signals were normalized to GAPDH using the comparative CT ($2^{-\Delta\Delta CT}$) method and expressed as fold-change versus GAPDH. (A, B) Cells from OVA-treated mice showed increased mRNA expression of Ym-1 and Arg-1, indicating M2 polarization. (C, D) Quantitative PCR results for prototypical M1 cytokines. (E–H) Quantitative PCR results for the M2 cytokines CCL22 and CCL24. (I, J) Protein levels from supernatants measured using ELISA ($n = 3$). *Different from PBS ($P < 0.05$). †Different from media ($P < 0.05$; one way ANOVA).

Figure 3. Effects of IL-4 on BMM surface markers and viral replication. (A–D) BMMs were pretreated with or without IL-4 and then stimulated with RV as described in Figure 1. Cells were fixed and stained with fluorescent antibodies against CD11c (A), CD11b (B), the mouse MHCII homolog IA/IE (C), and CD86 (D) and analyzed using flow cytometry. Results shown are representative of three individual experiments. *Closed black regions* = isotype control; *solid gray line* = PBS-pretreated, sham-infected BMMs; *dashed gray line* = PBS RV; *solid black line* = IL-4 sham; *dashed black line* = IL-4 RV. (E–H) Cell lysate and supernatants from BMMs, HeLa cells, and BAL macrophages were infected with RV1B at a multiplicity of infection of 5.0 and harvested at 1, 4, 6, 8, 12, and 24 hours after infection for measurement of positive-strand RV by quantitative PCR and viral titer by plaque assay. (E) Viral titers of two batches of RV compared with UV-irradiated virus. (F) HeLa cells show increased positive-strand RV compared with PBS and IL-4-pretreated BMMs. As expected, there was no increase in vRNA expression in cells inoculated with UV-irradiated replication-deficient RV. (G) BAL macrophages from PBS- and OVA-treated mice show no increase in RV copy number per μg of total RNA after RV infection. (H) HeLa cell but not BMM supernatants show increased viral titer after RV infection. Results shown are representative of three individual experiments. PFU, plaque-forming units.

Overall, our results demonstrate that RV elicits proinflammatory responses from BMMs without viral replication, consistent with our results above, showing similar cytokine responses to intact RV and replication deficient UV-RV.

BMM Production of Proinflammatory Cytokines in Response to RV Occurs Independently of Viral Endocytosis

Our data suggest that RV stimulates macrophages to produce proinflammatory cytokines independently of viral replication. To determine whether viral binding to the cell surface is sufficient for the RV-induced response, we pretreated BMMs with cytochalasin D, an agent that inhibits F-actin polymerization. To confirm that cytochalasin blocked viral entry, we infected vehicle and cytochalasin-treated BMMs

and stained them with fluorescent antibodies against viral capsid protein and CD68 (a macrophage marker). Confocal microscopy images showed substantially fewer RV particles within BMMs pretreated with cytochalasin compared with cells pretreated with a vehicle control (Figure 4A), suggesting that cytochalasin successfully blocked viral entry. We examined the effect of cytochalasin D pretreatment on the production of cytokines by BMMs in response to RV. mRNA expression of TNF- α and CXCL1 did not decrease in cells that were pretreated with cytochalasin (Figures 4B and 4C). Furthermore, cytokine expression was nearly equivalent for UV-RV- and RV-treated cells. Cytochalasin had modest inhibitory effects on CXCL1 protein secretion (Figure 4D). In IL-4-treated cells,

cytochalasin had modest inhibitory effects on RV-induced expression of CCL22 and was associated with increased CCL24 (Figures 4E and 4F). Finally, the endocytosis inhibitors brefeldin A and latrunculin similarly failed to attenuate RV-induced cytokine expression (Figure 4G). These data indicate that, although bound RV is internalized by macrophages, internalization is not necessary for RV-induced cytokine production in BMMs.

