Distinct TLR-mediated pathways regulate house dust mite–induced allergic disease in the upper and lower airways

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Background: Allergic rhinitis (AR) and asthma are 2 entities of allergic airway diseases that frequently occur together, which is referred to as *united airways*. In contrast to this general concept, we hypothesized that innate immunity of the upper and lower airways is respectively distinctive, because the immunologic conditions of the nasal and lung mucosa as well as the functions of the immune cells within their epithelia are different.

Objective: We wanted to identify distinctive mechanisms of innate immunity in the nose and lung mucosa, which are responsible for house dust mite (HDM)–induced AR and allergic asthma (AA), respectively.

Methods: We constructed a mouse model of AR or AA induced by sensitization and consequent provocation with HDM extracts.

Results: HDM-derived β-glucans, rather than LPS, were proven to be essential to activating innate immunity in the nasal mucosa and triggering AR, which depended on Toll-like receptor 2 (TLR2), but not on TLR4; however, the LPS/TLR4 signaling axis, rather than β-glucans/TLR2, was critical to HDM-induced AA. These differences were attributed to the specific role of β-glucans and LPS in inducing the surface expression of TLR2 and TLR4 and their translocation to lipid rafts in nasal and bronchial epithelial cells, respectively. We also showed that dual oxidase 2–generated reactive oxygen species mediate both β-glucan–induced TLR2 activation and LPS-induced TLR4 activation.

Conclusions: We describe a novel finding of distinctive innate immunity of the nose and lungs, respectively, which trigger AR and AA, by showing the critical role of HDM-induced TLR activation via dual oxidase 2–mediated reactive oxygen species. (J Allergy Clin Immunol 2013;131:549-61.)

Key words: *Allergic rhinitis, allergic asthma, innate immunity, house dust mite, pathogen associated molecular pattern, β-glucans, Toll-like receptor, epithelium, reactive oxygen species, dual oxidase 2*

Abbreviations used

AA:	Allergic asthma
AR:	Allergic rhinitis
BAL:	Bronchial lavage
DC:	Dendritic cell
DUOX2:	Dual oxidase 2
MβCD:	Methyl-β-cyclodextrin
HDM:	House dust mite
HDMΔβ-glucan:	HDM extracts pretreated with β-glucanase to degrade β-glucan structures
HDMΔLPS:	HDM extracts pretreated with polymyxin B to inactivate LPS
HDMΔprotease:	HDM extracts pretreated with protease inhibitor to inactivate proteases
MHCII:	Major histocompatibility complex class II
NAC:	N-acetylcysteine
NAL:	Nasal lavage
NHNE:	Normal human nasal epithelial
PAMP:	Pathogen associated molecular pattern
PRR:	Pattern recognition receptor
ROS:	Reactive oxygen species
TLR:	Toll-like receptor

Data accumulated over the past century have shown that allergic rhinitis (AR) and asthma often occur together and share a common genetic background, which is generally referred to as *united airways* or *one airway-one disease*.[1-3] However, immunologic conditions of the nasal and lung mucosa are not identical because the nasal mucosa is continuously exposed to various microorganisms and aeroallergens, whereas the lung mucosa is only occasionally infected with a few microbes.[4,5] In addition, the type, number, and immunologic function of barrier epithelial cells, the first line of

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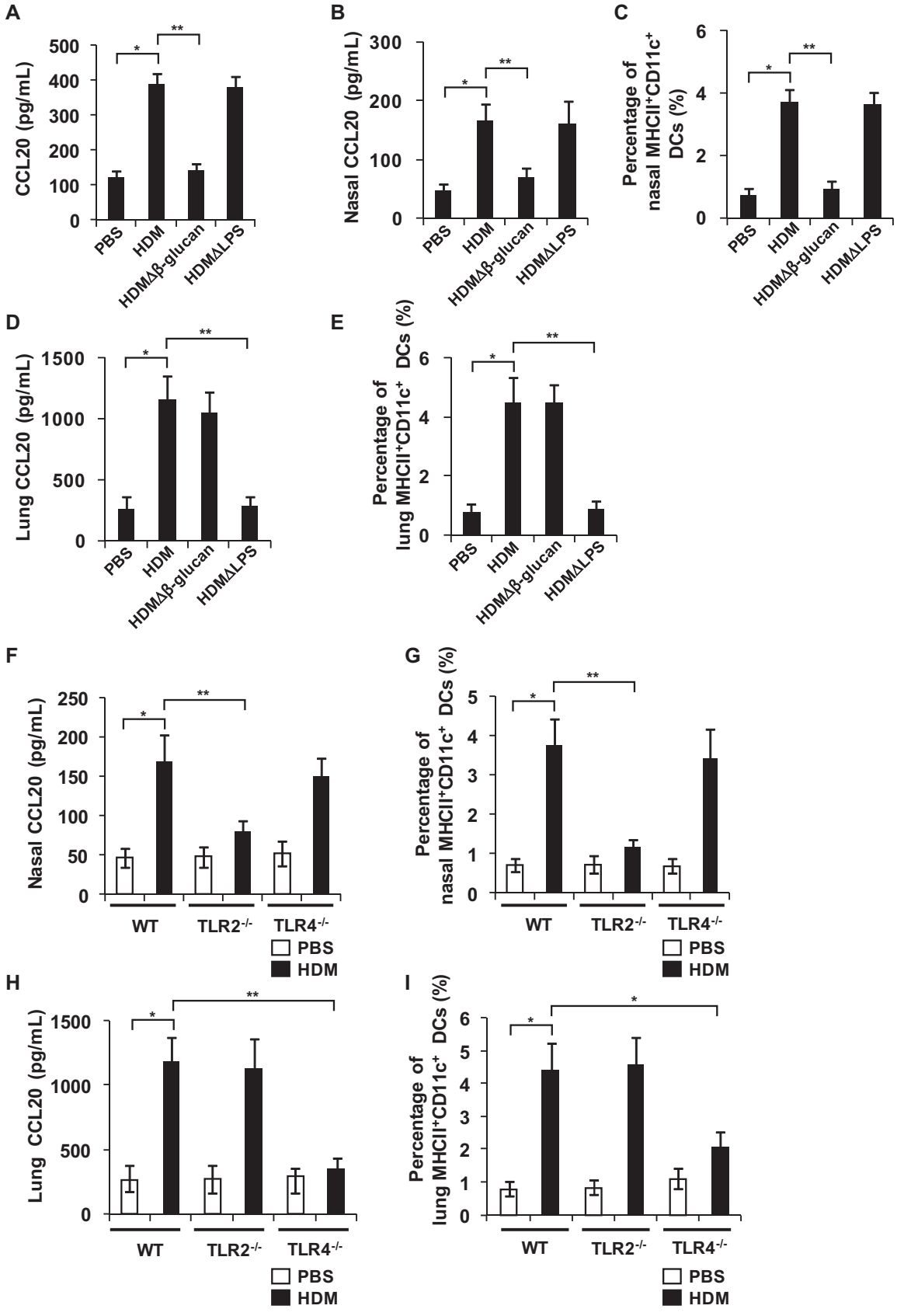


