

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

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 mice² 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 κ B 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

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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 × 10⁸ 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 ¹³⁷Cs-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. Lee¹⁶ 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

TABLE I. Primer sequences for real-time quantitative PCR

mRNA	Forward primer (5' to 3')	Reverse primer (5' to 3')
Rhinovirus	GCT GCC TGC ACA CCC TGA GGG GTGT	GAA ACA CGG ACA CCC AAA GTA GT
TNF- α	ATG CAC CAC CAT CAA GGA CTC AA	ACC ACT CTC CCT TTG CAG AAC TC
CXCL1	TGC ACC CAA ACC GAA GAA GTC AT	CAA GGG AGC TTC AGG GTC AAG
IFN- α	CCA TCC CTG TCC TGA GTG	CCA TGC AGC AGA TGA GTC CTT
IFN- β	GAC GGA GAT GAT GGA GAA GAG TTA G	CCA CCC AGT GCT GGA GAA
IL-13	TCC CTG ACC AAC ATC TCC AAT	ACA GAG GCC ATG CAA TAT CC
CCL24	ACC TCC AGA ACA TGG CGG GC	AGA TGC AAC ACG CGC AGG CT
TLR2	TCG TTC ATC TCT GGA GCA TC	TTG ACG CTT TGT CTG AGG TT
GAPDH	GTC GGT GTG AAC GGA TTT G	GTC GTT GAT GGC AAC AAT CTC

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

carboxyfluorescein diacetate succinimidyl ester (Life Technologies) for 10 minutes. Macrophages were transferred to mice intratracheally at 10^6 cells per mouse 24 hours before rhinovirus infection.

RESULTS

TLR2 is required for rhinovirus-induced cytokine expression in cultured mouse macrophages

We have shown that RV1B-induced cytokine expression is attenuated in TLR2^{-/-} bone marrow-derived macrophages.⁷ We repeated this experiment, substituting virus purified by means of sucrose gradient centrifugation for virus partially purified by means of ultrafiltration. Rhinovirus-induced expression of TNF- α and CXCL1 expression was blocked in TLR2-null cells (Fig 1, A).

TLR2 is required for rhinovirus-induced cytokine expression in cultured human macrophages

In this set of experiments, macrophages were generated from human CD14⁺ peripheral blood monocytes and infected with sucrose gradient-purified RV1B or RV39. Selected cultures were treated with IL-4 or anti-TLR2. RV1B- and RV39-infected human macrophages each produced CXCL2 and CXCL8 (Fig 1, B and C). Incubation with IL-4 shifted the macrophage response to rhinovirus toward an M2 polarization pattern, with infected cells producing the eosinophil chemoattractant CXCL11 and IL-13 (Fig 1, B and C). With both minor and major group rhinovirus, cytokine responses were blocked by anti-TLR2.

TLR2 knockout does not affect rhinovirus copy number

We sought to determine whether TLR2 is required for rhinovirus-induced responses *in vivo*. First, wild-type C57BL/6 and TLR2^{-/-} mice were infected with rhinovirus for up to 3 days to assess whether TLR2 plays a role in rhinovirus replication or clearance in the lung. Lungs were harvested at different time points after infection and processed for positive-strand viral RNA. Measurement of rhinovirus copy number between wild-type and TLR2^{-/-} mice showed no difference at each of the indicated time points (Fig 2, A).

Rhinovirus induces TLR2 mRNA expression

It has been shown previously that rhinovirus increases TLR2, TLR7, and TLR8 expression in cultured airway epithelial cells.⁸ In cells inoculated with replication-deficient UV-irradiated virus,

only TLR2 was induced. We tested whether TLR2 mRNA expression was increased in the lungs of rhinovirus-infected mice (Fig 2, B). TLR2 transcripts were increased 24 hours after infection and persisted to the 48-hour time point.

TLR2 knockout attenuates rhinovirus-induced airway inflammation

After rhinovirus infection of C57BL/6 mice, lung neutrophils significantly increased (Fig 2, C). Expression of CXCL1 and TNF- α mRNA was increased significantly beginning 4 hours after infection and remained so 48 hours after infection (Fig 2, D and E). Proinflammatory cytokine levels were significantly reduced in TLR2^{-/-} mice. The type I interferons IFN- α and IFN- β were induced after rhinovirus infection (Fig 2, F and G). Interferon levels were significantly reduced in TLR2^{-/-} mice.

Histologic sections were stained with hematoxylin and eosin to further examine differences in rhinovirus response between wild-type and TLR2^{-/-} mice. Lungs of wild-type animals revealed peribronchial inflammatory and intraluminal infiltrates (Fig 2, H). In contrast, rhinovirus-infected TLR2^{-/-} mice showed very little inflammation. Consistent with our previous findings, lung digests from infected wild-type mice showed a significant influx of CD11b⁺Ly6c⁺ exudative macrophages, whereas influx was absent from rhinovirus-infected TLR2^{-/-} mice (Fig 3).

Rhinovirus infection of OVA-sensitized and OVA-challenged mice induces a synergistic increase in airway eosinophil counts that is dependent on TLR2

Because TLR2 knockout resulted in a significant reduction in rhinovirus-mediated airway inflammation in naive mice, we next examined whether TLR2 plays a role in rhinovirus-mediated exacerbation of allergic airway disease. Wild-type and TLR2^{-/-} C57BL/6 mice were sensitized and challenged with OVA for 2 weeks and then infected with rhinovirus overnight. As shown previously,^{2,4} wild-type mice sensitized with OVA showed an influx of neutrophils and eosinophils into the bronchoalveolar lavage (BAL) fluid (Fig 4, A and B). Rhinovirus infection of allergen-sensitized and -challenged mice showed a synergistic increase in airway eosinophil counts; TLR2^{-/-} knockout significantly reduced rhinovirus-induced neutrophil and eosinophil counts but had no effect on airway inflammation induced by OVA alone. TLR2^{-/-} mice showed fewer eosinophils in the airway tissue (Fig 4, C). TLR2^{-/-} knockout also significantly attenuated rhinovirus-induced airways hyperresponsiveness in OVA-treated mice (Fig 4, D).