We also assessed the effect of cytochalasin D on RV-induced cytokine production in BAL macrophages from OVA-sensitized and -challenged mice. BAL macrophages isolated from OVA-treated mice and infected with RV *ex vivo* produce similar levels of proinflammatory cytokines in the presence or absence of cytochalasin pretreatment, similar to

Figure 4. RV stimulation of BMM cytokine production does not require phagocytosis/endocytosis. BMMs were pretreated with 2 μ M cytochalasin D (cytoD) or DMSO vehicle control for 45 minutes before RV stimulation at a multiplicity of infection of 5.0. (A) Confocal microscopy images of BMMs stimulated with RV for 30 minutes after DMSO or cytoD treatment (red, CD68; green, RV1B; blue, DAPI; white bar is 20 μ m). After infection, DMSO-treated cells show RV on the cell surface (colocalization with CD68 appears yellow-orange). Below the cell surface, cytoplasmic RV appears light blue. CytoD-treated cells show RV on the cell surface but not below. Results shown are representative of three individual experiments. (B–D) Quantitative PCR and ELISA analysis of RV1B-induced cytokine mRNA and protein expression in the presence or absence of cytoD treatment. mRNA signals were normalized to GAPDH using the comparative CT ($2^{-\Delta\Delta CT}$) method and expressed as fold-change versus GAPDH. (E–G) In IL-4-treated cells, cytochalasin had no effect on RV-induced expression of CCL22 or CCL24 ($n = 9$). *Different from PBS ($P < 0.05$). †Different from media ($P < 0.05$; one-way ANOVA). (H) Brefeldin A and latrunculin A failed to inhibit TNF- α -induced cytokine expression ($n = 3$). *Different from DMSO ($P < 0.05$; one-way ANOVA).

BMMs (Figure 5). Taken together, these data suggest that cell surface interactions between RV and macrophages (i.e., viral attachment) are sufficient for proinflammatory cytokine responses.

RV-Induced Cytokine Responses Are Dependent on TLR2/MyD88 Signaling and Independent of TLR3

We next determined which cell surface receptors are responsible for sensing RV in the context of RV-induced cytokine production. Members of the TLR family are involved in the sensing of RV by mammalian host cells (26, 27). Furthermore, we have found that inhibition

of TLR2 prevents RV-induced IRAK-1 degradation in airway epithelial cells (28). To test this, we examined the RV-induced responses of BMMs from MyD88/TRIF double KO mice. mRNA expression of TNF- α and IL-6 was up-regulated in RV- and UV-RV-stimulated BMMs from wild-type (WT) mice but not in cells from MyD88/TRIF double KO mice (Figures 6A and 6B). Similar results were found in MyD88 KO cells (Figures 6C and 6D). In contrast, polyI:C-induced cytokine expression was not blocked in MyD88 KO cells (Figure 6E), consistent with the notion that cytokine expression was independent of TRIF and TLR3. IL-

4-treated cells from MyD88 KO mice also showed reduced CCL22 and CCL24 expression (Figures 6F and 6G).

We found that RV does not replicate within BMMs, suggesting that TLR3, a receptor for viral double-stranded RNA, which is formed during viral replication (29), is not required for RV-induced cytokine production by BMMs. To test this, we measured cytokine mRNA expression in RV-infected BMMs from TLR3 KO mice (Figure 6H). Compared with cells from WT mice, there was no reduction in TNF- α mRNA expression. As expected, polyI:C-induced TNF- α mRNA expression was decreased in the TLR3 KO cells (Figure 6I).

Figure 5. RV stimulation of BAL macrophage cytokine production does not require phagocytosis/endocytosis. BAL macrophages harvested from OVA-sensitized and -challenged mice were allowed to adhere to plastic. Adherent cells were then pretreated with DMSO or 2 μ M cytoD for 45 minutes before RV stimulation. (A–D) Quantitative PCR analysis of cytokine mRNA production. mRNA signals were normalized to GAPDH using the comparative CT ($2^{-\Delta\Delta CT}$) method and expressed as fold-change versus GAPDH. (E, F) Cell supernatant cytokine levels assessed by ELISA ($n = 3$). *Different from PBS ($P < 0.05$). †Different from media ($P < 0.05$; one-way ANOVA).

