

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 2012;■■■:■■■-■■■.)

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
M β CD:	Methyl- β -cyclodextrin
HDM:	House dust mite
HDM $\Delta\beta$ -glucan:	HDM extracts pretreated with β -glucanase to degrade β -glucan structures
HDM Δ LPS:	HDM extracts pretreated with polymyxin B to inactivate LPS
HDM Δ protease:	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 defense against inhaled allergens, are different in the nasal and

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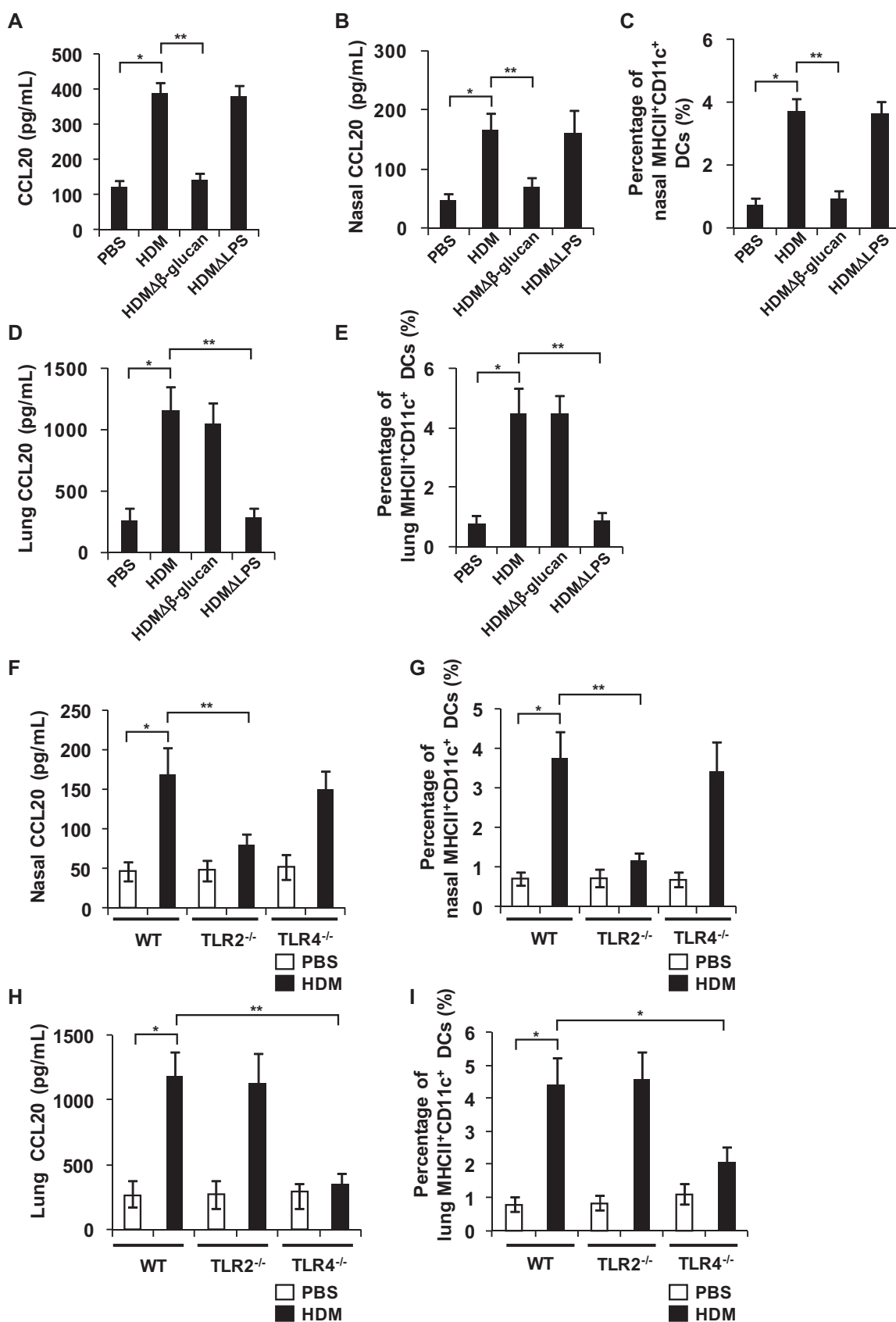


FIG 1. HDM-derived β -glucans and TLR2 and HDM-derived LPS and TLR4 are responsible for innate immunity in the nasal mucosa and the lung mucosa, respectively. **A**, Quantification of CCL20 secretion by treatment with HDM extracts (50 μ g/mL) for 24 hours in NHNE cells. HDM extracts pretreated with

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 (HDM $\Delta\beta$ -glucan) or polymyxin B to inactivate LPS (HDM Δ LPS) 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 HDM Δ LPS, whereas HDM $\Delta\beta$ -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 HDM $\Delta\beta$ -glucan but not by HDM Δ LPS (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 HDM $\Delta\beta$ -glucan but not by HDM Δ LPS (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 HDM $\Delta\beta$ -glucan but not on HDM Δ LPS (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 HDM $\Delta\beta$ -glucan but were decreased by HDM Δ LPS (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 HDM Δ LPS but not by HDM $\Delta\beta$ -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 (HDM $\Delta\beta$ -glucan) or polymyxin B (HDM Δ LPS). **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 $\pm$ SEMs and are representative of 3 independent experiments. Three to 5 mice were used per group.