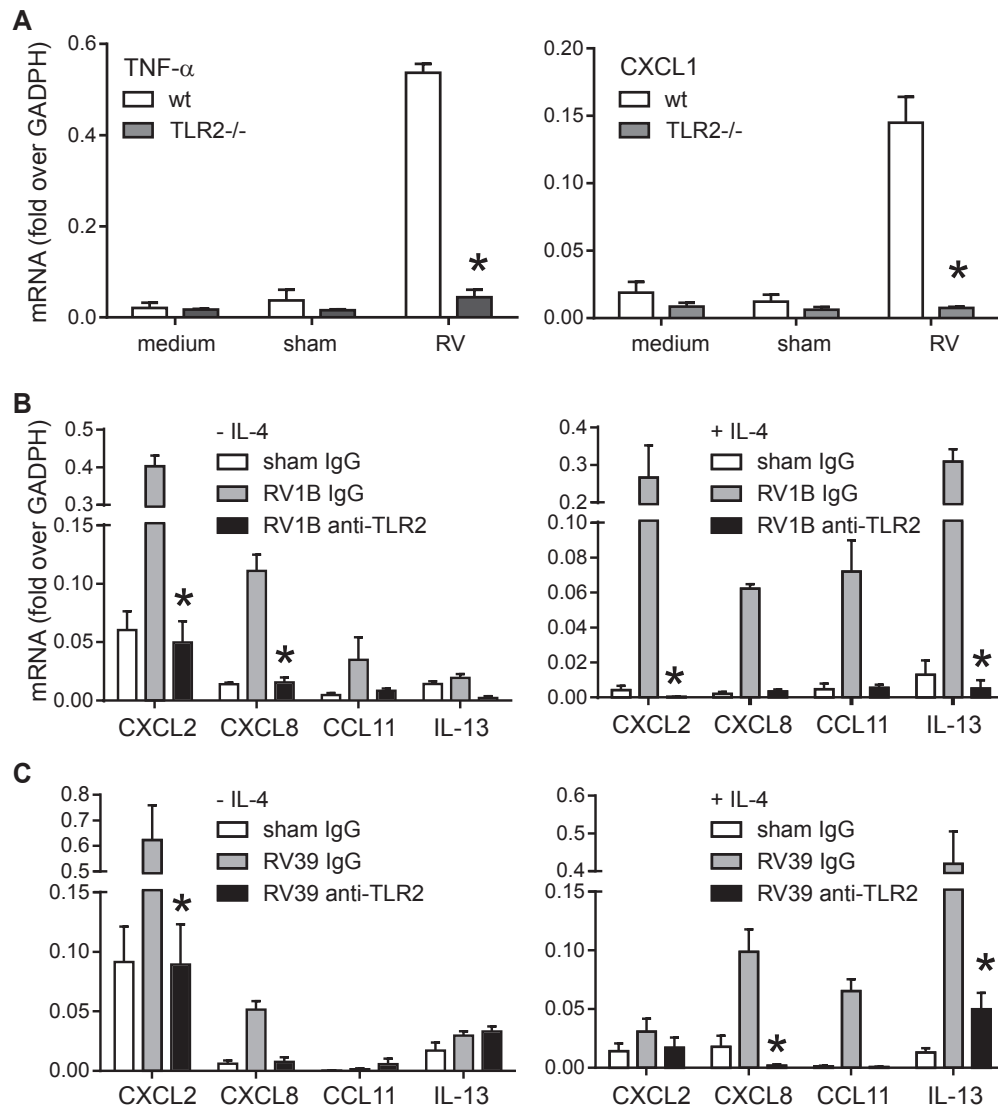


FIG 1. Infection of C57BL/6 and TLR2-null mice. **A**, RV1B infection of bone marrow–derived macrophages from wild-type C57BL/6 and TLR2^{-/-} mice. **B** and **C**, Rhinovirus infection of human macrophages derived from CD14⁺ peripheral blood monocytes. Macrophages were cultured in the presence or absence of IL-4 and infected with sucrose gradient-purified RV1B (Fig 1, **B**) or RV39 (Fig 1, **C**). Whole-lung mRNA expression was measured by using quantitative PCR. Signals were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) by using the comparative cycle threshold ($2^{-\Delta\Delta CT}$) method and expressed as fold change versus *GAPDH* ($n = 3$ samples per condition, mean $\pm$ SEM). *Different from wild-type or IgG control: $P < .05$, ANOVA. RV, Rhinovirus.

We also administered neutralizing antibody against TLR2 to determine the requirement of TLR2 for rhinovirus-induced responses. Antibody was administered just before rhinovirus infection. Lung neutrophils and eosinophils were identified by means of flow cytometry with the markers CD45, CD11b, Gr1, and Siglec-F.¹⁷ Anti-TLR2 significantly decreased rhinovirus-induced neutrophilic and eosinophilic airway inflammation (Fig 4, **E**). Consistent with this, OVA-treated and rhinovirus-infected mice showed additive or synergistic increases in CCL24 and CXCL1 mRNA expression, levels of which were decreased by treatment with anti-TLR2 neutralizing antibody administered before rhinovirus infection (Fig 4, **F** and **G**).