To test whether TLR2 is required for RV-induced cytokine production, cells were pretreated with a monoclonal antibody against TLR2 or isotype-control antibody before infection with RV. RV-induced production of TNF- α , CXCL1, IL-6, and IL-1 β was nearly abolished in the presence of the anti-TLR2 antibody (Figures 7A–7D). Cytokine production in response to PAM3cys, a known TLR2 agonist, was also abolished by the antibody, whereas responses generated by polyI:C, a TLR3 agonist, remained unchanged (data not shown). CCL22 mRNA expression by IL-4-treated cells was also partially blocked by anti-TLR2, but there was no effect on CCL24 (Figures 7E and 7F). To examine this point further, we examined BMMs isolated from TLR2 KO mice. RV-induced CXCL1, CCL22, and CCL24 expression were all blocked in the TLR2 null BMMs (Figures 7G–I). Thus, virus-induced type 1 and type 2 cytokine responses from M1- and M2-polarized macrophages were each dependent on TLR2 binding.

To further characterize the role of TLR2 in binding of RV to BMMs, cells were pretreated with anti-TLR2 antibody or

isotype control and then exposed to RV for 30 minutes. Fluorescence microscopy images illustrated that, in the absence of anti-TLR2, many cells showed colocalization of RV and CD68 on the cell surface or in the cytoplasm (Figure 7J). However, in the presence of the anti-TLR2, there was a substantial loss of RV capsid protein (Figure 7K), indicating a reduction in RV attachment and internalization. Finally, immunofluorescence staining of lung sections from OVA-sensitized and -challenged mice infected with RV1B showed colocalization of TLR2 and RV in epithelial cells and CD68-positive macrophages (Figures 7L–7O). Together with the cytokine data presented above, these results show that TLR2 expressed on the surface of macrophages serves as a platform for RV attachment and subsequent production of proinflammatory cytokines.

Discussion

RV is responsible for the majority of virus-induced asthma exacerbations (1, 2).

However, the precise mechanisms underlying RV-induced asthma exacerbations are not known. Recently, we demonstrated that, in allergen-sensitized and -challenged mice, RV colocalizes with eotaxin- and IL-4-positive lung macrophages (9), suggesting that lung macrophages may play a role in the airway inflammatory response to RV *in vivo*. Furthermore, prior allergen treatment changed the activation of lung macrophages to an M2 polarized state. In the present study, we sought to determine the mechanisms underlying RV-induced cytokine expression in BMMs, a commonly used model for the study of resident macrophages in the innate immune system. Using BMMs as our model system, we showed that, although RV is internalized by macrophages, the RV-induced proinflammatory responses occur through a cell surface-mediated, replication-independent mechanism that is dependent on TLR2/MyD88 signaling.

Several studies have examined the infection of monocytic cells by RV *in vitro* (3–8). A small amount of viral replication has been noted in RV-infected peripheral blood monocyte-derived macrophages but not in BAL-derived macrophages (3, 4). Nevertheless, in these studies, *ex vivo* exposure to UV-irradiated, replication-deficient RV induced similarly robust production of TNF- α (3) and IP-10 (6). In our study, positive-strand viral RNA and viral titer failed to increase in viral-infected BMMs as they did in HeLa cells. However, cytokine responses remained robust in cells infected with UV-irradiated, replication-deficient virus. Taken together, these data indicate that RV does not replicate inside BMMs and that RV-induced cytokine responses are independent of viral replication. In addition, we show for the first time that cytochalasin D, an inhibitor of endocytosis, fails to block cytokine expression. Brefeldin A and latrunculin A had similar effects, demonstrating that internalization of virus is similarly not required for RV-induced BMM responses.