FIG 1. HDM-derived β-glucans and TLR2 and HDM-derived LPS and TLR4 are responsible for innate immunity in the nasal mucosa and the lung mucosa, respectively. **A**, Quantification of CCL20 secretion by treatment with HDM extracts (50 μg/mL) for 24 hours in NHNE cells. HDM extracts pretreated with

defense against inhaled allergens, are different in the nasal and lung mucosae.[6] Thus, several studies have endeavored to clarify the differences between AR and asthma and to determine the relation between them according to epidemiologic and clinical data.[7-9] However, the distinction between the two can be difficult to make because symptom perception of patients with AR and/or asthma is widely variable. To overcome the clinical limitations, several studies have been attempted to discover the association between the 2 diseases by using ovalbumin-driven AR or allergic asthma (AA) mouse models.[10-12] They have shown that dendritic cells (DCs) are critical in T_H2 cell activation in both AR and AA,[10,13-15] and that ovalbumin-induced AR mice are more susceptible to AA after lower airway challenge, which is mediated by circulating T_H2 effector cells.[12] LPS, a pathogen-associated molecular pattern (PAMP) molecule found in house dust mite (HDM) extracts, was shown to activate innate immunity in airway epithelial cell and/or DCs via Toll-like receptor 4 (TLR4), resulting in T_H2-mediated allergic inflammation in AA mouse model systems.[13,16] Given that early immune responses in airway epithelial cells regulate the activation of DCs, leading to T_H2-mediated allergic inflammation,[13,14,16-21] identifying innate immune function in nasal and lung epithelial cells might be relevant to not only understanding the regulatory mechanisms of AR and AA themselves but also appreciation of the link between the two. Herein, we described the different mechanisms of innate immunity of the nose and lungs activated by HDM, a ubiquitous indoor allergen, and their role in triggering AR and AA, using *in vivo* mouse model systems. We show that HDM-derived β-glucans, rather than LPS, are the main PAMP molecules responsible for activating innate immunity in nasal mucosa and triggering AR, which depends on TLR2 but not on TLR4. In contrast, LPS, rather than β-glucans, is required for HDM-induced innate immunity in lung mucosa and AA, which relies on TLR4 but not on TLR2. These differences were ascribed to the specific role of β-glucans and LPS in inducing TLR2 and TLR4 surface expression and their translocation into lipid rafts in nasal and bronchial epithelial cells, respectively. In addition to HDM-derived PAMP-dependent TLR activation in a tissue-specific manner, we show that dual oxidase 2 (DUOX2)–mediated reactive oxygen species (ROS) regulates innate immunity via mediating both β-glucan–induced TLR2 activation and LPS-induced TLR4 activation, leading to AR and AA.

METHODS

For details on methods, see this article's Methods section (in the Online Repository available at www.jacionline.org).

RESULTS

β-Glucans and LPS within HDM-triggered innate immunity in the upper and lower airways, respectively

Because β-glucans and LPS are known to be the main PAMPs within HDM extracts that activate innate immunity in the airway epithelium,[13,17,19,22] we examined their roles in the HDM-induced innate immune responses in airway epithelial cells. We pretreated HDM extracts with either β-glucanase to degrade β-glucan structures (HDMΔβ-glucan) or polymyxin B to inactivate LPS (HDMΔLPS) before treating primary normal human nasal epithelial (NHNE) cells. First, we measured the level of CCL20 secretion from NHNE cells, because CCL20 is a chemokine secreted from airway epithelial cells by HDM exposure,[17] and its expression level is upregulated in AR.[23,24] Interestingly, the increased CCL20 secretion seen after HDM exposure was not affected by HDMΔLPS, whereas HDMΔβ-glucan dramatically decreased HDM-induced CCL20 secretion (Fig 1, *A*). Consistent with the results in NHNE cells, increased CCL20 secretion from the nasal mucosa of HDM-challenged mice was also decreased by HDMΔβ-glucan but not by HDMΔLPS (Fig 1, *B*). In addition to CCL20, we measured the concentration of HDM-induced DC-activating cytokines, including GM-CSF, thymic stromal-derived lymphopoietin, IL-25, and IL-33 from nasal lavage (NAL) fluid. However, only IL-33 was detected, and HDM-induced IL-33 levels were decreased by HDMΔβ-glucan but not by HDMΔLPS (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org). The recruitment of MHC class II (MHCII)–positive $CD11c^{hi}$ DCs to the nasal mucosa also depended on HDMΔβ-glucan but not on HDMΔLPS (Fig 1, *C*). However, recent data have shown that HDM-derived LPS is required to stimulate innate immunity in the lung mucosa.[13,16] Thus, we examined whether HDM-derived LPS played an essential role in innate immune response in lung mucosa in our experimental condition, and whether HDM-derived β-glucans are also required for activating innate immunity in lung mucosa. Interestingly, HDM-induced CCL20 secretion and DC recruitment in the lung mucosa were not affected by HDMΔβ-glucan but were decreased by HDMΔLPS (Fig 1, *D* and *E*). In addition, the increased levels of GM-CSF, thymic stromal-derived lymphopoietin, and IL-33 from bronchial lavage (BAL) fluid were significantly decreased by HDMΔLPS but not by HDMΔβ-glucan (see Fig E1, *B*). These results indicated that β-glucans, rather than LPS, plays a pivotal role in HDM-induced innate immunity in the nasal mucosa, whereas LPS, rather than β-glucans, plays a critical role for that in lung mucosa.

TLR2 and TLR4 are required for HDM-induced innate immune response in the nasal and lung mucosa, respectively

To determine the pattern recognition receptors (PRRs) that are responsible for HDM-induced innate immunity in nasal epithelial cells, we measured the level of HDM-induced CCL20 in NHNE cells in which gene expression of Dectin1, TLR2, and TLR4 were knocked down, because TLR2 and Dectin1 are the main PRRs activated by β-glucans, and TLR4 is the main PRR for LPS.[13,25-31] HDM-induced CCL20 secretion was not affected by knockdown of Dectin-1 or TLR4 expression, whereas

β-glucanase (HDMΔβ-glucan) or polymyxin B (HDMΔLPS). **B-I**, Mice were intranasally challenged without anesthesia (Fig 1, *B*, *C*, *F*, and *G*) or with anesthesia (Fig 1, *D*, *E*, *H*, and *I*) and analyzed 24 hours after the challenge. **B** and **D**, CCL20 secretion from NAL fluids (Fig 1, *B*), or BAL fluid (Fig 1, *D*) of wild-type *(WT)* mice. **C** and **E**, Percentage of $MHCII^+CD11c^+$ DCs in the nasal mucosa (Fig 1, *C*) or in the lung mucosa (Fig 1, *E*). **F** and **H**, CCL20 secretion from NAL (Fig 1, *F*) or BAL (Fig 1, *H*) fluid of WT, $TLR2^{-/-}$, $TLR4^{-/-}$ mice. **G** and **I**, $MHCII^+CD11c^+$ DCs in the nasal (Fig 1, *G*) or the lung (Fig 1, *I*) mucosa. $^*P < .05$ and $^{**}P < .01$. All results are shown as means ± SEMs and are representative of 3 independent experiments. Three to 5 mice were used per group.