Bone marrow chimeras

To determine the relative contributions of TLR2⁺ bone marrow–derived and structural cells to rhinovirus-induced airways inflammation and hyperresponsiveness, we performed adoptive transfer of wild-type bone marrow into TLR2^{-/-} and wild-type mice and, conversely, transfer of TLR2^{-/-} bone marrow into TLR2^{-/-} and wild-type mice. Six weeks after transfer, mice were sensitized and challenged with OVA, as described above, and exposed to sham treatment or rhinovirus. BAL fluid was harvested for cell counts. All mice showed lung neutrophils in response to OVA exposure and sham infection (Fig 5, **A**). However, only wild-type and TLR2^{-/-} mice reconstituted with wild-type bone marrow showed a significant

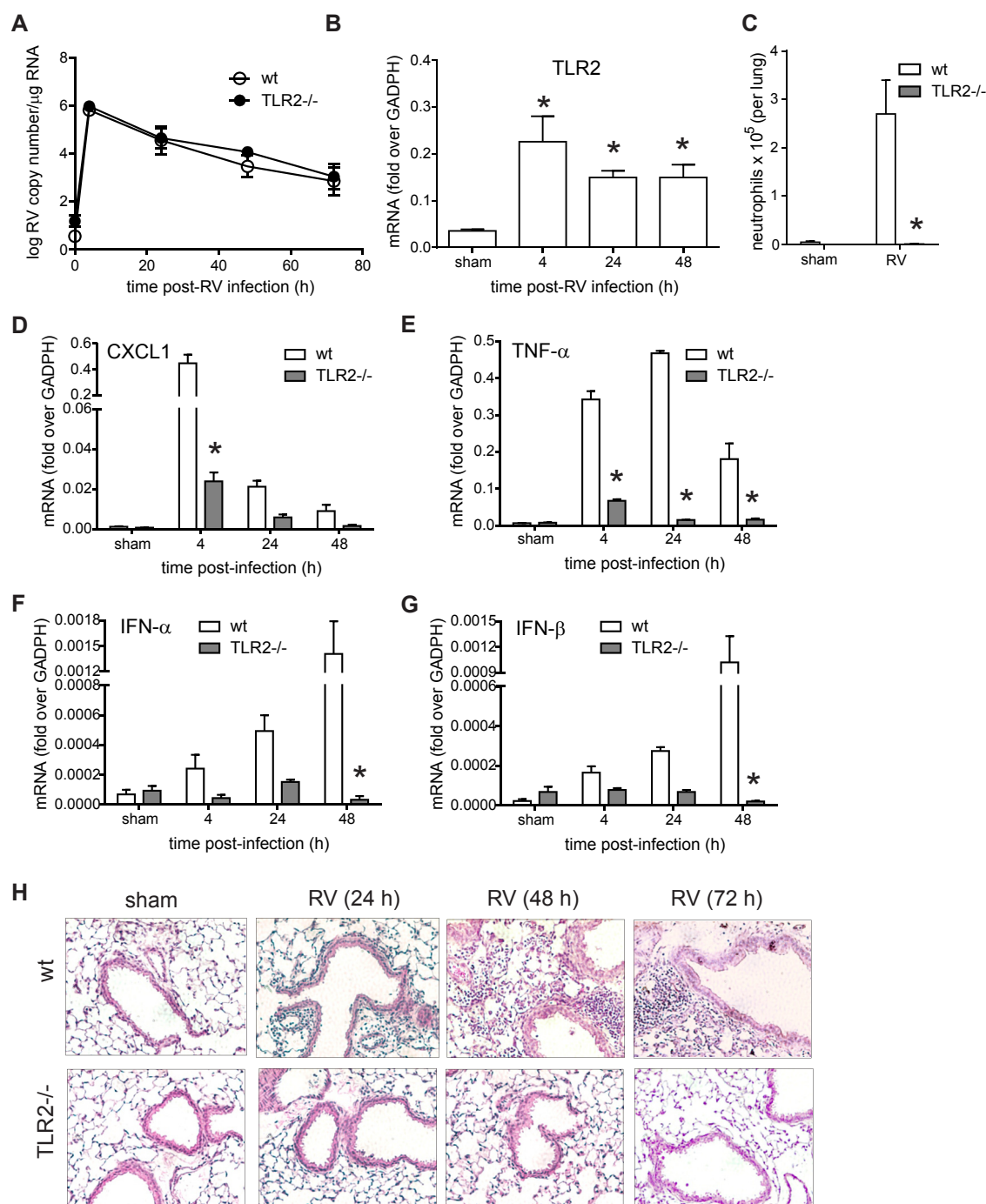


FIG 2. Rhinovirus (RV)-induced airway inflammation is TLR2 dependent. Wild-type (wt) and TLR2^{-/-} mice were infected intranasally with rhinovirus or sham HeLa cell lysate control and killed, and lungs were harvested at different time points. **A**, Rhinovirus positive-strand RNA was assessed 24 to 72 hours after infection and normalized by total micrograms of RNA. **B**, Transcript levels of TLR2 from lungs of mice infected with rhinovirus. **C**, BAL neutrophils. **D-G**, Transcript levels of the proinflammatory cytokines CXCL1 and TNF- α (Fig 2, D and E) and the type I interferons IFN- α and IFN- β (Fig 2, F and G) were assessed by using quantitative PCR, and results were expressed as fold change over glyceraldehyde-3-phosphate dehydrogenase (GADPH). For Fig 2, A-G, there were 3 mice per group (mean $\pm$ SEM). *Different from wild-type: $P < .05$, ANOVA. **H**, Hematoxylin and eosin staining of lung sections from sham- and rhinovirus-infected mice harvested 24 hours after inoculation (magnification $\times 200$).

increase in BAL neutrophil counts after rhinovirus infection. A similar pattern was observed with BAL eosinophils. Only wild-type and TLR2^{-/-} mice reconstituted with wild-type bone marrow showed a significant increase in BAL eosinophil

counts after rhinovirus infection. Wild-type mice reconstituted with TLR2^{-/-} bone marrow showed a trend toward increased airway eosinophil counts after rhinovirus infection. TLR2^{-/-} mice reconstituted with TLR2^{-/-} bone marrow showed rare