TLRs are an essential component of the innate immune system's ability to detect viral pathogens. TLR3 and TLR7/8, located in endosomes, respond to viral double-stranded and single-stranded RNA, respectively. All TLRs signal through the adaptor protein MyD-88 except for TLR3, which signals through TIR-domain

Figure 6. RV-induced cytokine production from BMMs is dependent on myeloid differentiation factor (MyD)88 and independent of Toll-like receptor (TLR)3 signaling. (A, B) BMMs were isolated from MyD88/TRIF double knockout (KO) BALB/c mice, expanded, and stimulated with RV1B or UV-RV. mRNA levels were assessed using quantitative PCR (qPCR). mRNA signals were normalized to GAPDH using the comparative CT (2^{-ΔΔCT}) method and expressed as fold-change versus GAPDH. (C, D) BMMs were isolated from wild-type (WT) or MyD88 KO mice and stimulated with RV1B, UV-RV, or polyI:C. TNF-α mRNA levels were assessed by qPCR. (E–G) IL-4-treated BMMs from MyD88 KO mice also showed reduced cytokine expression ($n = 3–9$). *Different from PBS ($P < 0.05$; one-way ANOVA). (H–J) BMMs isolated from WT or TLR3 KO mice were stimulated with polyI:C, and cytokine mRNA levels were assessed by qPCR ($n = 9$). *Different from PBS ($P < 0.05$). †Different from media ($P < 0.05$; one-way ANOVA).

containing adapter-inducing interferon β (TRIF). Cytoplasmic dsRNA is also recognized by RNA helicases, such as the melanoma-differentiation-associated 5 gene and retinoic acid-inducible gene I (26, 27, 30). In the present study, cytokine expression in BMMs from MyD88/TRIF KO mice was significantly lower compared with BMMs from WT mice, indicating the requirement for TLRs. RV- but not polyI:C-induced cytokine production was also reduced in BMMs from MyD88 KO mice, suggesting that MyD88 is required for RV-induced signaling. Finally, RV-induced cytokine expression was unaffected in BMMs from TLR3 KO mice, confirming that TLR3 is dispensible for RV-induced macrophage responses.

Recent evidence suggests that TLR2 plays a role in RV recognition. TLR2 expression is induced in RV-stimulated

airway epithelial cells, and siRNA against TLR2 decreased RV-induced IL-6 and IL-8 cytokine expression (28, 31). In the present study, based on studies in cells from TLR2 KO mice, we show for the first time that TLR2 is required for the cytokine response to RV in cells of monocyte/macrophage origin. Furthermore, TLR2 receptor antibody blocked viral attachment as assessed by fluorescence microscopy. Because TLR2 is localized to the cell surface, these data are consistent with our findings that the RV responses do not require viral endocytosis. Furthermore, these data suggest that macrophage TLR2 recognizes and responds to a molecular pattern on the viral capsid. Although the picornaviruses are nonenveloped, they interact with cellular proteins during entry into the cell and virus particle assembly. For example, RV and other picornavirus

particles are specifically modified by having myristic acid covalently bound to a capsid protein (32). The interaction between TLR2 and the LDL family receptors, the canonical minor group viral receptors, is unclear. Other respiratory viruses have been shown to interact with TLR2. Macrophages from TLR2 KO mice produce lower levels of intracellular TNF- α than WT cells after RSV infection (33), suggesting a major role for TLR2 in the induction of proinflammatory cytokines after RSV stimulation. Although it has been demonstrated that the RSV F protein binds with TLR4 (34), the precise interaction with TLR2 has not been defined. On the other hand, in contrast to TLR4 null mice, which are protected from influenza-induced mortality, TLR2 null mice show a similar susceptibility to mouse-adapted influenza virus infection as WT mice (35).