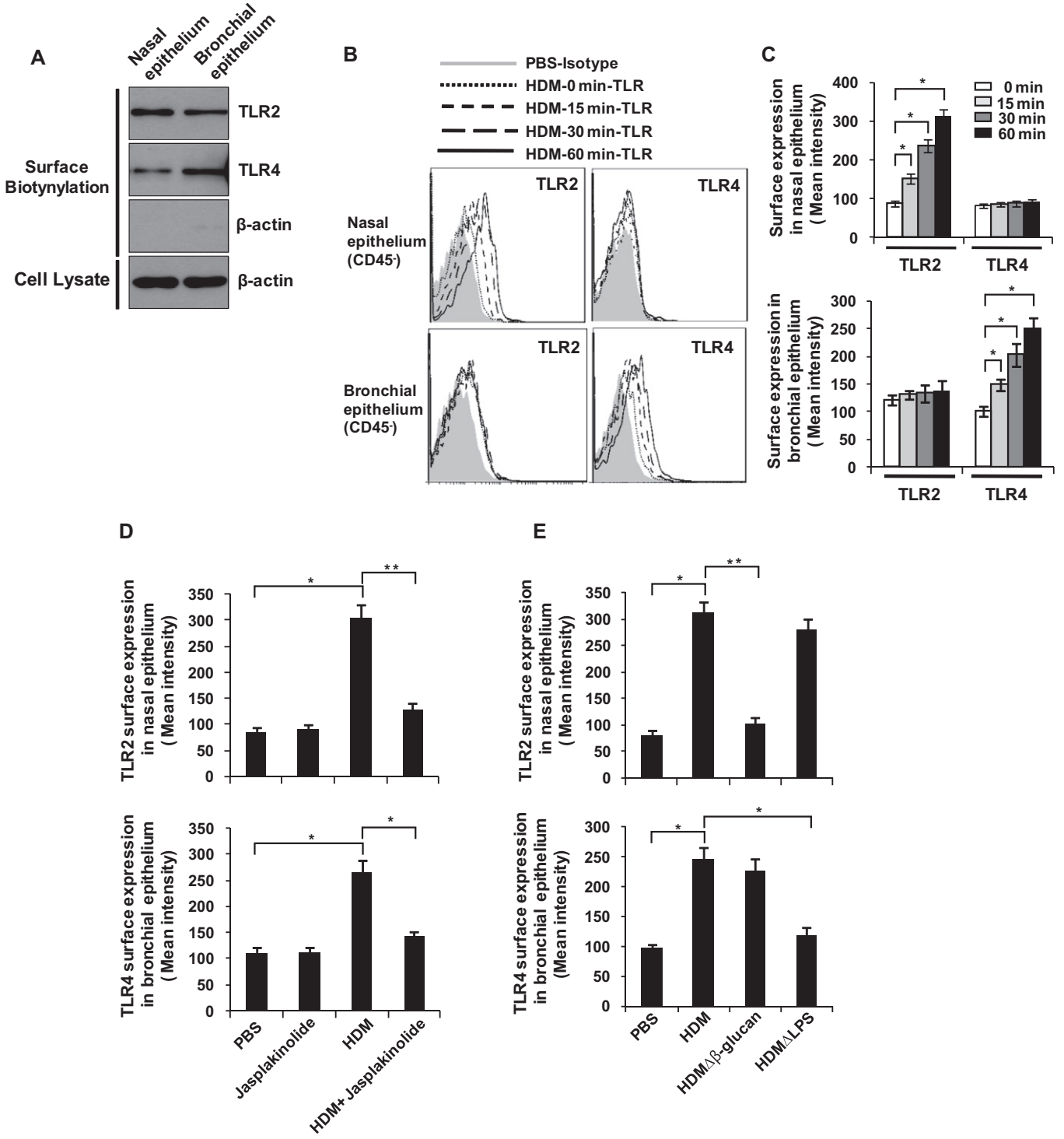


FIG 2. β-Glucans and LPS within HDM induce TLR2 and TLR4 surface expressions in nasal and bronchial epithelial cells, respectively. The nasal epithelium or bronchial epithelium isolated from the murine airway mucosa was taken and analyzed. **A**, Basal surface expression level of TLR2 and TLR4 by Western blot analysis. **B**, TLR2 and TLR4 surface expressions induced by HDM challenge (0, 15, 30, 60 minutes) by fluorescence-activated cell sorting analysis. **C**, Mean intensity of TLR2 and TLR4 surface expressions from panel *B*. **D**, TLR2 and TLR4 surface expressions by HDM challenge (1 hour) in the nasal and bronchial epithelia in the presence (1 hour) or absence of jasplakinolide (1 μmol/L). **E**, TLR2 and TLR4 surface expressions by challenge with HDM, HDMΔβ-glucan, and HDMΔLPS. $^{*}P < .05$ and $^{**}P < .01$. All results are shown as means ± SEMs and are representative of 3 independent experiments.

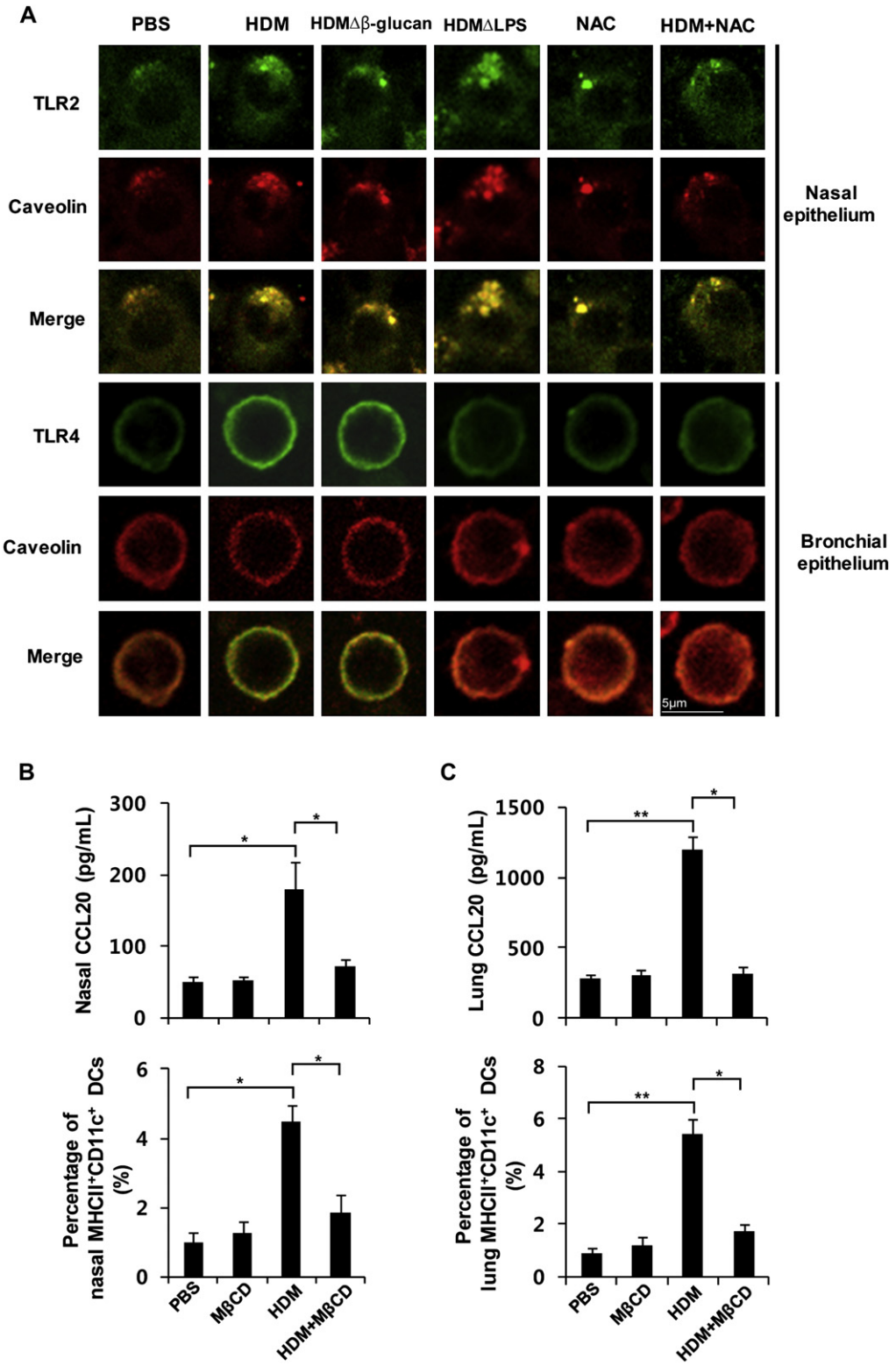


FIG 3. β-Glucans and LPS within HDM induce TLR2 and TLR4 translocation to lipid rafts in cells of nasal and bronchial epithelia, respectively. **A**, TLR2 *(green)* and TLR4 *(green)* translocation to lipid rafts (caveolin; *red*) in cells of nasal and bronchial epithelia, respectively, by challenge (1 hour) with HDM in the presence (1 hour) or absence of NAC (10 mmol/L), or with HDMΔβ-glucan, HDMΔLPS, respectively (50 μg/mL). **B**, CCL20 secretion and $MHCII^+CD11c^+$ DCs in the nasal mucosa by HDM challenge (24 hours) in the presence or absence of methyl-beta-cyclodextrin (MβCD) (1 mmol/L). **C**, CCL20 secretion and $MHCII^+CD11c^+$ DCs in the lung mucosa by HDM challenge in the presence or absence of MβCD (1 mmol/L). $^*P < .05$ and $^{**}P < .01$. All results are shown as means ± SEMs and are representative of 3 independent experiments. Three to 5 mice were used per group.