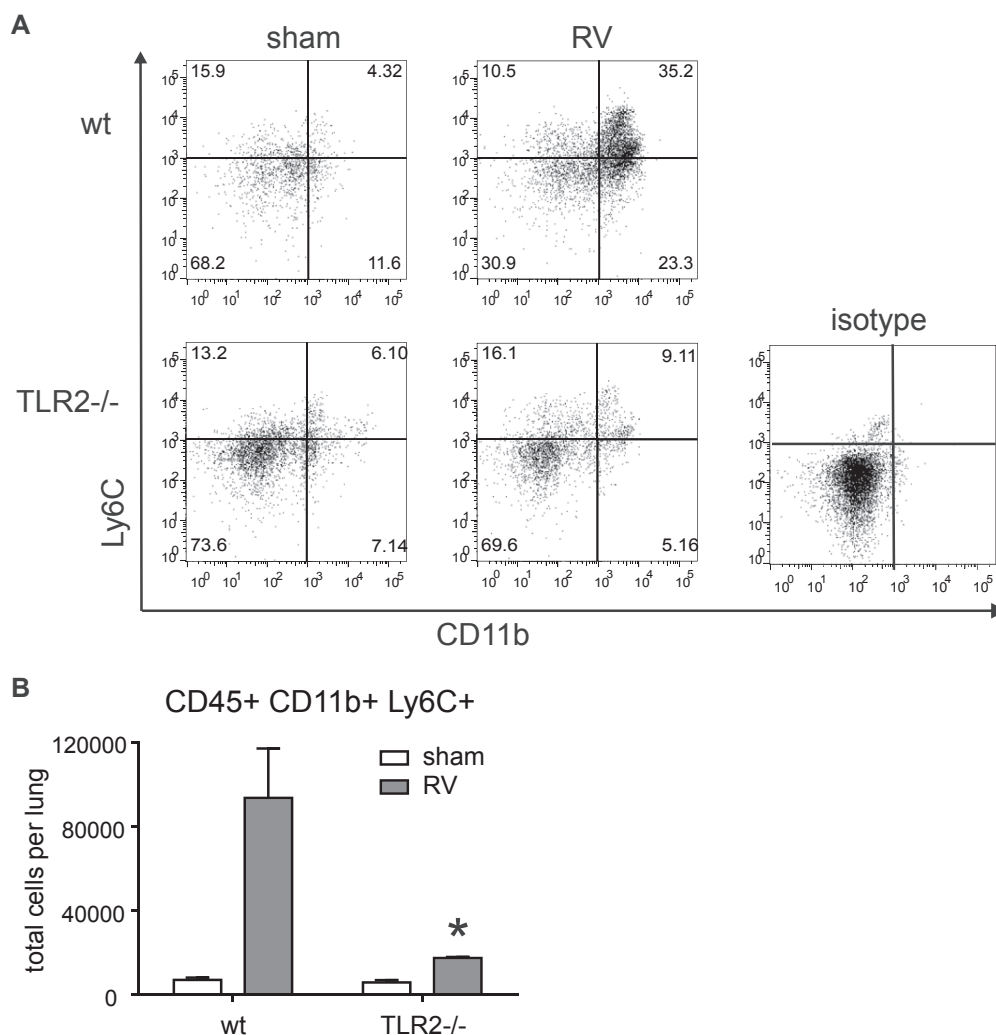


FIG 3. Rhinovirus (RV)-induced infiltration of CD11b⁺Ly6c⁺ exudative macrophages into the airways is TLR2 dependent. **A**, Lung single-cell suspensions were gated for cell viability, surface expression of CD45 and F4/80, and then CD11b and Ly6c. **B**, Group mean data for total CD45⁺, F4/80, CD11b⁺, and Ly6c⁺ cells per lung (n = 3). *Different from wild-type: $P < .05$, ANOVA. wt, Wild-type.

eosinophils after OVA treatment either before or after rhinovirus infection.

Next, we assessed airways cholinergic responsiveness (Fig 5, B). Mice undergoing irradiation and bone marrow transfer followed by OVA exposure and rhinovirus infection tended to have lower airway responses than nonirradiated mice (compare with Fig 4), leading us to increase the maximal concentration of methacholine. Only wild-type and TLR2^{-/-} mice reconstituted with wild-type bone marrow showed a significant increase in airways responsiveness after rhinovirus infection. Similar to BAL cell counts, wild-type mice reconstituted with TLR2^{-/-} bone marrow showed a trend toward increased responsiveness after rhinovirus infection.

Adaptive transfer of wild-type macrophages to the airways of TLR2^{-/-} mice

Because we have previously shown that macrophages are required for rhinovirus-induced airway inflammation^{2,4} and that TLR2 is essential for rhinovirus-induced cytokine production

by macrophages,⁷ we tested whether transferring TLR2⁺ wild-type bone marrow-derived macrophages would be sufficient to increase airway inflammation in TLR2^{-/-} mice infected with rhinovirus. Bone marrow-derived macrophages from wild-type or TLR2^{-/-} mice were transferred to TLR2^{-/-} mice intranasally. Twenty-four hours later, animals were infected with rhinovirus. Transferred carboxyfluorescein diacetate succinimidyl ester-stained macrophages were detectable in recipient animals (Fig 6, A). TLR2^{-/-} mice receiving untreated wild-type macrophages showed a significant increase in neutrophil influx in response to rhinovirus compared with mice receiving TLR2^{-/-} macrophages (Fig 6, B and C). There was no change in airway eosinophil counts. Next, to simulate prior antigen sensitization and challenge, bone marrow macrophages were treated with 20 μ g/mL IL-4 and then transferred to TLR2^{-/-} hosts. We have previously shown that IL-4 treatment is sufficient to polarize bone marrow-derived macrophages to an alternatively activated M2 phenotype, which is characterized in part by type 2 cytokine production.⁷ TLR2^{-/-} mice receiving IL-4-polarized wild-type macrophages showed significant increases in both neutrophil