Figure 7. TLR2 is required for RV-induced cytokine expression in BMMs. (A–F) BMMs were treated with sham or RV. BMMs were pretreated with 1 μ g/ml anti-TLR2 blocking antibody or isotype control for 30 minutes before infection with RV1B or UV-RV. mRNA expression was measured by qPCR. mRNA signals were normalized to GAPDH using the comparative CT ($2^{-\Delta\Delta CT}$) method and expressed as fold-change versus GAPDH. In control (A–D) and IL-4-treated cells (E, F), TLR2 blockade caused inhibition of cytokine mRNA expression ($n = 3$). *Different from IgG ($P < 0.05$). [†]Different from media ($P < 0.05$; one-way ANOVA). (G–I) BMMs were isolated from TLR2 KO mice, expanded, and stimulated with RV1B or UV-RV. TLR2 KO abolished RV-induced CXCL1, CCL22, and CCL24 mRNA expression. (J, K) Confocal images of BMMs pretreated with 1 μ g/ml isotype control antibody or anti-TLR2 blocking antibody 30 minutes before infection with RV at a multiplicity of infection of 5.0 (RV1B, red; CD68, green; DAPI, blue). (J) Isotype antibody-treated cells show RV on the cell surface (red or, colocalizing with CD68, yellow). (K) Anti-TLR2 treated cells show decreased RV on the cell surface. (L–O) Immunofluorescent images of an airway from an OVA-treated, RV-infected mouse (RV1B, red; TLR2, blue; CD68, green; white bar is 50 μ m). Merged image (L) shows colocalization of TLR2 and RV in airway epithelial cells (magenta) and macrophages (white).

We have previously demonstrated that adherent BAL cells from an OVA-sensitized and -challenged mice express higher levels of the M2 macrophage markers Arg-1, Mgl-2, and Ym-1. Inoculation of BAL cells induced the expression of type 2 cytokines, including eotaxin, IL-4, and IL-13, in contrast to cells from PBS-treated mice, which preferentially expressed TNF- α (9). RV infection of OVA-treated mice increased BAL concentrations of IL-4, consistent with previous studies showing that IL-4 drives macrophages to differentiate into an M2 or alternatively activated state (36, 37). To test this, we inoculated BMMs with RV in the absence or presence of IL-4. Untreated BMMs expressed the type 1 cytokines TNF- α , CXCL1, and IL-6. In contrast, in the presence of IL-4, BMMs expressed the alternative activation markers Ym-1 and Arg-1 and, in response to RV stimulation, the type 2 cytokines CCL17, CCL22, and CCL24. These macrophage-derived

chemokines have been shown to be involved in the recruitment of Th2 cells and eosinophils (23, 24, 38, 39). The results of IL-4 pretreatment of BMMs paralleled those of BAL macrophages from OVA-sensitized mice. Together, these data suggest that IL-4 is sufficient to induce macrophage alternative activation, thereby skewing the cytokine expression profile in response to RV infection.

Macrophages may play a role in antigen presentation and activation of naive T cells. Macrophages regulate the T-cell response via presentation of antigen fragments on MHC class II as well as and other costimulatory molecules, such as CD80 (B7-1) and CD86 (B7-2). Previous studies have shown alveolar macrophage HLA-DR and HLA-B7 isoform up-regulation in asthma and in response to IL-4 exposure (40–42). We examined the expression of BMM cell surface markers by flow cytometry. Whereas IL-4 increased the expression of CD11c and MHCII in both

BMMs, there was no change in the expression of CD11b, CD11c, MHCII, and CD86 after RV exposure. Thus, unlike IL-4, RV exposure does not appear to enhance the antigen-presenting or T-cell-activating capacity of mature macrophages.

In conclusion, RV elicits a proinflammatory cytokine response in BMMs through a cell-surface-mediated, TLR2-dependent mechanism that does not require viral endocytosis or replication. Interestingly, TLR2 variants have been associated with in atopic wheezing (43) and asthma development (44) in children. Additional studies examining RV-induced macrophage responses may provide insight into viral-induced asthma exacerbations. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Dr. Gabriel Nunez (University of Michigan Medical School) for his gift of a mouse macrophage MyD88/TRIF knockout cell line.