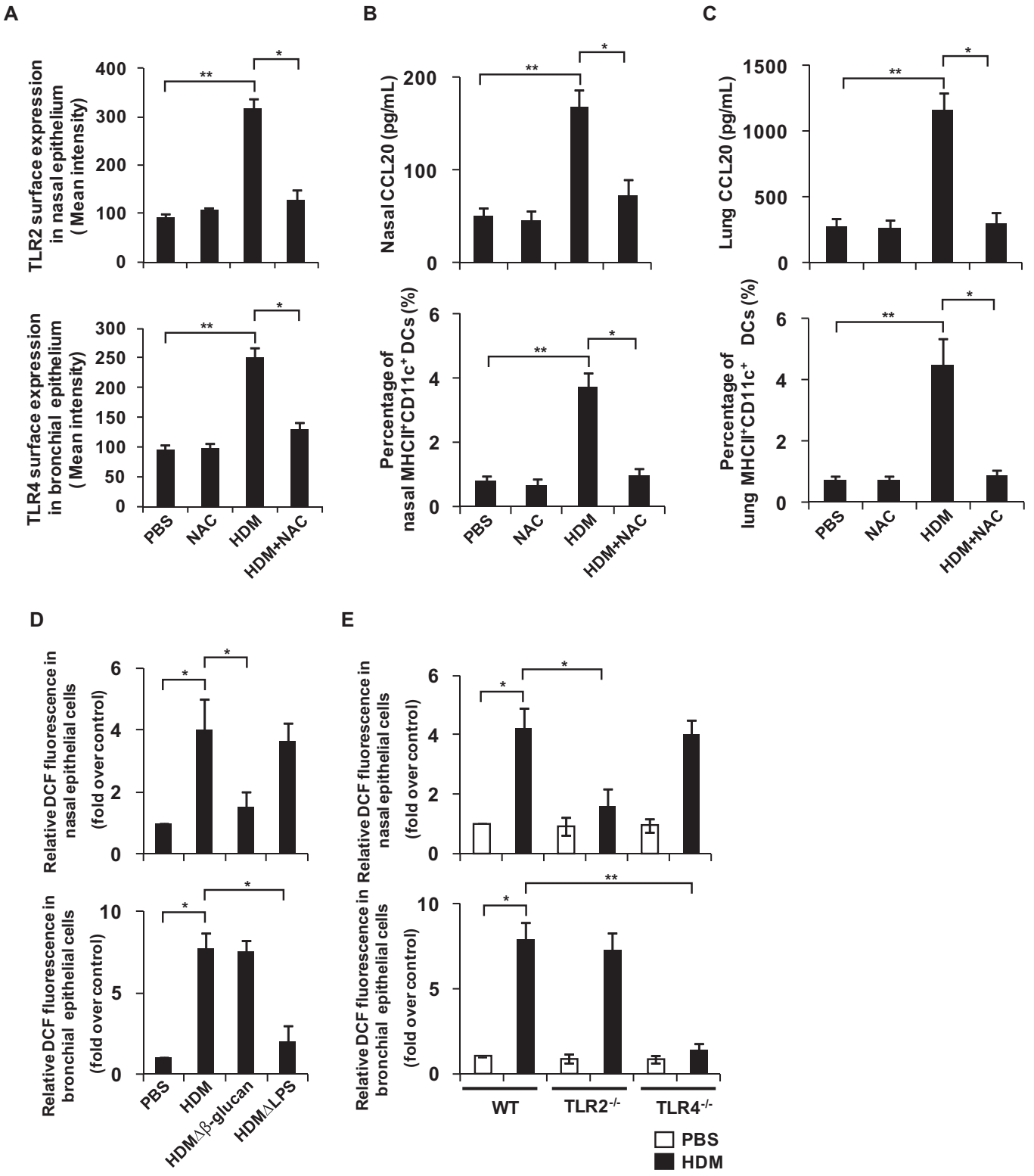


FIG 4. HDM-induced ROS is responsible for innate immunity in both the nasal and the bronchial epithelium. **A**, TLR2 and TLR4 surface expressions by HDM challenge in the nasal and bronchial epithelia in the presence or absence of NAC (10 mmol/L). **B**, CCL20 secretion and $MHCII^+CD11c^+$ DCs in the nasal mucosa by HDM challenge in the presence or absence of NAC. **C**, CCL20 secretion and $MHCII^+CD11c^+$ DCs in the lung mucosa by HDM challenge in the presence or absence of NAC. **D** and **E**, ROS generation was analyzed in primary nasal and bronchial epithelial cells cultured from the wild-type *(WT)* murine airway epithelium (Fig 4, *D*), or WT, $TLR2^{-/-}$, and $TLR4^{-/-}$ airway epithelia (Fig 4, *E*). $^*P < .05$ and $^{**}P < .01$. All results are shown as means ± SEMs and are representative of 3 independent experiments. Three to 5 mice were used per group.