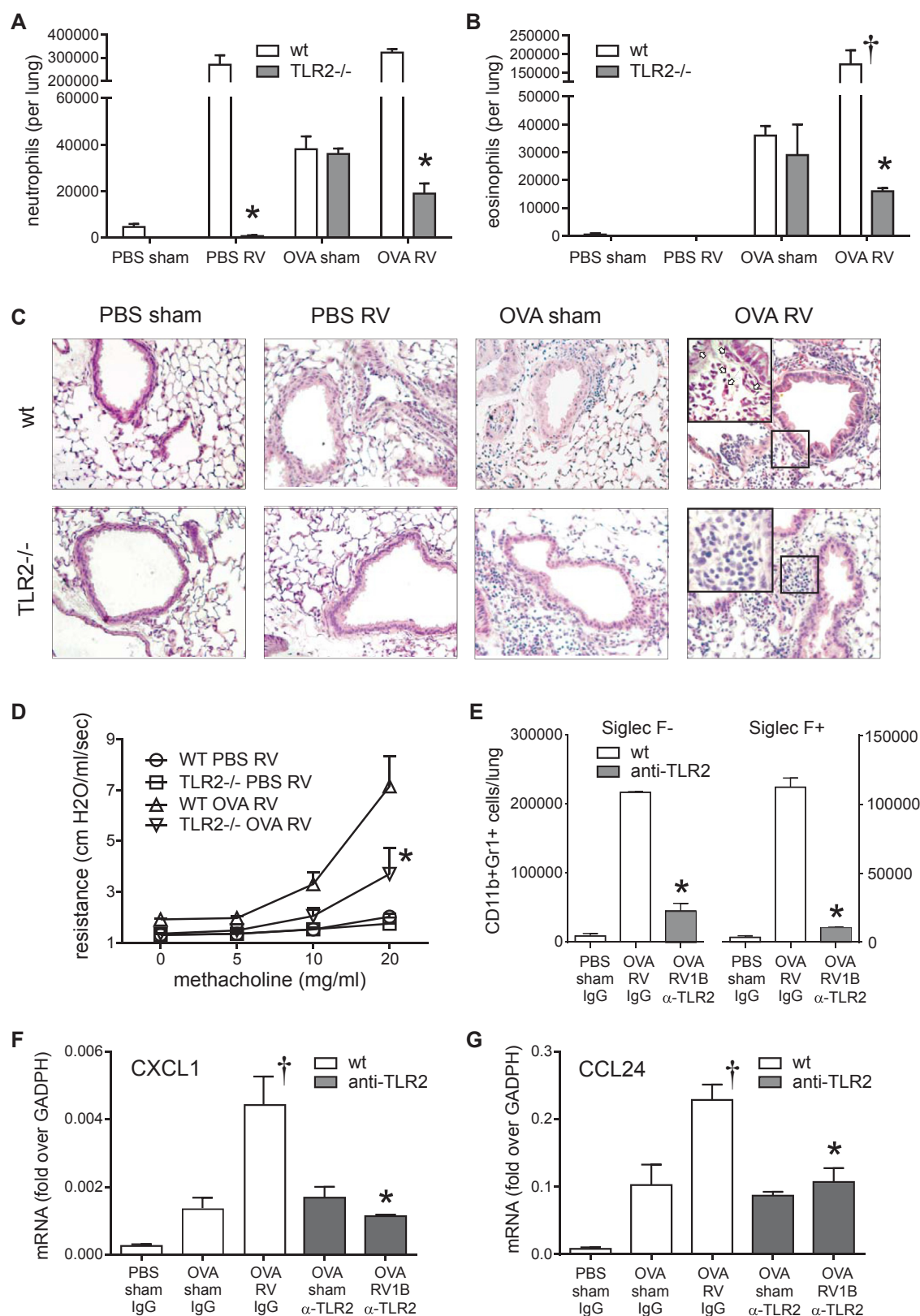


FIG 4. TLR2 is required for rhinovirus (RV)-induced airway inflammation and responsiveness in OVA-sensitized and OVA-challenged mice. **A–D**, Female C57BL/6 wild-type or TLR2-null mice were exposed to either PBS or OVA before infection with rhinovirus or sham control for 24 hours. **A** and **B**, BAL neutrophils (Fig 4, A) and eosinophils (Fig 4, B; $n = 3$ mice per group, mean $\pm$ SEM). *Different from wild-type and †different from OVA/sham; $P < .05$, ANOVA. **C**, Lung sections stained with hematoxylin and eosin are shown (original magnification $\times 200$; arrows in inset indicate eosinophils). **D**, Airways responsiveness was