References

1. Johnston SL, Pattemore PK, Sanderson G, Smith S, Lampe F, Josephs L, Symington P, O'Toole S, Myint SH, Tyrrell DA, *et al.* Community study of role of viral infections in exacerbations of asthma in 9–11 year old children. *BMJ* 1995;310:1225–1229.
2. Nicholson KG, Kent J, Ireland DC. Respiratory viruses and exacerbations of asthma in adults. *BMJ* 1993;307:982–986.
3. Gern JE, Dick EC, Lee WM, Murray S, Meyer K, Handzel ZT, Busse WW. Rhinovirus enters but does not replicate inside monocytes and airway macrophages. *J Immunol* 1996;156:621–627.
4. Laza-Stanca V, Stanciu LA, Message SD, Edwards MR, Gern JE, Johnston SL. Rhinovirus replication in human macrophages induces NF- κ B-dependent tumor necrosis factor alpha production. *J Virol* 2006;80:8248–8258.
5. Hall DJ, Bates ME, Guar L, Cronan M, Korpi N, Bertics PJ. The role of p38 MAPK in rhinovirus-induced monocyte chemoattractant protein-1 production by monocytic-lineage cells. *J Immunol* 2005;174:8056–8063.
6. Korpi-Steiner NL, Bates ME, Lee W-M, Hall DJ, Bertics PJ. Human rhinovirus induces robust IP-10 release by monocytic cells, which is independent of viral replication but linked to type I interferon receptor ligation and STAT1 activation. *J Leukoc Biol* 2006;80:1364–1374.
7. Johnston SL, Papi A, Monick MM, Hunninghake GW. Rhinoviruses induce interleukin-8 mRNA and protein production in human monocytes. *J Infect Dis* 1997;175:323–329.
8. Khaitov MR, Laza-Stanca V, Edwards MR, Walton RP, Rohde G, Contoli M, Papi A, Stanciu LA, Kotenko SV, Johnston SL. Respiratory virus induction of alpha-, beta- and lambda-interferons in bronchial epithelial cells and peripheral blood mononuclear cells. *Allergy* 2009;64:375–386.
9. Nagarkar DR, Bowman ER, Schneider D, Wang Q, Shim J, Zhao Y, Linn MJ, McHenry CL, Gosangi B, Bentley JK, *et al.* Rhinovirus infection of allergen-sensitized and -challenged mice induces eotaxin release from functionally polarized macrophages. *J Immunol* 2010;185:2525–2535.
10. Bentley JK, Sajjan US, Dzaman MB, Jarjour NN, Lee W-M, Gern JE, Hershenson MB. Rhinovirus colocalizes with CD68- and CD11b-positive macrophages after experimental infection in humans. *J Allergy Clin Immunol* 2013;758–761:2013.
11. Papi A, Johnston SL. Rhinovirus infection induces expression of its own receptor intercellular adhesion molecule 1 (ICAM-1) via increased NF-kappaB-mediated transcription. *J Biol Chem* 1999;274:9707–9720.
12. Newcomb DC, Sajjan U, Nanua S, Jia Y, Goldsmith AM, Bentley JK, Hershenson MB. Phosphatidylinositol 3-kinase is required for rhinovirus-induced airway epithelial cell interleukin-8 expression. *J Biol Chem* 2005;280:36952–36961.
13. Martin S, Casasnovas JM, Staunton DE, Springer TA. Efficient neutralization and disruption of rhinovirus by chimeric ICAM-1/immunoglobulin molecules. *J Virol* 1993;67:3561–3568.
14. Weischenfeldt J, Porse B. Bone marrow-derived macrophages (BMM): isolation and applications. *CSH Protoc* 2008;2008:pdb.prot5080.
15. Nagpal K, Plantinga TS, Sirois CM, Monks BG, Latz E, Netea MG, Golenbock DT. Natural loss-of-function mutation of the myeloid differentiation protein 88 disrupts its ability to form Myddosomes. *J Biol Chem* 2011;286:11875–11882.
16. Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, Rock KL, Fitzgerald KA, Latz E. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol* 2008;9:847–856.
17. Contoli M, Message SD, Laza-Stanca V, Edwards MR, Wark PA, Bartlett NW, Keadze T, Mallia P, Stanciu LA, Parker HL, *et al.* Role of deficient type III interferon-lambda production in asthma exacerbations. *Nat Med* 2006;12:1023–1026.
18. Newcomb DC, Sajjan US, Nagarkar DR, Wang Q, Nanua S, Zhou Y, McHenry CL, Hennrick KT, Tsai WC, Bentley JK, *et al.* Human rhinovirus 1B exposure induces phosphatidylinositol 3-kinase-dependent airway inflammation in mice. *Am J Respir Crit Care Med* 2008;177:1111–1121.
19. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. *Front Biosci* 2008;13:453–461.
20. Krausgruber T, Blazek K, Smallie T, Alzabin S, Lockstone H, Sahgal N, Hussell T, Feldmann M, Udalova IA. IRF5 promotes inflammatory macrophage polarization and Th1-Th17 responses. *Nat Immunol* 2011;12:231–238.
21. Ying S, O'Connor B, Ratoff J, Meng Q, Mallett K, Cousins D, Robinson D, Zhang G, Zhao J, Lee TH, *et al.* Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with