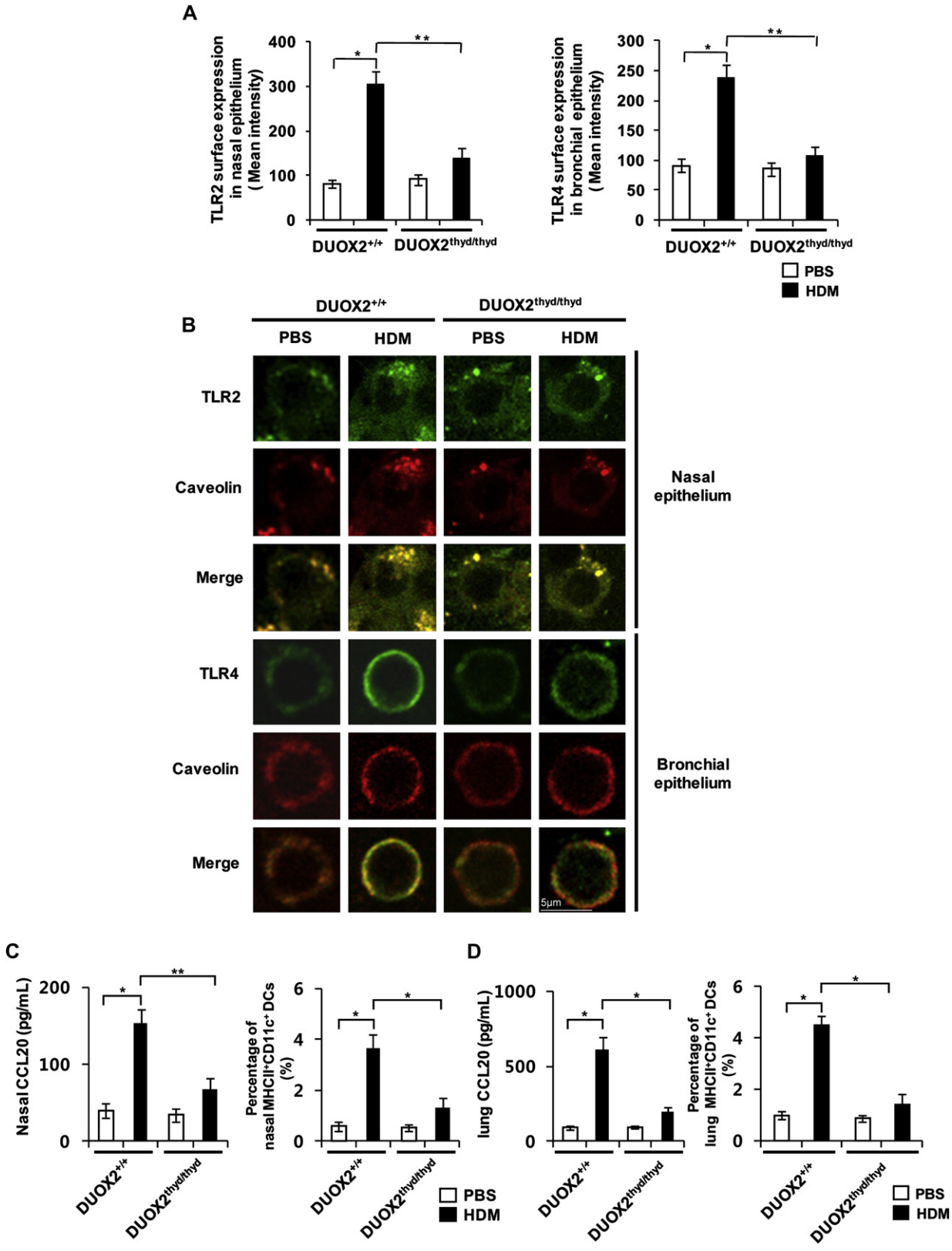
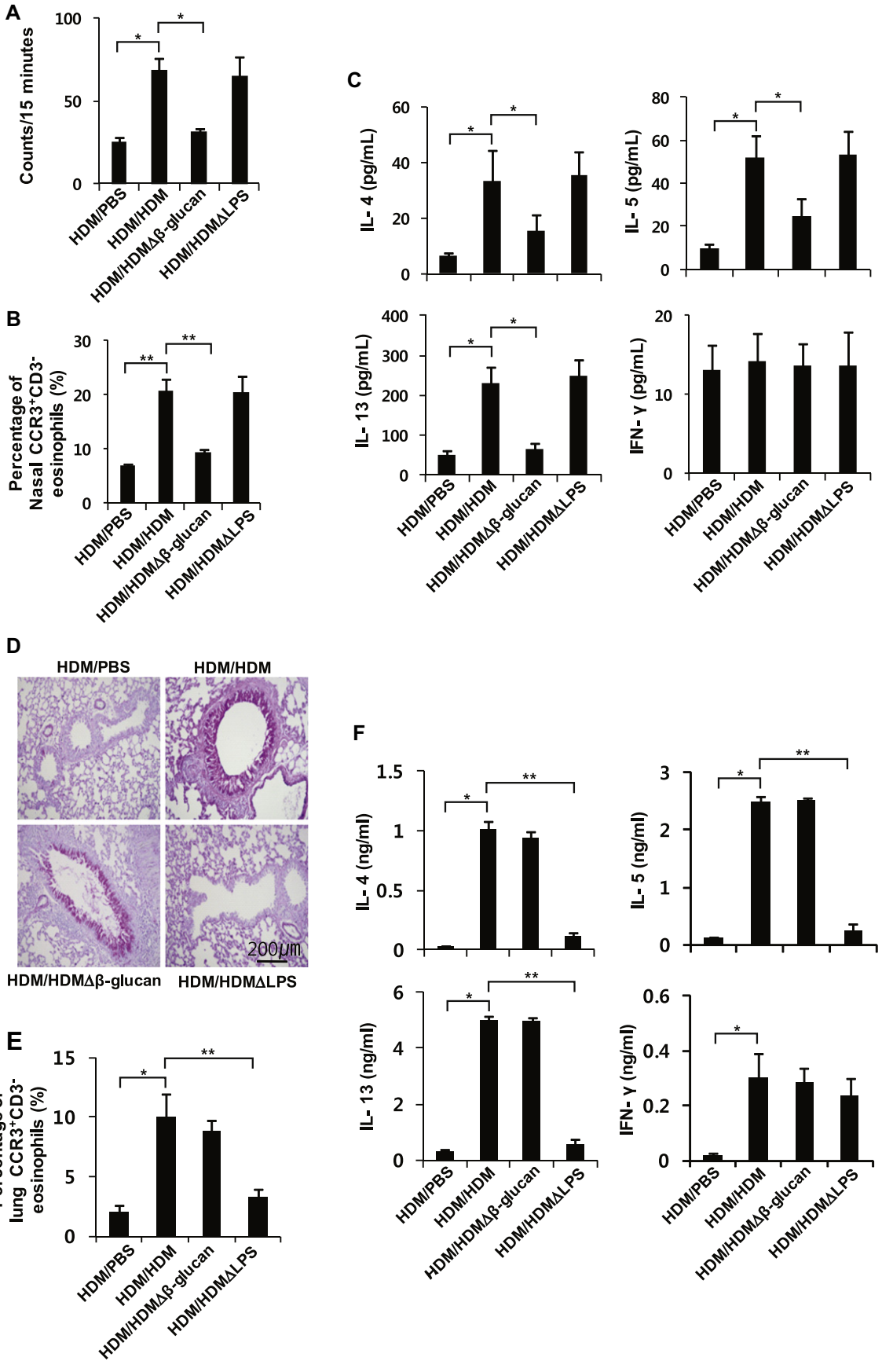


FIG 5. DUOX2 is responsible for HDM-induced innate immunity in both nasal and bronchial epithelium. **A**, TLR2 and TLR4 surface expressions by HDM in the nasal and bronchial epithelia isolated from the DUOX2$^{+/+}$ or DUOX2$^{thyd/thyd}$ airway mucosa. **B**, TLR2 *(green)* and TLR4 *(green)* translocation to lipid rafts (caveolin; *red*) in cells of nasal and bronchial epithelia, respectively, by HDM challenge. **C** and **D**, CCL20 secretion and MHCII^{+}CD11c^{+} DCs by HDM challenge in the nasal mucosa (Fig 5, *C*) and lung mucosa (Fig 5, *D*) of DUOX2$^{+/+}$ or DUOX2$^{thyd/thyd}$ mice. $^{*}P < .05$ and $^{**}P < .01$. All results are shown as means ± SEMs and are representative of 3 independent experiments. Three to 5 mice were used per group.


A
Counts/15 minutes
100
50
0
HDM/PBS
HDM/HDM
HDM/HDMΔβ-glucan
HDM/HDMΔLPS
B
Percentage of Nasal CCR3+CD3- eosinophils (%)
30
20
10
0
C
IL- 4 (pg/mL)
60
40
20
IL- 5 (pg/mL)
80
IL- 13 (pg/mL)
400
300
200
100
IFN- γ (pg/mL)
D
HDM/PBS
HDM/HDM
HDM/HDMΔβ-glucan
HDM/HDMΔLPS
200μm
E
Percentage of lung CCR3+CD3- eosinophils (%)
15
10
5
F
IL- 4 (ng/ml)
1.5
1
0.5
IL- 5 (ng/ml)
3
2
IL- 13 (ng/ml)
6
4
IFN- γ (ng/ml)
0.6
0.4
0.2

knockdown of TLR2 expression dramatically decreased the HDM-induced CCL20 secretion (see Fig E2 in this article's Online Repository at www.jacionline.org). To confirm this result *in vivo*, we examined CCL20 secretion levels and DC recruitment in the nasal and lung mucosa of $TLR2^{-/-}$ or $TLR4^{-/-}$ mice. HDM-induced CCL20 secretion (Fig 1, *F*) and DC recruitment to the nasal mucosa (Fig 1, *G*) were attenuated in $TLR2^{-/-}$ mice but not in $TLR4^{-/-}$ mice, whereas HDM-induced CCL20 secretion (Fig 1, *H*) and DC recruitment to the lung mucosa (Fig 1, *I*) were diminished in $TLR4^{-/-}$ mice but not in $TLR2^{-/-}$ mice. Taken together, the β-glucan/TLR2 signaling axis was essential in mediating HDM-induced innate immune responses in nasal mucosa. In contrast, LPS/TLR4 signaling was required in the lung mucosa.