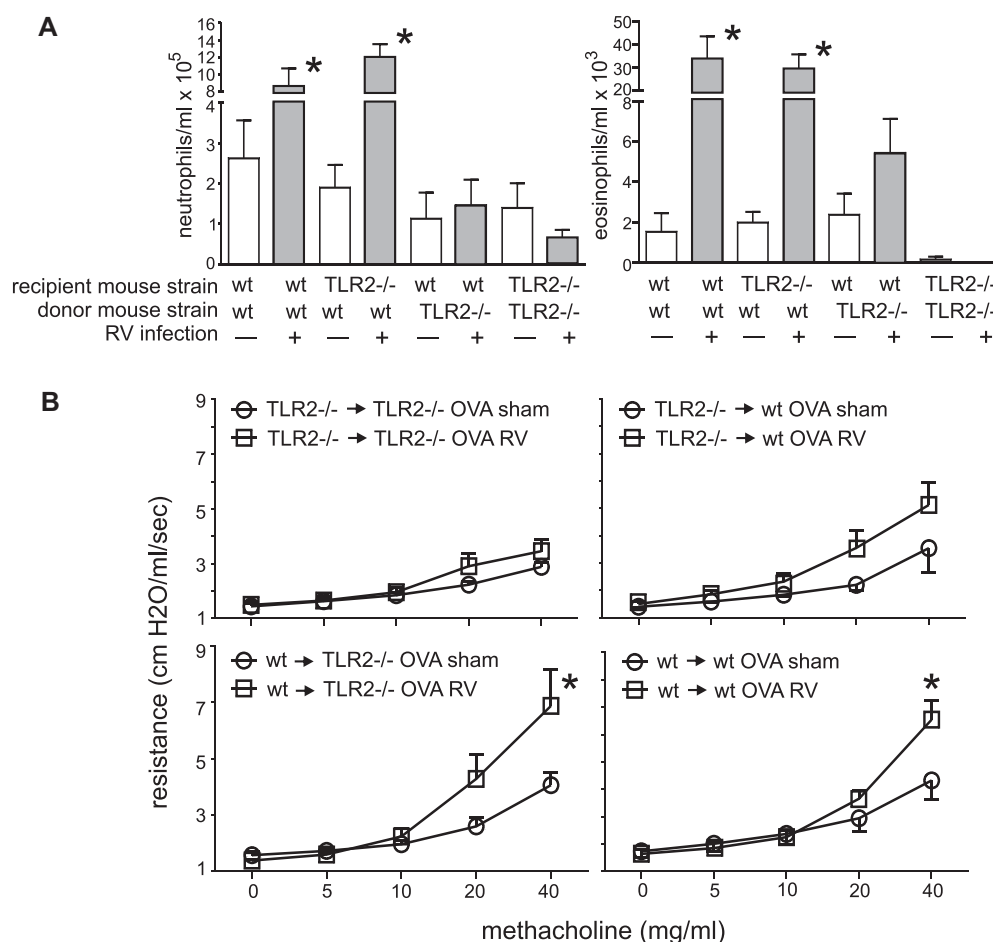


FIG 5. Bone marrow chimeras. After 13 Gy ^{137}Cs -gamma-irradiation, wild-type bone marrow was transferred to TLR2^{-/-} and wild-type mice, and conversely, TLR2^{-/-} bone marrow was transferred to TLR2^{-/-} and wild-type mice. Six to 8 weeks after transfer, mice were sensitized and challenged with OVA and exposed to sham or rhinovirus (RV). **A**, BAL fluid was harvested for cell counts. **B**, Airways responsiveness was assessed by means of exposure to increasing doses of inhaled methacholine. Total respiratory resistance was measured with a Buxco plethysmograph ($n = 6$, mean $\pm$ SEM). *Different from sham: $P < .05$, 1-way or 2-way ANOVA, as applicable.

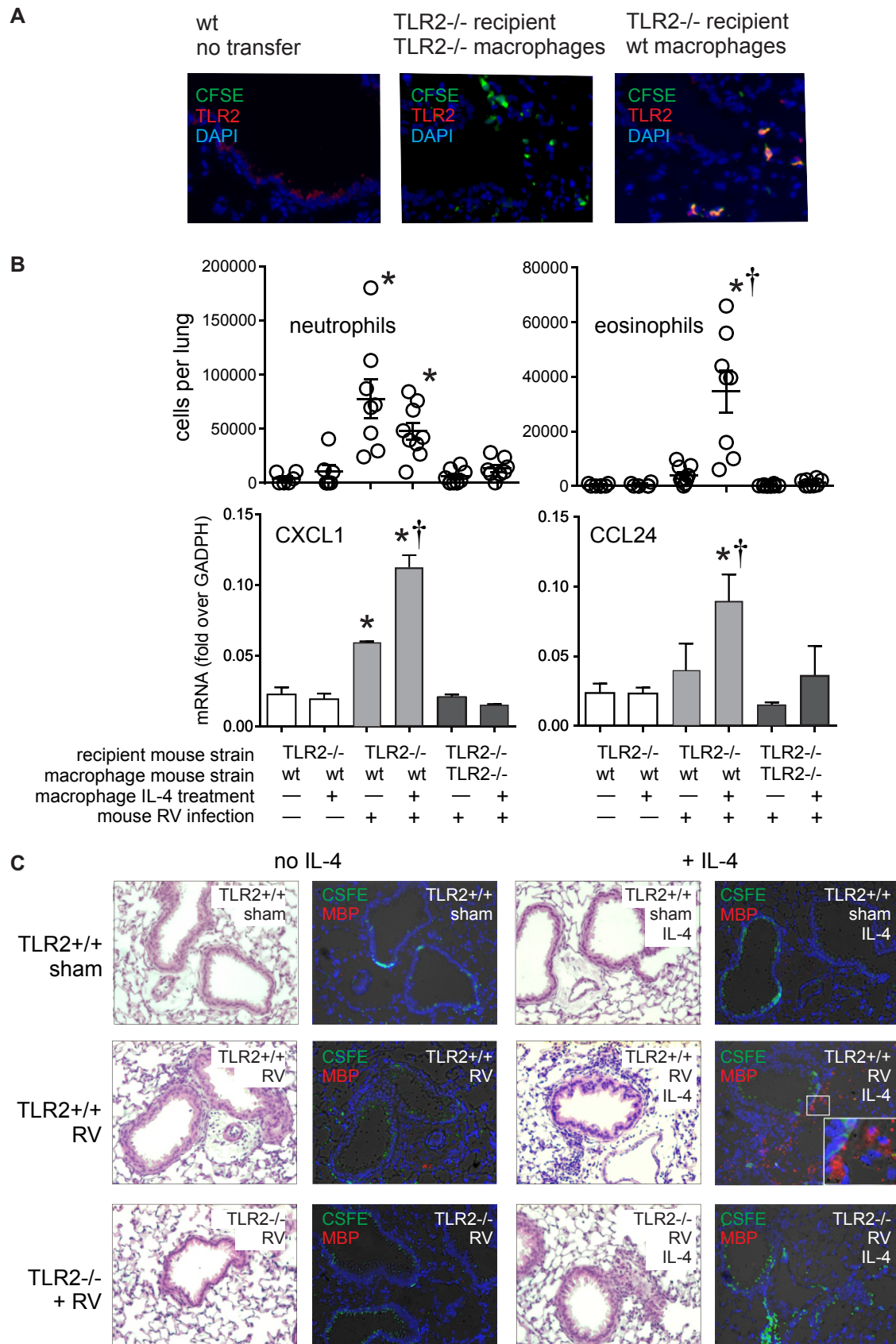
and eosinophil counts in response to subsequent rhinovirus infection. The presence of eosinophils was verified by staining with myelin basic protein (Fig 6, C). Transfer of IL-4-treated TLR2^{-/-} macrophages had no effect. It should be noted that the level of inflammation was lower than that of wild-type mice infected with rhinovirus (Fig 4). Transfer of untreated macrophages increased whole-lung mRNA expression of the neutrophil chemoattractant CXCL1, whereas transfer of IL-4-treated macrophages increased expression of both CXCL1 and the eosinophil chemoattractant CCL24 (Fig 6, B). Transfer of IL-4-treated wild-type macrophages to TLR2^{-/-} mice induced mucous metaplasia, as evidenced by periodic acid-Schiff staining (Fig 6, D). Transfer of untreated wild-type macrophages or IL-4-treated TLR2^{-/-} macrophages had no effect (data not shown).