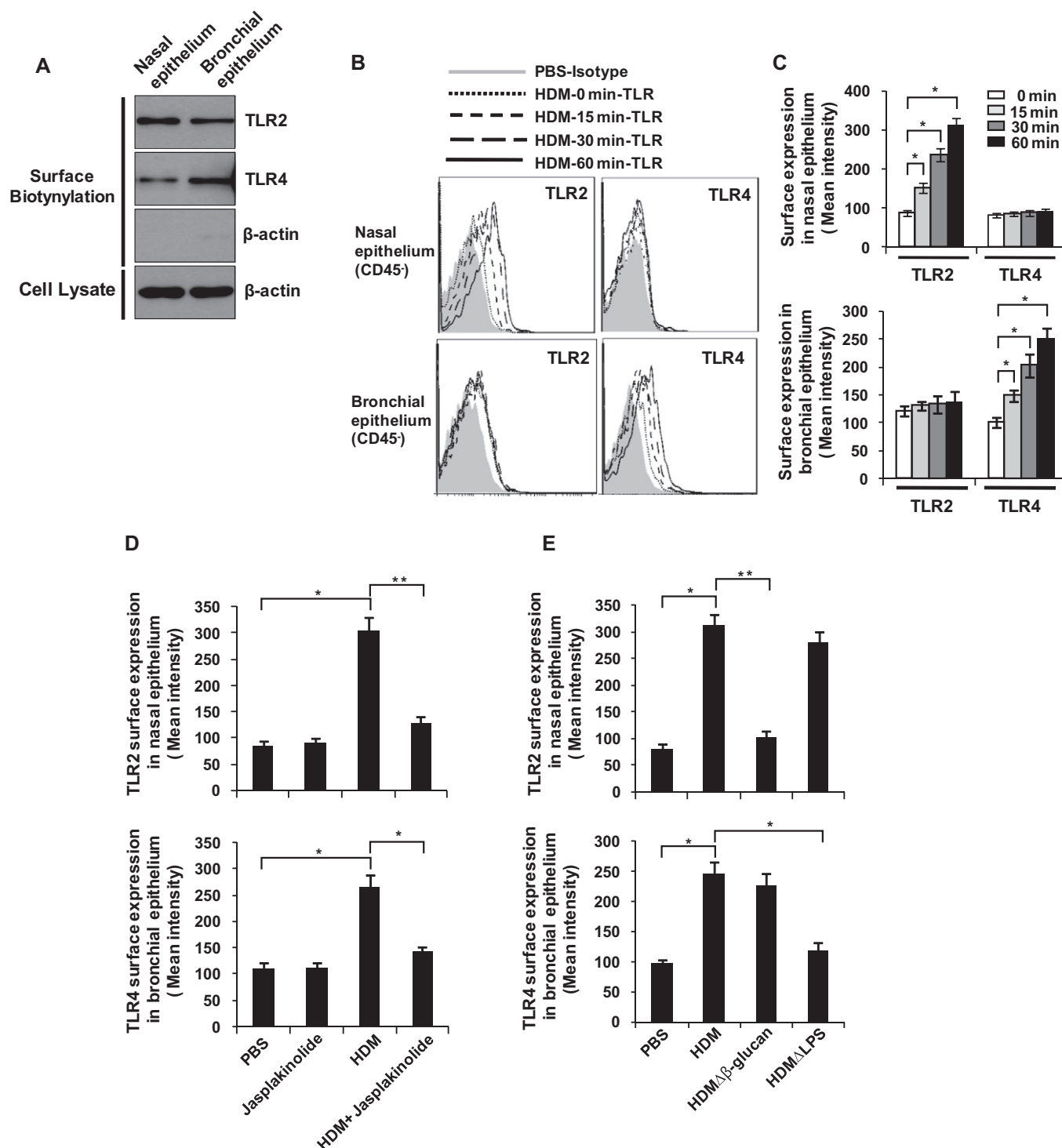


FIG 2. β -Glucans and LPS within HDM induce TLR2 and TLR4 surface expressions in nasal and bronchial epithelial cells, respectively. The nasal epithelium or bronchial epithelium isolated from the murine airway mucosa was taken and analyzed. **A**, Basal surface expression level of TLR2 and TLR4 by Western 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 μ mol/L). **E**, TLR2 and TLR4 surface expressions by challenge with HDM, HDM $\Delta\beta$ -glucan, and HDM Δ LPS. * P < .05 and ** P < .01. All results are shown as means $\pm$ SEMs and are representative of 3 independent experiments.