HDM-derived β-glucans induced TLR2 surface expression of epithelial cells in nasal mucosa, whereas HDM-derived LPS induced TLR4 surface expression of those in bronchial mucosa

To understand the reason why HDM-derived β-glucans and TLR2 are critical to the activation of innate immunity in the nasal mucosa and why HDM-derived LPS and TLR4 are critical in the lung mucosa, we first checked the basal level of surface expression of TLR2 and TLR4 in the epithelium obtained from the murine nasal and bronchial mucosa. Interestingly, basal surface expression of TLR2 or TLR4 in the nasal and bronchial epithelia was almost the same (Fig 2, *A*). In addition, intracellular proteins of TLR2 or TLR4 in the nasal and bronchial epithelia were not increased by HDM challenge (see Fig E3 in this article's Online Repository at www.jacionline.org). Thus, we investigated the induction of surface expression of TLR2 and TLR4 in cells ($CD45^-$ cells) within the nasal and bronchial epithelia by HDM challenge. Fluorescence-activated cell sorting analysis showed that TLR2 surface expression was increased rapidly at 15 minutes and was maximally induced at 1 hour in nasal epithelium after HDM challenge, but TLR4 surface expression was not. TLR4 surface expression was enhanced at 15 minutes and maximally induced at 1 hour in bronchial epithelia after HDM challenge, whereas TLR2 surface expression was not (Fig 2, *B* and *C*). Dectin-1 surface expression was not affected in nasal and bronchial epithelia by HDM challenge (see Fig E4 in this article's Online Repository at www.jacionline.org). These data indicate that signal-dependent TLR2 and TLR4 surface expression in nasal and bronchial epithelia, respectively, might be a possible mechanism in how TLR2 and TLR4 have a pivotal role in HDM-induced innate immunity in a tissue-specific manner. Consistent with data obtained from the nasal and bronchial epithelia, TLR2 and TLR4 surface expression were increased by HDM in primary epithelial cells cultured from the nasal and bronchial epithelia, respectively (see Fig E5 in this article's Online Repository at www.jacionline.org), suggesting that HDM-induced TLR overexpression in the nasal and bronchial epithelia mainly occurred in epithelial cells, rather than in hematopoietic cells such as macrophages and DCs. To check that TLRs surface expression was caused by HDM-induced exocytosis, we pretreated mice with jasplakinolide, a marine sponge toxin that was shown to physically interfere with exocytosis,[32,33] and examined TLR surface expression after HDM challenge. Jasplakinolide decreased HDM-induced TLR2 and TLR4 surface expressions in the nasal and bronchial epithelia, respectively (Fig 2, *D*). We also showed that HDM-induced TLR2 and TLR4 surface expression in the nasal and bronchial epithelia (Fig 2, *E*) or primary nasal and bronchial epithelial cells (see Fig E6 in this article's Online Repository at www.jacionline.org) depended on HDM-derived β-glucans and LPS, respectively.

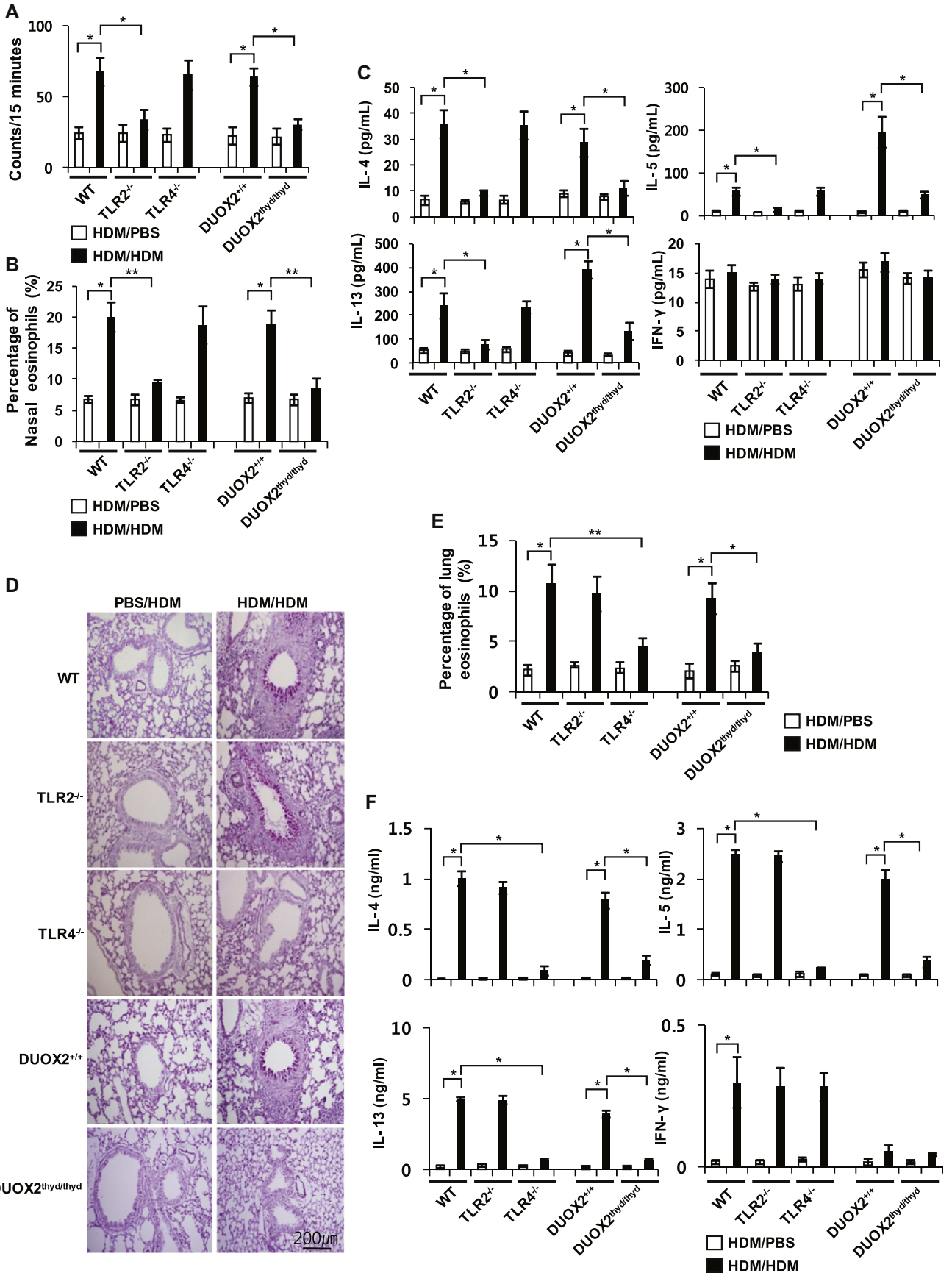
β-Glucans and LPS within HDM were responsible for TLR2 and TLR4 translocation to lipid rafts of nasal and bronchial epithelial cells, respectively

Next, we examined whether HDM-derived β-glucans and LPS induced TLR2 and TLR4 translocation to lipid rafts in cells within nasal and bronchial epithelia, respectively. HDM challenge induced TLR2 translocation to lipid rafts (stained with caveolin) in cells of nasal epithelium and TLR4 translocation in cells of bronchial epithelium in a time-dependent manner (see Fig E7 in this article's Online Repository at www.jacionline.org). The increased TLR2 and TLR4 translocation to lipid rafts were prohibited by HDMΔβ-glucan and HDMΔLPS, respectively (Fig 3, *A*). We also showed that disruption of lipid rafts by methyl-β-cyclodextrin (MβCD) caused a significant decrease of CCL20 secretion and DC recruitment in both nasal and bronchial mucosa (Fig 3, *B* and *C*), indicating that intact lipid rafts in airway epithelial cells were required for HDM-induced innate immune responses.