Finally, we examined the effect of IL-4-treated macrophages on rhinovirus-induced airways responsiveness (Fig 6, E). Compared with IL-4-treated TLR2^{-/-} macrophages, transfer of IL-4-treated wild-type macrophages caused a small but significant increase in methacholine responsiveness. Together, these data suggest that the TLR2⁺ M2-polarized macrophage is sufficient for the type 2 cytokine response to rhinovirus.

DISCUSSION

We have found that TLR2 is required for rhinovirus-induced airway responses in both naive and allergen-sensitized mice. TLR2^{-/-} mice had significantly attenuated airway inflammation and responsiveness in response to rhinovirus infection. In

measured in tracheotomized animals exposed to increasing doses of methacholine ($n = 3$ mice per group, mean $\pm$ SEM). *Different from wild-type OVA/RV mice: $P < .05$, 2-way ANOVA. **E-G**, C57BL/6 wild-type mice were exposed to either PBS or OVA before treatment with IgG or anti-TLR2 and infection with rhinovirus or sham control. Fig 4, E, Lung neutrophil and eosinophil counts were assessed by using flow cytometry gating on high-complexity, CD45⁺Gr1⁺Siglec-F⁺ cells. Whole-lung mRNA expression of CXCL1 (Fig 4, F) and CCL24 (Fig 4, G) was measured by using quantitative PCR ($n = 3$ mice per group, mean $\pm$ SEM). *Different from IgG and †different from OVA/sham: $P < .05$, ANOVA. wt, Wild-type.



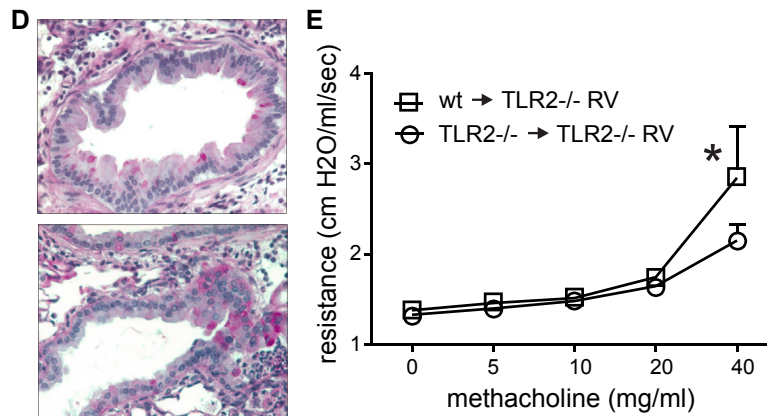


FIG 6. (Continued).

addition, transfer of TLR2-expressing bone marrow cells, as well as IL-4-treated bone marrow–derived macrophages, to TLR2^{-/-} mice was sufficient to induce airway inflammation and hyperresponsiveness on subsequent infection with rhinovirus. These data not only demonstrate a significant role for TLR2 in the innate immune response to rhinovirus but also identify the airway macrophage as a critical cell target and effector of rhinovirus infection.

Recent studies have uncovered a potential role for TLR2 in sensing rhinovirus infection. TLR2, a membrane surface receptor that recognizes bacterial lipopeptides and lipoteichoic acid, was recently shown to be required and sufficient for rhinovirus-induced nuclear factor κ B activation in cultured airway epithelial cells and HEK cells, respectively.⁸ TLR2 inhibition also blocked responses to replication-deficient UV-irradiated virus. Similarly, we previously found that rhinovirus-induced cytokine expression and viral attachment were abolished in bone marrow–derived macrophages from TLR2 knockout mice,⁷ suggesting a specific requirement of TLR2 in macrophage-mediated responses. TLR2-dependent cytokine expression did not depend on viral endocytosis or replication. We also colocalized rhinovirus and TLR2 on the macrophage cell surface. In the present study we confirmed that TLR2 is required for rhinovirus-induced cytokine

expression in macrophages, this time by using a highly purified viral preparation. Finally, we found that TLR2 was required for rhinovirus-induced airway responses *in vivo*.

Together, these data suggest that surface TLR2 responds to a molecular pattern on the viral capsid rather than to double-stranded viral RNA, a product of viral replication. At first blush, such an interaction is surprising because picornaviruses are nonenveloped and their capsid does not include a lipid component. On the other hand, picornaviruses interact with cellular proteins during entry into the cell and virus particle assembly, interactions that alter the structure of the future viral capsid. For example, rhinovirus and other picornavirus particles are specifically modified by having myristic acid covalently bound to the VP4 capsid protein.^{18,19} Such a modification might serve as a ligand for TLR2. Although VP4 normally resides on the capsid inner surface, binding of rhinovirus to its cellular receptor induces conformational changes in the virus, including translocation of VP4 to the exterior viral surface.²⁰ This altered particle interacts directly with membranes without further involvement of the cellular virus receptor.²¹ It is also conceivable that there is no direct interaction between rhinovirus and TLR2; viral capsid could bind with one of TLR2's many coreceptors, including CD14, scavenger receptors, integrins, dectin-1, or