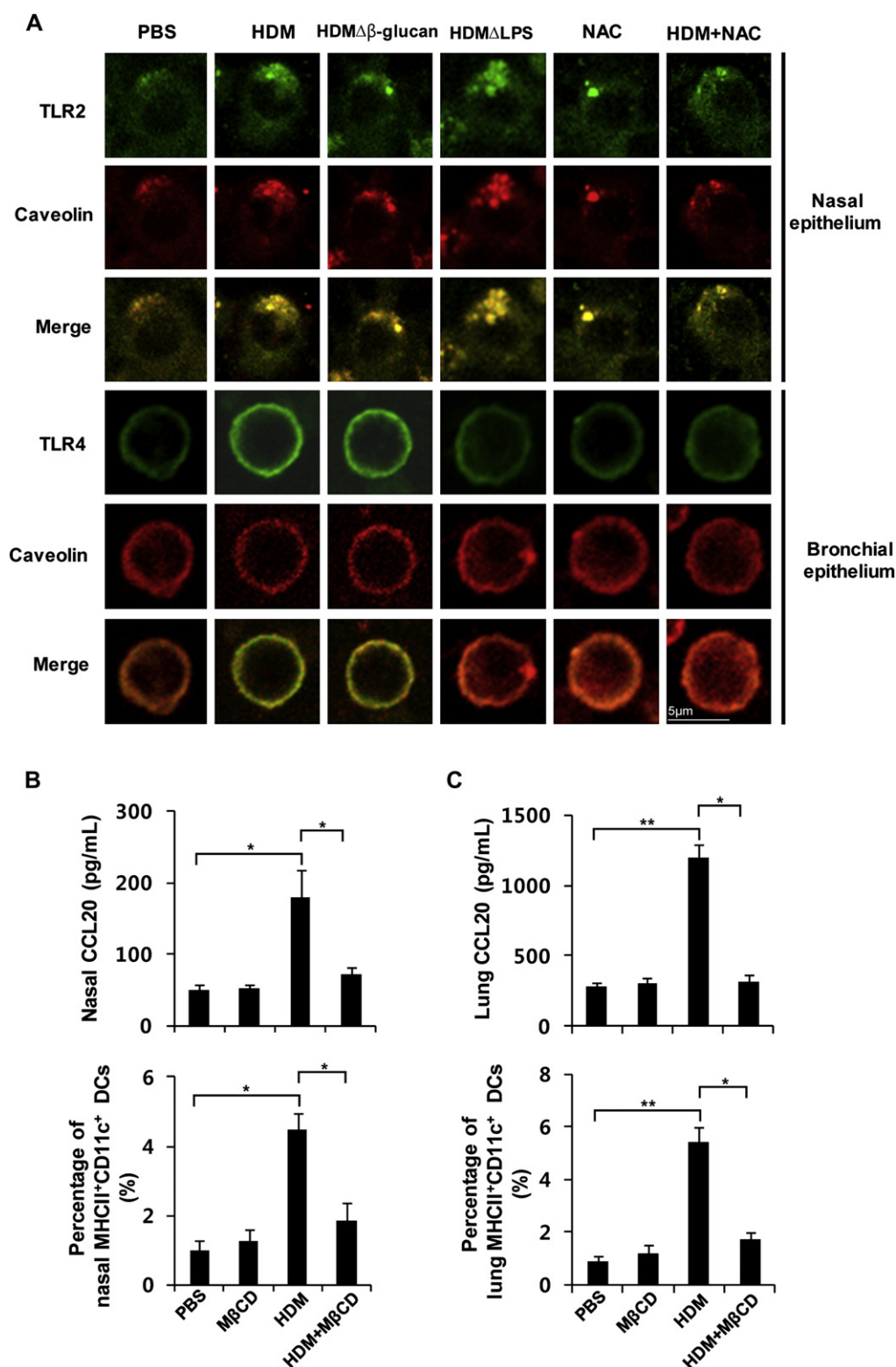


FIG 3. β -Glucans and LPS within HDM induce TLR2 and TLR4 translocation to lipid rafts in cells of nasal and bronchial epithelia, respectively. **A**, TLR2 (green) and TLR4 (green) translocation to lipid rafts (caveolin; red) in cells of nasal and bronchial epithelia, respectively, by challenge (1 hour)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