- expression of Th2-attracting chemokines and disease severity. *J Immunol* 2005;174:8183–8190.
22. Ravensberg AJ, Ricciardolo FLM, van Schadewijk A, Rabe KF, Sterk PJ, Hiemstra PS, Mauad T. Eotaxin-2 and eotaxin-3 expression is associated with persistent eosinophilic bronchial inflammation in patients with asthma after allergen challenge. *J Allergy Clin Immunol* 2005;115:779–785.
 23. Crapster-Pregont M, Yeo J, Sanchez RL, Kuperman DA. Dendritic cells and alveolar macrophages mediate IL-13-induced airway inflammation and chemokine production. *J Allergy Clin Immunol* 2012;129:1621–1627, e3.
 24. Staples KJ, Hinks TSC, Ward JA, Gunn V, Smith C, Djukanović R. Phenotypic characterization of lung macrophages in asthmatic patients: overexpression of CCL17. *J Allergy Clin Immunol* 2012;130:1404–1412, e7.
 25. Oliver BG, Lim S, Wark P, Laza-Stanca V, King N, Black JL, Burgess JK, Roth M, Johnston SL. Rhinovirus exposure impairs immune responses to bacterial products in human alveolar macrophages. *Thorax* 2008;63:519–525.
 26. Wang Q, Miller DJ, Bowman ER, Nagarkar DR, Schneider D, Zhao Y, Linn MJ, Goldsmith AM, Bentley JK, Sajjan US, et al. MDA5 and TLR3 initiate pro-inflammatory signaling pathways leading to rhinovirus-induced airways inflammation and hyperresponsiveness. *PLoS Pathog* 2011;7:e1002070.
 27. Wang Q, Nagarkar DR, Bowman ER, Schneider D, Gosangi B, Lei J, Zhao Y, McHenry CL, Burgens RV, Miller DJ, et al. Role of double-stranded RNA pattern recognition receptors in rhinovirus-induced airway epithelial cell responses. *J Immunol* 2009;183:6989–6997.
 28. Unger BL, Faris AN, Ganesan S, Comstock AT, Hershenson MB, Sajjan US. Rhinovirus attenuates non-typeable *Hemophilus influenzae*-stimulated IL-8 responses via TLR2-dependent degradation of IRAK-1. *PLoS Pathog* 2012;8:e1002969.
 29. Sha Q, Truong-Tran AQ, Plitt JR, Beck LA, Schleimer RP. Activation of airway epithelial cells by toll-like receptor agonists. *Am J Respir Cell Mol Biol* 2004;31:358–364.
 30. Slater L, Bartlett NW, Haas JJ, Zhu J, Message SD, Walton RP, Sykes A, Dahdaleh S, Clarke DL, Belvisi MG, et al. Co-ordinated role of TLR3, RIG-I and MDA5 in the innate response to rhinovirus in bronchial epithelium. *PLoS Pathog* 2010;6:e1001178.
 31. Triantafilou K, Vakakis E, Richer EA, Evans GL, Villiers JP, Triantafilou M. Human rhinovirus recognition in non-immune cells is mediated by Toll-like receptors and MDA-5, which trigger a synergetic pro-inflammatory immune response. *Virulence* 2011;2:22–29.
 32. Hellstrom M, Kalén M, Lindahl P, Abramsson A, Betsholtz C. Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development* 1999;126:3047–3055.