FIG 6. Adoptive transfer of bone marrow–derived macrophages from wild-type C57BL/6 mice to TLR2^{-/-} mice partially restores the inflammatory response to rhinovirus (RV). Bone marrow cells from either wild-type or TLR2^{-/-} mice were cultured in L929 media for 5 days, incubated in the presence or absence of IL-4 overnight, pulsed with carboxyfluorescein diacetate succinimidyl ester (CFSE), and instilled intratracheally into TLR2^{-/-} mice. **A**, Immunofluorescence images showing CFSE-pulsed cells in the airways (green, CFSE; red, TLR2; blue, 4'-6-diamidino-2-phenylindole dihydrochloride; magnification $\times 200$). **B** and **C**, BAL cell counts, whole-lung mRNA expression, and airway histology from mice 24 hours after sham or rhinovirus infection. Transfer of untreated macrophages from wild-type mice to TLR2^{-/-} mice was sufficient for rhinovirus-induced neutrophilic airway inflammation, and transfer of IL-4-treated wild-type macrophages was sufficient for rhinovirus-induced eosinophilic inflammation ($n = 6-9$; as pooled value from t3 separate experiments, each with 3 mice per group; mean $\pm$ SEM). *Different from transfer of TLR2^{-/-} cells and †different from transfer of untreated wild-type cells: $P < .05$, ANOVA. **C**, Airway sections from mice described in Fig 6, **B**, were stained with hematoxylin and eosin and anti-MBP. **D**, Effect of macrophage adoptive transfer on airway mucus, as demonstrated by periodic acid–Schiff staining. **E**, Effect of macrophage transfer on airway cholinergic responsiveness. TLR2^{-/-} mice treated with IL-4-treated wild-type or TLR2^{-/-} macrophages and then infected with rhinovirus ($n = 6$, mean $\pm$ SEM). *Different from transfer of TLR2^{-/-} cells: $P < .05$, 2-way ANOVA. CFSE, Carboxyfluorescein diacetate succinimidyl ester; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; wt, Wild-type.

CXCR4.²² Finally, TLR2 could be activated by rhinovirus-mediated release of a damage-associated molecular pattern.

There is increasing evidence that genetic differences in TLR2 expression influence the development of asthma. Peripheral blood monocytes from farmers' children with reduced risk of atopy express significantly higher amounts of CD14 and TLR2 than those from nonfarmers' children.²³ Later, the TLR2/−16934 polymorphism, a marker for a group of highly linked TLR2 single nucleotide polymorphisms, was associated with reduced risk of asthma in children of farmers.²⁴ Although the functional significance of this polymorphism is not known, it was suggested that this variant could result in increased TLR2 protein expression. On the other hand, the −16934 polymorphism has also been associated with wheeze and bronchial hyperresponsiveness in day care attendees,²⁵ which is consistent with the notion that TLR2 expression heightens the inflammatory response to rhinovirus and other viral respiratory tract infections. We speculate that an increase in TLR2 expression or function could heighten inflammatory responses to rhinovirus infection, whereas a loss of TLR2 could protect against rhinovirus exacerbations.

Concerning the cellular source of TLR2 required and sufficient for rhinovirus-induced airway responses, we found that transfer of TLR2⁺ bone marrow cells to TLR2^{−/−} mice was sufficient to confer airways inflammation and responsiveness to rhinovirus. Intranasal transfer of IL-4-treated wild-type macrophages to TLR2^{−/−} mice was also sufficient for rhinovirus-induced airway inflammation and hyperresponsiveness. Taken together, these data implicate the macrophage as the primary cell responsible for rhinovirus-induced airway responses. However, airway epithelial cells are also likely to play a role. First, inflammatory and functional responses to rhinovirus after transfer of IL-4-treated TLR2⁺ macrophages did not reach the level of wild-type mice. Second, wild-type mice reconstituted with TLR2^{−/−} bone marrow maintained an intermediate airway eosinophil count and methacholine responsiveness to rhinovirus infection. Airway epithelial cells are a source of chemokines after rhinovirus infection.^{2,26} Also, because significant viral replication does not occur in macrophages, we speculate that epithelial cells are needed as a reservoir for new macrophage-binding viral particles.

Although transfer of TLR2-expressing macrophages to TLR2^{−/−} mice was sufficient to induce airway inflammation on subsequent infection with rhinovirus, untreated macrophages elicited only neutrophilic inflammation, whereas IL-4-treated macrophages elicited both neutrophilic and eosinophilic inflammation. These data are consistent with the notion that *ex vivo* IL-4 treatment induces M2 polarization, leading to production of eosinophil-attracting chemokines on rhinovirus infection *in vivo*. We have shown that allergen sensitization and challenge induces alternative activation of lung macrophages, as evidenced by expression of the M2 markers arginase-1, Ym-1, CD206/Mrc1, and CD301/Mgl-2, as well as secretion of the M2 cytokines CCL11, CCL17, CCL22, and CCL24.^{2,4,7} In the present study IL-4 treatment increased rhinovirus-induced CCL11 and IL-13 expression. Increasing evidence from animal models^{9,27-30} and human subjects^{31,32} have implicated macrophages with features of M2 polarization in the pathogenesis of allergic asthma. Based on previous work showing colocalization of rhinovirus with CD68⁺ and CD11b⁺ macrophages in the airways of human asthmatic patients experimentally infected with rhinovirus,² we propose that interactions between rhinovirus and polarized macrophages are responsible, at least in part, for the

increased airway inflammation observed after rhinovirus infection of asthmatic patients.

There are limitations to our current study. First, although we determined the requirement of TLR2-expressing bone marrow cells for rhinovirus-induced airway responses, we did not determine the specific requirement of TLR2-expressing macrophages, which would require a tissue-specific knockout. Second, the absence of strong viral replication in the rhinovirus mouse model¹⁰ could minimize the apparent role of airway epithelial cells, which would ordinarily be needed to sustain viral responses by serving as a reservoir for viral replication.

We conclude that in mice with allergic airways disease, TLR2 is required for early inflammatory responses induced by rhinovirus. Macrophage TLR2 expression was also partially sufficient for rhinovirus-induced airway inflammation. Future studies examining the mechanism of TLR2 regulation could provide a new therapeutic target for virus-induced asthma exacerbation.

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Key messages

- In cultured human macrophages cytokine responses to either RV1B or RV39 are blocked by neutralizing antibody to TLR2.
- RV1B-induced airway inflammation is blocked 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 restored them.
- Transfer of wild-type macrophages to TLR2^{−/−} mice is sufficient to confer airway neutrophilic inflammation after rhinovirus infection, and transfer of IL-4-treated macrophages (to shift cells to an M2 phenotype) is associated with neutrophilic and eosinophilic inflammation, mucous metaplasia, and airways hyperresponsiveness analogous to that seen in rhinovirus-infected mice with allergic airways disease.

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