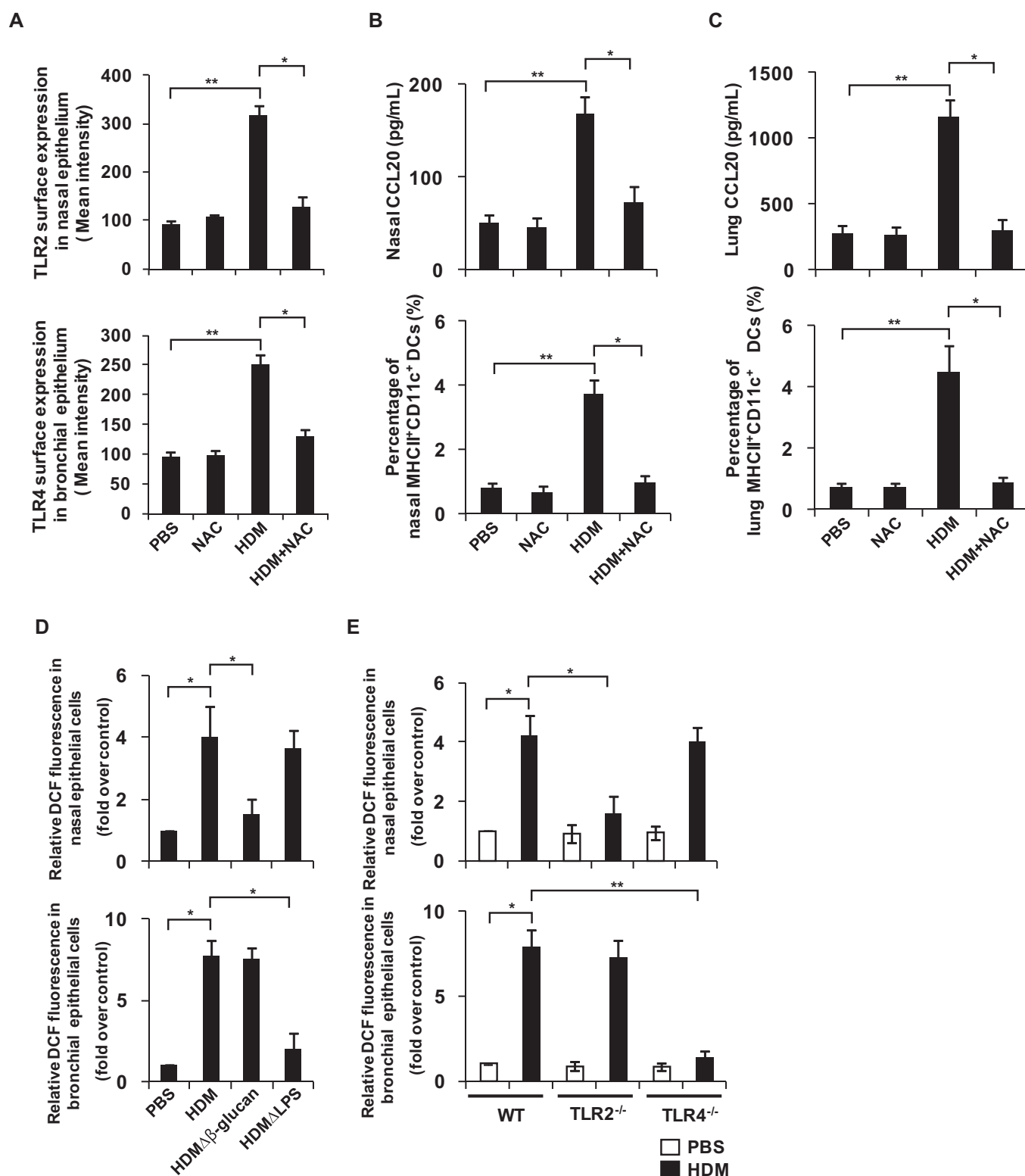


FIG 4. HDM-induced ROS is responsible for innate immunity in both the nasal and the bronchial epithelium. **A**, TLR2 and TLR4 surface expressions by HDM challenge in the nasal and bronchial epithelia in the presence or absence of NAC (10 mmol/L). **B**, CCL20 secretion and MHCII⁺CD11c⁺ DCs in the nasal 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 $\pm$ SEMs and are representative of 3 independent experiments. Three to 5 mice were used per group.