 33. Murawski MR, Bowen GN, Cerny AM, Anderson LJ, Haynes LM, Tripp RA, Kurt-Jones EA, Finberg RW. Respiratory syncytial virus activates innate immunity through Toll-like receptor 2. *J Virol* 2009;83:1492–1500.
 34. Kurt-Jones EA, Popova L, Kwinn L, Haynes LM, Jones LP, Tripp RA, Walsh EE, Freeman MW, Golenbock DT, Anderson LJ, et al. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol* 2000;1:398–401.
 35. Shirey KA, Lai W, Scott AJ, Lipsky M, Mistry P, Pletneva LM, Karp CL, McAlees J, Gioannini TL, Weiss J, et al. The TLR4 antagonist Eritoran protects mice from lethal influenza infection. *Nature* 2013;497:498–502.
 36. Stein M, Keshav S, Harris N, Gordon S. Interleukin 4 potentially enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med* 1992;176:287–292.
 37. Muller U, Stenzel W, Kohler G, Werner C, Polte T, Hansen G, Schutze N, Straubinger RK, Blessing M, McKenzie AN, et al. IL-13 induces disease-promoting type 2 cytokines, alternatively activated macrophages and allergic inflammation during pulmonary infection of mice with *Cryptococcus neoformans*. *J Immunol* 2007;179:5367–5377.
 38. Yamashita U, Kuroda E. Regulation of macrophage-derived chemokine (MDC, CCL22) production. *Crit Rev Immunol* 2002;22:105–114.
 39. Ben-Yehuda C, Bader R, Puxeddu I, Levi-Schaffer F, Breuer R, Berkman N. Airway eosinophil accumulation and eotaxin-2/CCL24 expression following allergen challenge in BALB/c mice. *Exp Lung Res* 2008;34:467–479.
 40. Tang C, Ward C, Reid D, Bish R, O'Byrne PM, Walters EH. Normally suppressing CD40 coregulatory signals delivered by airway macrophages to TH2 lymphocytes are defective in patients with atopic asthma. *J Allergy Clin Immunol* 2001;107:863–870.
 41. Hofer MF, Jirapongsananuruk O, Trumble AE, Leung DY. Upregulation of B7.2, but not B7.1, on B cells from patients with allergic asthma. *J Allergy Clin Immunol* 1998;101:96–102.
 42. Agea E, Forenza N, Piattoni S, Russano A, Monaco A, Flenghi L, Bistoni O, Gillies DA, Azuma M, Bertotto A, et al. Expression of B7 co-stimulatory molecules and CD1a antigen by alveolar macrophages in allergic bronchial asthma. *Clin Exp Allergy* 1998;28:1359–1367.
 43. Eder W, Klimecki W, Yu L, von Mutius E, Riedler J, Braun-Fahrlander C, Nowak D, Martinez FD. Toll-like receptor 2 as a major gene for asthma in children of European farmers. *J Allergy Clin Immunol* 2004;113:482–488.
 44. Custovic A, Rothers J, Stern D, Simpson A, Woodcock A, Wright AL, Nicolaou NC, Hankinson J, Halonen M, Martinez FD. Effect of day care attendance on sensitization and atopic wheezing differs by Toll-like receptor 2 genotype in 2 population-based birth cohort studies. *J Allergy Clin Immunol* 2011;127:390–397, e1–9.