Toll-like receptor 2–expressing macrophages are required and sufficient for rhinovirus-induced airway inflammation

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Background: We have shown that rhinovirus, a cause of asthma exacerbation, colocalizes with CD68$^+$ and CD11b$^+$ airway macrophages after experimental infection in human subjects. We have also shown that rhinovirus-induced cytokine expression is abolished in Toll-like receptor (TLR2)$^{-/-}$ bone marrow–derived macrophages.

Objective: We hypothesize that TLR2$^+$ macrophages are required and sufficient for rhinovirus-induced airway inflammation *in vivo*.

Methods: Naive and ovalbumin (OVA)–sensitized and challenged C57BL/6 wild-type and TLR2$^{-/-}$ mice were infected with RV1B, followed by IgG or anti-TLR2, to determine the requirement and sufficiency of TLR2 for rhinovirus-induced airway responses. Bone marrow chimera experiments using OVA-treated C57BL/6 and TLR2$^{-/-}$ mice were also performed. Finally, naive TLR2$^{-/-}$ mice underwent intranasal transfer of bone marrow–derived wild-type macrophages.

Results: RV1B infection of naive wild-type mice induced an influx of airway neutrophils and CD11b$^+$ exudative macrophages, which was reduced in TLR2$^{-/-}$ mice. After allergen exposure, rhinovirus-induced neutrophilic and eosinophilic airway inflammation and hyperresponsiveness were reduced in TLR2$^{-/-}$ and anti-TLR2–treated mice. Transfer of TLR2$^{-/-}$ bone marrow into wild-type, OVA-treated C57BL/6 mice blocked rhinovirus-induced airway responses, whereas transfer of wild-type marrow to TLR2$^{-/-}$ mice restored them. Finally, transfer of wild-type macrophages to naive TLR2$^{-/-}$ mice was sufficient for neutrophilic inflammation after rhinovirus infection, whereas macrophages treated with IL-4 (to induce M2 polarization) were sufficient for eosinophilic inflammation, mucous metaplasia, and airways hyperresponsiveness.

Conclusions: TLR2 is required for early inflammatory responses induced by rhinovirus, and TLR2$^+$ macrophages are sufficient to confer airway inflammation to TLR2$^{-/-}$ mice, with the pattern of inflammation depending on the macrophage activation state. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

***Key words:** Alternative activation, asthma, CD11b, exacerbation, M2 macrophage*

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Rhinovirus is the most common cause of asthma exacerbations in children and adults. However, the precise mechanisms by which rhinovirus induces disease exacerbation are not completely known. Although airway epithelial cells are thought to be a major target of rhinovirus, infection of epithelial cells is spotty after experimental infection.[1] Cytokine-producing cells of the monocyte/macrophage lineage can also interact with rhinovirus. We have noted colocalization of rhinovirus with CD68, a sialoadhesin associated with macrophage lineage cells in the lungs of ovalbumin (OVA)–sensitized and OVA-challenged rhinovirus-infected mice[2] and human asthmatic patients experimentally infected with rhinovirus.[3] In OVA-treated mice depletion of macrophages decreased rhinovirus-induced airway inflammation and hyperresponsiveness, suggesting that macrophages play a direct role in the response to rhinovirus.[2] In this model rhinovirus infection induces an influx of CD45$^+$CD68$^+$F4/80$^+$Ly6c$^+$CD11b^{high} inflammatory monocytes and exudative macrophages into the lung, which, if preceded by OVA treatment, express the M2 alternate activation markers CD206 and CD301 and produce IL-13 and other type 2 cytokines.[4]