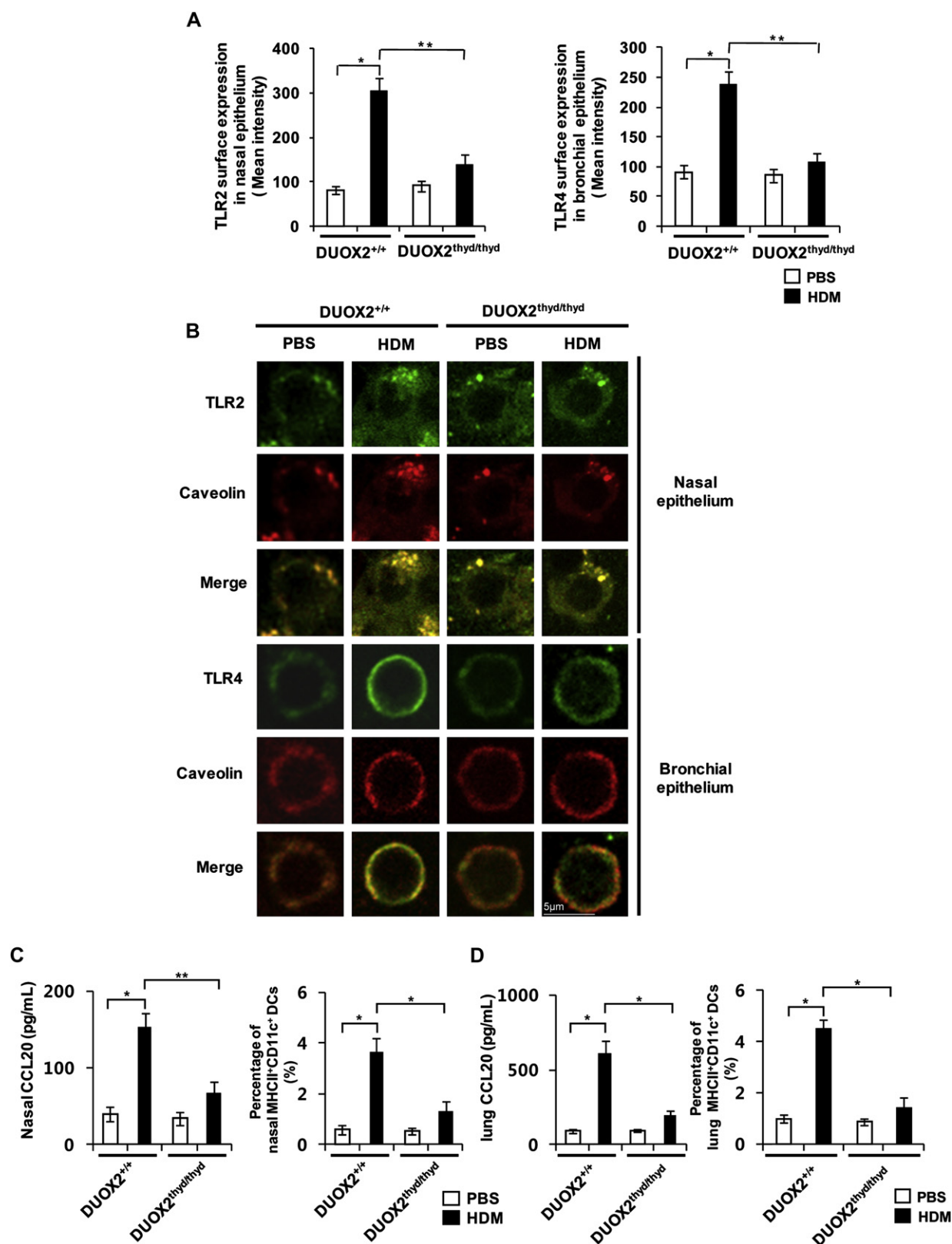