β-Glucan/TLR2 and LPS/TLR4 signaling axes were required for HDM-induced ROS generation which mediates innate immunity in the nasal and bronchial mucosa, respectively

Several studies have shown that oxidative stress induces recruitment of TLR4 or TLR2 into lipid rafts of immune cells stimulated by LPS or lipoprotein.[34,35] Thus, we pretreated the airway mucosa with N-acetylcysteine (NAC), a ROS scavenging chemical, and measured HDM-induced TLR overexpression and its translocation to lipid rafts. ROS removal by NAC treatment decreased TLR2 and TLR4 surface expression (Fig 4, *A*) and translocation to lipid rafts (Fig 3, *A*). We also showed that NAC treatment downregulated CCL20 secretion and DC recruitment in both nasal and lung mucosa (Fig 4, *B* and *C*), suggesting

FIG 6. β-Glucans and LPS within HDM are responsible for HDM-induced AR and AA, respectively. **A-C**, Wild-type *(WT)* mice were sensitized with HDM extract and consequent provocation by intranasal challenging with PBS *(HDM/PBS)*, HDM extracts *(HDM/HDM)*, HDMΔβ-glucan *(HDM/HDMΔβ-glucan)*, and HDMΔLPS *(HDM/HDMΔLPS)*, without anesthesia. **A**, Frequencies of nose scratching. **B**, $CCR3^+CD3^-$ eosinophils in the nasal mucosa. **C**, Cytokine production in cervical lymph node cells restimulated *ex vivo* for 4 days with PBS, HDM, HDMΔβ-glucan, and HDMΔLPS. **D-F**, WT mice were sensitized as in panels *A-C* and consequent provocation by intranasal challenging under anesthesia. **D**, Periodic acid-Schiff staining of lung tissue. **E**, $CCR3^+CD3^-$ eosinophils from the BAL fluid. **F**. Cytokine production in the mediastinal lymph node cells restimulated as in panel *C*. $^*P < .05$ and $^{**}P < .01$. All results shown are means ± SEMs and are representative of 4 independent experiments. Three to 5 mice were used per group.


A
Counts/15 minutes
100
50
0
WT
TLR2-/-
TLR4-/-
DUOX2+/+
DUOX2thyd/thyd
HDM/PBS
HDM/HDM
B
Percentage of Nasal eosinophils (%)
25
20
15
10
5
0
C
IL-4 (pg/mL)
IL-5 (pg/mL)
IL-13 (pg/mL)
IFN-γ (pg/mL)
D
PBS/HDM
HDM/HDM
WT
TLR2-/-
TLR4-/-
DUOX2+/+
DUOX2thyd/thyd
200μm
E
Percentage of lung eosinophils (%)
F
IL-4 (ng/ml)
IL-5 (ng/ml)
IL-13 (ng/ml)
IFN-γ (ng/ml)

that HDM-induced ROS generation was required for TLR2 and TLR4 activation, leading to innate immune responses in the airway epithelium. We next investigated whether β-glucans and LPS were required for HDM-induced ROS generation in nasal and bronchial epithelial cells, respectively. ROS generation was maximally increased at 5 minutes after treatment with 50 μg/mL HDM in both primary bronchial epithelial cells (see Fig E8 in this article's Online Repository at www.jacionline.org) and nasal epithelial cells (data not shown). Increased ROS generation in nasal epithelial cells was dramatically decreased by HDMΔβ-glucan, but not HDMΔLPS, whereas ROS induction in bronchial epithelial cells was reduced by HDMΔLPS but not HDMΔβ-glucan (Fig 4, *D*). In addition, HDM-induced ROS generation was significantly decreased in $TLR2^{-/-}$ but not $TLR4^{-/-}$ nasal epithelial cells, whereas it was decreased in $TLR4^{-/-}$ but not $TLR2^{-/-}$ bronchial epithelial cells (Fig 4, *E*). Collectively, β-glucan/TLR2 and LPS/TLR4 signaling axes were required for HDM-induced ROS generation, and increased ROS positively regulated TLR2 and TLR4 surface expression and translocation to lipid rafts to the nasal and bronchial epithelial cells, respectively.

DUOX2-induced ROS mediated HDM-induced TLR activation which led to innate immunity in both nasal and bronchial mucosa

To determine the generation source of HDM-induced ROS in nasal epithelial cells, we knocked down gene expressions of DUOX1 and DUOX2, the main ROS sources activated by PAMP stimulation in airway epithelial cells,[36-39] and measured HDM-induced ROS generation in NHNE cells. Only knockdown of DUOX2 gene expression significantly decreased ROS induction, whereas knockdown of DUOX1 had no effect (see Fig E9 in this article's Online Repository at www.jacionline.org). Similar to the results obtained with the NAC treatment, TLR2 and TLR4 surface expression (Fig 5, *A*) and translocation to lipid rafts (Fig 5, *B*) were fundamentally attenuated in nasal and bronchial epithelia of $DUOX2^{thyd/thyd}$ mutant mice, respectively. In addition, knockdown of DUOX2 gene expression in NHNE cells decreased HDM-induced TLR2 surface expression, but TLR4 surface expression itself was not affected by HDM (see Fig E10 in this article's Online Repository at www.jacionline.org). On the basis of previous reports,[40,41] which suggested that a physical interaction between TLR and NADPH oxidase isozyme play an essential role in ROS-induced immune responses, we examined the interaction between endogenous TLR and DUOX2 in NHNE cells. Cell lysates were subjected to immunoprecipitation with DUOX2 antibody. Immunoblot analysis of the resulting precipitates with TLR2 and TLR4 antibodies found that TLR2 strongly interact with DUOX2, whereas TLR4 weakly interacted with DUOX2 (see Fig E11 in this article's Online Repository at www.jacionline.org). However, the interaction between TLRs and DUOX2 was not increased in response to HDM, suggesting that DUOX2 localized with TLR signaling complexes to efficiently activate DUOX2-mediated signaling pathway. In addition, CCL20 secretion and DCs recruitment to the nasal and lung mucosa were decreased in $DUOX2^{thyd/thyd}$ mice (Fig 5, *C* and *D*), suggesting that DUOX2 was responsible for HDM-induced innate immunity through generating ROS production that induced activation of both TLR2 and TLR4, respectively.

β-Glucan/TLR2 and LPS/TLR4 signaling axes were critical for allergic inflammation in HDM-induced AR and AA mouse models, respectively