Pattern-recognition receptors, including members of the Toll-like receptor (TLR) family, appear to play a key role in sensing rhinovirus infection. Inhibition of TLR3, an endosomal receptor that interacts with double-stranded RNA, decreases rhinovirus-induced interferon expression in cultured airway epithelial cells.[5] TLR3-null mice infected with RV1B show normal interferon responses and unchanged viral titers but reduced lung inflammatory responses and airways responsiveness.[6] On the other hand, rhinovirus-induced cytokine expression and viral attachment were abolished in bone marrow–derived macrophages from TLR2, but not TLR3, knockout mice,[7] suggesting a specific requirement for TLR2 in macrophage-mediated responses. TLR2-dependent cytokine expression did not depend on viral endocytosis or replication. TLR2, a membrane surface receptor that recognizes microbe membrane constituents, such as lipoteichoic acids, peptidoglycan, and lipopeptides, was recently shown to be required and sufficient for rhinovirus-induced nuclear factor κB activation in cultured airway epithelial cells and HEK cells, respectively.[8] TLR2 inhibition also blocked responses to replication-deficient UV-irradiated virus. In addition, we have shown that TLR2 is required for rhinovirus-induced IL-1 receptor–associated kinase

Abbreviations used
BAL: Bronchoalveolar lavage
MBP: Major basic protein
OVA: Ovalbumin
TLR: Toll-like receptor

1 degradation in airway epithelial cells.[9] Together, these data suggest that TLR2 recognizes some component of the rhinovirus viral capsid.

In the present study we hypothesized that $TLR2^+$ macrophages are responsible for rhinovirus-induced airway inflammation *in vitro* and *in vivo*. To test this, we examined the requirement of TLR2 for rhinovirus-induced responses in human peripheral blood monocyte–derived macrophages. Next, we infected naive and allergen-challenged $TLR2^{-/-}$ and anti-TLR2–treated mice with RV1B, a minor group virus that replicates in mouse airways.[10] Bone marrow chimera experiments with OVA-treated C57BL/6 and $TLR2^{-/-}$ mice were also performed. Finally, to specifically examine the role of TLR2 expressed on airway macrophages, we transferred wild-type bone marrow–derived macrophages to TLR2 knockout mice and examined their effects on rhinovirus-induced airway responses.

METHODS

Macrophages

Bone marrow was isolated and cultured with L929 mouse fibroblast supernatants as a source of macrophage colony-stimulating factor, as described, to obtain mouse macrophages.[7,11] $CD14^+$ peripheral blood monocytes (Precision Bioservices, Frederick, Md) were incubated with 50 ng/mL macrophage colony-stimulating factor (PeproTech, Rocky Hill, NJ) for 7 days to obtain human macrophages. Selected cultures were also incubated with 50 ng/mL IL-4 (PeproTech), 30 μg/mL anti-TLR2 neutralizing mAb (clone T2.5; eBioscience, San Diego, Calif), or isotype control antibody.

Animals

C57BL/6 mice wild-type and $TLR2^{-/-}$ (B6.129-$Tlr2^{tm1Kir}$/J TLR2 knockout) mice were purchased from Jackson Laboratory (Bar Harbor, Me). All animal use was approved by the Institutional Animal Care and Use Committee and followed guidelines set forth in the Principles of Laboratory Animal Care from the National Society for Medical Research. Mice were housed in the pathogen-free facility within the Unit for Laboratory Animal Medicine at the University of Michigan.

Rhinovirus infection and allergen sensitization

RV1B (ATCC, Manassas, Va), a minor group virus that infects mouse cells,[12] was partially purified from infected HeLa cell lysates by means of ultrafiltration with a 100-kDa cutoff filter and titered by using a plaque assay.[10,13] Intact virus does not go through the filter and is concentrated. Retentates from uninfected HeLa cells were used for sham infections. Viral preparations tested negative for *Mycoplasma* species (Mycoprobe Mycoplasma Detection Kit; R&D Systems, Minneapolis, Minn) or LPS (Pierce Endotoxin Quantitation Kit; Thermo Scientific, Rockford, Ill). For infection of cultured mouse macrophages, RV1B was purified by means of sucrose gradient centrifugation.[13] Finally, human macrophages were infected with either sucrose gradient-purified RV1B or RV39 (ATCC), a major group virus. Viral proteins were detected by using silver staining and anti-RV16 antibody (QED Biosciences, San Diego, Calif).