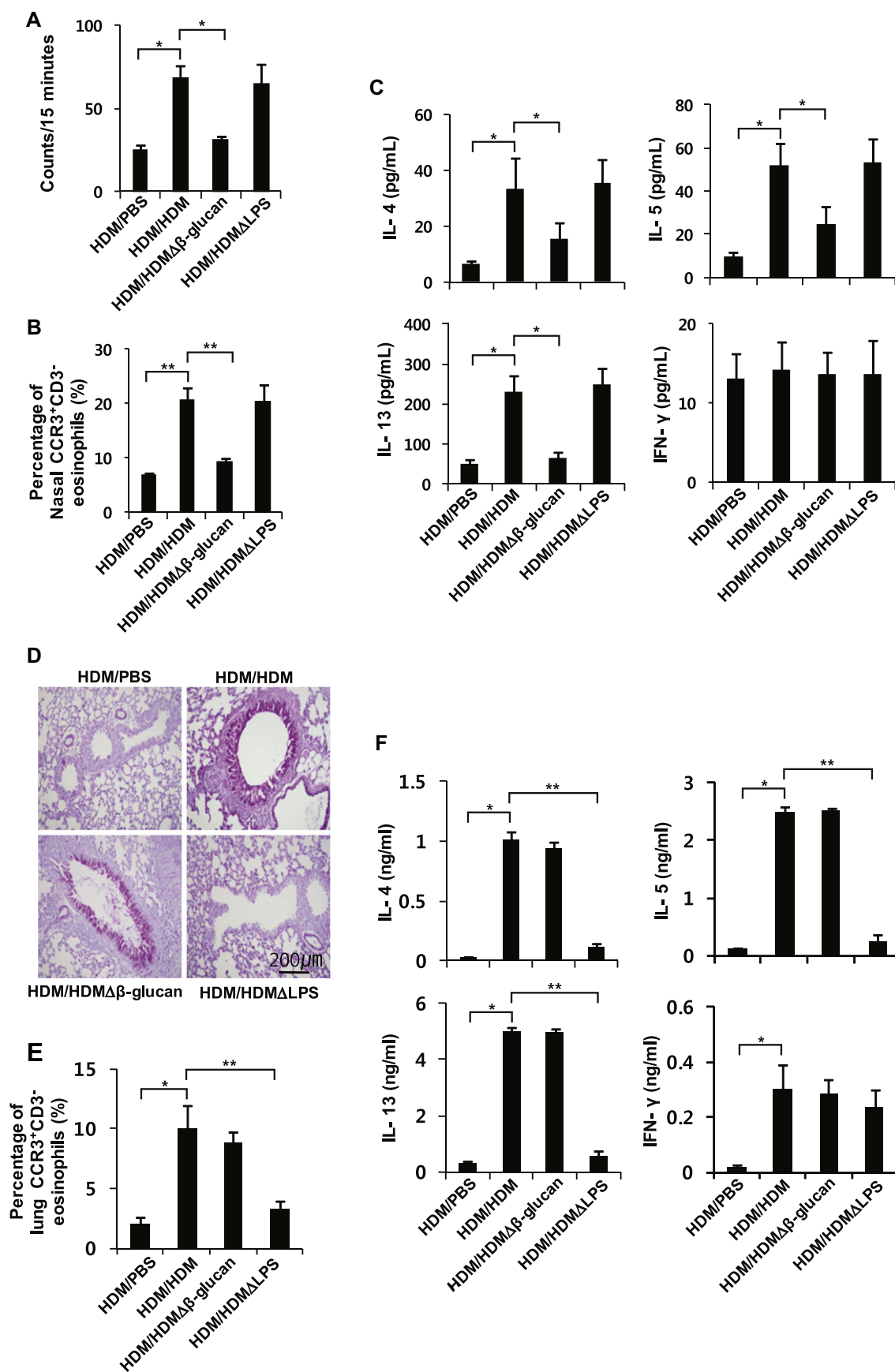