Given the role of β-glucan/TLR2 and LPS/TLR4 signaling axes in HDM-induced innate immunity in the nasal and lung mucosa, respectively, we next examined the influence of mucosal innate immunity on allergic inflammatory diseases with the use of HDM-induced AR and AA mouse models. First, we constructed a mouse model of AR or AA induced by sensitization with HDM extracts and consequent provocation of PBS (HDM/PBS) or HDM extracts (HDM/HDM) (see Fig E12 in this article's Online Repository at www.jacionline.org). In our AR mouse model, AR-associated behavior such as nose scratching (Fig 6, *A*) and eosinophils in the nasal mucosa (Fig 6, *B*) were increased in the HDM/HDM AR mice. Furthermore, in the lymphoid cells of the cervical lymph nodes, into which the lymphatics of the nose drain, levels of T_H2 cell–associated cytokines, including IL-4, IL-5, and IL-13, were increased in the HDM/HDM AR mice, whereas levels of IFN-γ were unaffected (Fig 6, *C*). The efficacy of the AA mouse model was confirmed as several features of AA such as peribronchial inflammation and goblet cell hyperplasia (Fig 6, *D*); eosinophil increase from bronchial lavage fluid (Fig 6, *E*); and increase of IL-4, IL-5, and IL-13 in mediastinal lymph nodes (Fig 6, *F*). The allergic features of AA (see Fig E13, *A-C*, in this article's Online Repository at www.jacionline.org) and airway hyperresponsiveness to the bronchoconstrictor methacholine (see Fig E14 in this article's Online Repository at www.jacionline.org) in the AR mouse model were not observed, whereas the allergic features of AR in the AA mouse model were not detected (data not shown). Allergic features of AR were severely reduced in HDM/HDMΔβ-glucan but not in HDM/HDMΔLPS, compared with HDM/HDM (Fig 6, *A-C*). To investigate the contribution of proteases within HDM on AR features, we pretreated HDM extracts with protease inhibitor to inactivate proteases (HDMΔprotease) and examined the allergic features of the AR models in the presence of HDM/PBS, HDM/HDM, and HDM/HDMΔprotease. The allergic features of AR were not reduced in the presence of HDM/HDMΔprotease, suggesting that HDM-derived proteases were dispensable for the development of AR under HDM-sensitized conditions in our model (see Fig E15 in this article's Online Repository at www.jacionline.org). Allergic features of AA were significantly attenuated in HDM/HDMΔLPS but not in HDM/HDMΔβ-glucan

FIG 7. TLR2 and TLR4 are required for HDM-induced AR and AA, respectively, and DUOX2 is responsible for both HDM-induced AR and AA. **A-F**, Wild-type *(WT)*, $TLR2^{-/-}$, $TLR4^{-/-}$, $DUOX2^{+/+}$, and $DUOX2^{thyd/thyd}$ mice were used in HDM-induced AR (Fig 7, *A-C*) or AA (Fig 7, *D-F*) as in Fig 6. **A**, Nose scratching. **B**, $CCR3^{+}CD3^{-}$ eosinophils in the nasal mucosa. **C**, Cytokine production in restimulated cervical lymph node cells. **D**, Periodic acid–Schiff staining of lung tissue. **E**, $CCR3^{+}CD3^{-}$ eosinophils from the BAL fluid. **F**, Cytokine production in restimulated mediastinal lymph node cells. $^{*}P < .05$ and $^{**}P < .01$. All results are shown as means ± SEMs and are representative of 4 independent experiments. Three to 5 mice were used per group.

(Fig 6, *D-F*). These data suggested that HDM-derived β-glucan and LPS were important for the development of AR and AA, respectively, under HDM-sensitized conditions. We next examined the influence of TLR2 or TLR4 deficiency on an HDM-induced AR or AA mouse model. Features of AR were attenuated in $TLR2^{-/-}$ HDM/HDM mice but not in $TLR4^{-/-}$ HDM/HDM mice (Fig 7, *A-C*), whereas allergic features of AA were attenuated in $TLR4^{-/-}$ HDM/HDM mice but not in $TLR2^{-/-}$ HDM/HDM mice (Fig 7, *D-F*).

DUOX2-mediated ROS play a pivotal role for both HDM-induced AR and AA

Having shown that DUOX2-mediated ROS was critical for HDM-induced innate immunity in both the nasal and lung mucosae, we next attempted to verify the function of DUOX2 on HDM-induced AR or AA. Allergic characteristics of both AR and AA were dramatically reduced in $DUOX2^{thyd/thyd}$ mice (Fig 7, *A-F*). Taken together, DUOX2/ROS was a common signaling pathway that mediated both HDM-induced AR and AA, although the β-glucans/TLR2 and LPS/TLR4 signaling axes were required for AR and AA in a tissue-specific manner (see Fig E16 in this article's Online Repository at www.jacionline.org).

DISCUSSION

Here, we clearly showed that HDM-derived β-glucans and TLR2 were critical for activating innate immunity in the nasal mucosa and subsequent AR by showing that CCL20 secretion, DC recruitment, and HDM-induced AR were attenuated in HDMΔβ-glucan challenge or $TLR2^{-/-}$ mice. In contrast, LPS/TLR4 signaling axis, rather than β-glucan/TLR2, played a pivotal role in activating innate immunity in the lung mucosa and resultant AA in our study, in agreement with previous studies that TLR4, rather than TLR2, was the main PRR for HDM-induced AA[42] and that TLR4 triggering in lung structural cells by LPS and HDM was responsible for HDM-induced AA.[13,16]

However, we did not determine the intrinsic mechanism for why β-glucan/TLR2 and LPS/TLR4 signaling axes are selectively activated to HDM exposure in a tissue-specific manner, although HDM contains both β-glucan and LPS and because the surface expression levels of TLR2 and TLR4 in nasal and bronchial epithelial cells are almost the same. Speculatively, distinctive TLR-related signaling modules might exist in nasal and bronchial epithelial membranes, respectively, to aid in the interaction between specific PAMPs and TLRs, as well as to regulate signal-dependent TLR activation in a tissue-specific manner. Further studies will be required to determine the specific signaling modules that are differently controlled by HDM exposure in nasal and bronchial epithelia. In contrast to distinctive innate immunity activated by PAMP-dependent TLR activation, DUOX2-mediated ROS served as a common signaling substance that regulated innate immunity, because HDM-induced CCL20 secretion and DC recruitment in both the nasal and lung mucosa were significantly attenuated in NAC-treated mice or $DUOX2^{thyd/thyd}$ mice. However, triggering ROS generation depended on HDM-derived PAMP-dependent TLR recognition, because HDM-induced ROS production was decreased by HDMΔβ-glucan and was attenuated in $TLR2^{-/-}$ nasal epithelial cells. In contrast, increased ROS production was decreased by HDMΔLPS and reduced in $TLR4^{-/-}$ bronchial epithelial cells. Although several studies have shown that LPS and lipoprotein induce recruitment of TLR4 and TLR2 to lipid rafts through signal-dependent ROS production in macrophage cells,[34,35,43] the exact mechanisms by which DUOX2 is activated by the β-glucans/TLR2 or LPS/TLR4 signaling axis and how the activated DUOX2/ROS system mediates their surface expression and translocation to lipid rafts need to be further studied. The role of ROS in allergic inflammation was presented according to the finding that nicotinamide adenine dinucleotide phosphate oxidase within allergen pollen grain increased the level of ROS in airway epithelial cells and consequent airway inflammation in experimental animals.[44] These data support our results that HDM-induced ROS in airway epithelial cells play an essential role in activating innate immunity and subsequent AR and AA.

Although we showed that CCL20 is secreted from nasal or bronchial epithelial cells by HDM exposure, it was shown that CCL20 is induced by TLR ligands or allergen in nonepithelial cells such as fibroblasts and primary peribronchial lymph node cells.[15,45] Although CCL20 secretion sources differ, the main role of CCL20 in an allergic response is the recruitment of myeloid DCs into mucosal epithelia by immune stimuli.[46,47] Allergen-stimulated signal molecules from epithelial cells and consequent activation of DCs in airway epithelium were shown to play a critical role in mediating innate immunity to T_H2-medaited allergic inflammation.[10,14,15] Thus, identifying the signaling mechanism between epithelial cells and DCs might be significant to understanding allergic diseases.

Taken together, we describe the distinctive mechanisms of innate immunity in upper and lower airways and their role in mediating allergic diseases by showing that β-glucans/TLR2-mediated DUOX2/ROS and LPS/TLR4-activated DUOX2/ROS systems regulate innate immunity in the nasal and bronchial mucosa, which trigger HDM-induced AR and AA, respectively. These findings suggest that targeted inactivation of distinctive innate immune signals to allergen exposure in the upper and lower airways may potentially be useful to the development of specific therapeutics for AR and AA.

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Clinical implications: Targeted inactivation of distinctive innate immune signals to allergen exposure in the upper and lower airways may be useful to the development of specific therapeutics for AR and/or AA.

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