Six- to 8-week-old wild-type and $TLR2^{-/-}$ mice, each on the C57BL/6 genetic background, were inoculated with 50 μL of 1×10^8 platelet-forming units of RV1B or an equivalent volume of sham HeLa cell lysate. In certain experiments mice were sensitized intraperitoneally with 50 μL of 2 mg/mL endotoxin-free OVA in PBS mixed with alum or PBS control on days 0 and 7, followed by intranasal challenge with 50 μL of 2 mg/mL OVA or PBS on days 7, 12, and 13. These mice were inoculated with RV1B or sham on day 14 of the protocol. Lungs were harvested for analysis 1 to 3 days after infection. Finally, selected mice were treated intranasally with anti-TLR2 (100 μg in 50 μL) after the last OVA challenge.

Bone marrow chimeras

Chimeric mice were used to determine the source of TLR2 critical to rhinovirus-induced airways inflammation and hyperresponsiveness. Adoptive transfer of bone marrow from wild-type to TLR2-null and wild-type mice and, conversely, transfer of $TLR2^{-/-}$ bone marrow into $TLR2^{-/-}$ and wild-type mice were performed, as previously described.[14,15] Briefly, mice underwent ^{137}Cs-gamma-irradiation (13 Gy) from a Gammacell 40 Exactor Irradiator (Best Theratronics, Ottawa, Ontario, Canada) located in the Unit for Laboratory Animal Medicine–managed Experimental Irradiation Core facility. After a stabilization period of 6 to 8 weeks to allow clonal repopulation of the lung and periphery by transferred stem cells, mice were exposed to OVA with or without rhinovirus.

Histology and immunofluorescence microscopy

Lungs were fixed with 4% formaldehyde overnight. Five-micrometer-thick paraffin sections were processed for histology or fluorescence microscopy, as previously described.[2] Lung sections were stained with hematoxylin and eosin or periodic acid–Schiff. Anti-TLR2 was labeled with Alexa Fluor 555 succinimidyl ester (Life Technologies, Waltham, Mass). Eosinophil major basic protein (MBP) was visualized by using rat monoclonal anti-mouse MBP (MT-12.7). This antibody was obtained from Dr James J. Lee[16] and directly conjugated to Alexa Fluor 555.

Determination of viral copy number

Lungs were homogenized, and RNA was extracted with TRIzol (Sigma-Aldrich, St Louis, Mo). Positive-strand RNA was measured by using quantitative 1-step PCR. Copy numbers of positive-strand viral RNA were normalized to 18S RNA, which was similarly amplified with gene-specific primers and probes. Primer sequences are shown in Table I.

Measurement of cytokine mRNA expression

Lung RNA was extracted with TRIzol (Sigma-Aldrich) and analyzed for cytokine gene expression by using quantitative real-time PCR with specific primers and probes (Table I). Signals were normalized to glyceraldehyde-3-phosphate dehydrogenase (GADPH) by using the comparative ΔΔ cycle threshold (CT) method.

Flow cytometry

Mouse lungs were perfused with 5 mmol/L EDTA in PBS, minced, and digested in collagenase. Lung cell suspensions were processed and stained with fluorescent-labeled antibodies against CD45, F4/80, CD11b, CD11c, Ly6c, Gr1, and Siglec-F, as described previously.[4] All antibodies were purchased from BioLegend (San Diego, Calif), and flow data were analyzed with FlowJo software (TreeStar, Ashland, Ore).

Measurement of airway responsiveness

Mice were anesthetized, intubated, and ventilated with a Buxco FinePoint System (Wilmington, NC). Mice were administered increasing doses of nebulized methacholine to assess airways responsiveness, as previously described.[10]

Adoptive transfer of macrophages to the airways

Mouse macrophages were cultured in L929 medium for 5 days, incubated in the presence or absence of IL-4 overnight, and then pulsed with

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