FIG 5. DUOX2 is responsible for HDM-induced innate immunity in both nasal and bronchial epithelium. **A**, TLR2 and TLR4 surface expressions by HDM in the nasal and bronchial epithelia isolated from the DUOX2^{+/+} or DUOX2^{th/dth/d} airway mucosa. **B**, TLR2 (green) and TLR4 (green) translocation 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^{th/dth/d} 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.



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.

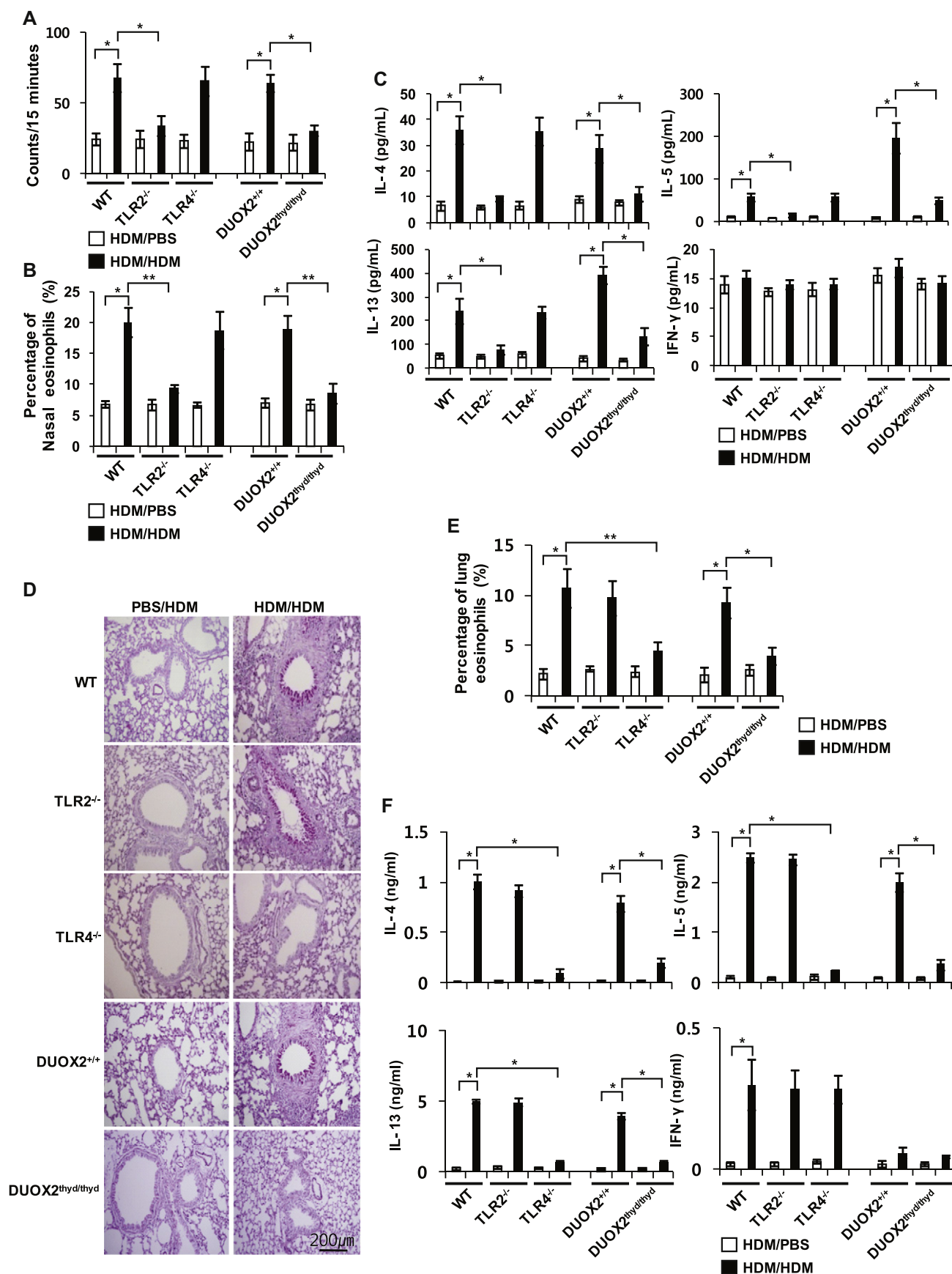
β -Glucans and LPS within HDM were responsible for TLR2 and TLR4 translocation to lipid rafts of nasal and bronchial epithelial cells, respectively

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 HDM $\Delta\beta$ -glucan and HDM Δ LPS, respectively (Fig 3, A). We also showed that disruption of lipid rafts by methyl- β -cyclodextrin (M β CD) 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), HDM $\Delta\beta$ -glucan (HDM/HDM $\Delta\beta$ -glucan), and HDM Δ LPS (HDM/HDM Δ LPS), 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, HDM $\Delta\beta$ -glucan, and HDM Δ LPS. **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 $\pm$ SEMs and are representative of 4 independent experiments. Three to 5 mice were used per group.



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 μ g/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 HDM $\Delta\beta$ -glucan, but not HDM Δ LPS, whereas ROS induction in bronchial epithelial cells was reduced by HDM Δ LPS but not HDM $\Delta\beta$ -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/HDM $\Delta\beta$ -glucan but not in HDM/HDM Δ LPS, 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 (HDM Δ protease) and examined the allergic features of the AR models in the presence of HDM/PBS, HDM/HDM, and HDM/HDM Δ protease. The allergic features of AR were not reduced in the presence of HDM/HDM Δ protease, 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/HDM Δ LPS but not in HDM/HDM $\Delta\beta$ -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 $\pm$ 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 HDM $\Delta\beta$ -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 AA⁴² 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 HDM $\Delta\beta$ -glucan and was attenuated in TLR2^{-/-} nasal epithelial cells. In contrast, increased ROS production was decreased by HDM Δ LPS 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-mediated 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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Online Repository

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

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Online Repository Methods

Mice

Wild-type BALB/c mice (aged 6-12 weeks) were purchased from Koatech. We obtained DUOX2 mutant mice from the Jackson Laboratory ¹. The new, recessive *thyd* mutation arose spontaneously in a B6 (129)-*Duox2*^{thyd}/J mouse (Jackson Laboratory; Stock no. 005543). TLR2^{-/-} and TLR4^{-/-} (BALB/c background) mice were provided by Shizuo Akira (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan). All experiments were approved by the Institutional Review Board of Yonsei University College of Medicine.

HDM extract preparation

HDMs (*Dermatophagoides pteronyssinus*) were obtained from the Arthropods of Medical Importance Bank (Yonsei University, Seoul, Korea). Mites were cultured in an insect rearing facility in the AMJB, as described previously ²⁻⁴. Briefly, 30 g of frozen dust mites were pulverized in liquid nitrogen, and defatted samples in 200 ml of a 1:1 volume of ethyl ether/ethyl acetate were extracted with slow stirring at 4°C overnight in a 100 mM phosphate-buffered saline (PBS) solution (pH 7.4). The extracts were then centrifuged at 10,000 g for 30 minutes at 4°C and the supernatants were finally filtered

through a 0.45µm filter. The HDM extracts had a final concentration of 1 mg/ml. Endotoxin levels were measured using the Limulus ameocyte lysate (LAL) QCL-1000 test (Lonza, MD), and the average endotoxin level was determined to be 4.661 EU/mg (approximately 0.5 ng/ml). (1→3)-β-glucans were also measured using the alternative LAL assay system, and the concentration of (1→3)-β-glucans in the HDM extracts in our study was approximately 4.5 ng/ml.

Generation of AR and AA mouse model

In the AR mouse model, we sensitized mice by intraperitoneal injection with 100 µg of house dust mite (HDM) extract in 2 mg of aluminium hydroxide weekly for 3 weeks.

One week after the last sensitization, the mice were intranasally challenged with 50 µg of HDM extract every day for 4 days without anesthesia (See Fig E7 in the Online Repository). We sacrificed mice 24 h after the last challenge. In the AA mouse model, we sensitized mice by intraperitoneal injection with 100 µg of HDM extracts in 2 mg of aluminium hydroxide weekly for 2 weeks. One week after the last sensitization, the mice were intranasally challenged with 30 µg of HDM extracts every day for 3 days under light isoflurane anesthesia (See Fig E7 in the Online Repository). We sacrificed mice 48 h after the last challenge.

Normal human nasal epithelial (NHNE) cell culture

Upon approval of the study protocol by the Institutional Review Board of Yonsei University College of Medicine, human nasal middle turbinate specimens were obtained from healthy volunteers. The culture system used for NHNE cells has been described previously⁵⁻⁶. Briefly, passage-2 NHNE cells (1×10^5 cells/culture) were seeded in 0.5 ml of culture medium onto a 24.5-mm, 0.45- μ m-pore Transwell-clear culture insert (Costar Co.). The cells were cultured in a 1:1 mixture of basal epithelial growth medium and Dulbecco's Modified Eagle's Medium (DMEM) containing all the supplements described previously⁶. Cultures were grown for the first 9 days in submerged conditions and during this period, the culture medium was changed on day 1 and every other day thereafter. An air-liquid interface (ALI) was created on day 9 by removing the apical medium and feeding the cultures from only the basal compartment. The culture medium was changed daily after the ALI was created. All experiments used NHNE cells 3 days after the creation of the ALI.

Reagents

β -Glucanase, polymyxin B, N-acetyl-L-cysteine (NAC), jasplakinolide, methyl- β -

cyclodextrin (M β CD), pronase, DNase, and collagenase were purchased from Sigma.

Antibodies

We purchased antibodies for either flow cytometry or blocking experiments. We used antibody to MHC class II [BD pharmingen; (I-A/I-E)-phycoerythrin (PE)-labeled; clone 2G9], antibody to CD11c [eBioscience; allophycocyanin (APC)-labeled; clone N413], antibody to CCR3 (BD pharmingen; AlexaFluor647-labeled; clone 83103), antibody to CD3 [BD pharmingen; fluorescein isothiocyanate (FITC)-labeled; clone 17A2], antibody to TLR2 (eBioscience; PE-labeled; 12-9021-82), antibody to TLR4 (eBioscience; PE-labeled; 12-9924-82), antibody to Dectin-1 (AbD serotec; Alexa fluor 647-labeled; MCA2289A647), and antibody to CD45R (eBioscience; APC-labeled; 17-0452-82), along with the appropriate isotype controls for flow cytometry analysis. We used antibodies to TLR2 (abcam; ab24192) and TLR4 (abcam; ab22048) for western blot analysis. We also used antibodies to TLR2 (Santa Cruz; sc-12504), TLR4 (Santa Cruz; sc 30002), and caveolin (BD biosciences; 610057) for lipid rafts staining. Anti-DUOX2 antibody was obtained as a gift from Dr. Yun Soo Bae (Department of Life Science, Ewha Woman's University, Seoul, Korea)⁷.

Flow cytometry

For detecting dendritic cells (DCs) in the nasal and lung mucosae, we collected the nasal and lung mucosae from the mice and prepared single-cell suspensions by treating the mucosa with collagenase⁸. Cells were double-stained with anti-CD11c and anti-MHCII. For detection of eosinophils in the nasal mucosa and BAL fluid of the lung, cells were double-stained with anti-CCR3 and anti-CD3. For detection of TLR2, TLR4 and Dectin-1 surface expressions in nasal and bronchial epithelial cells, *in vivo*, mouse nasal and bronchial tissues were incubated with collagenase (0.14 mg/ml) for 15 minutes at 37 °C until the epithelium could be mechanically dislodged from pieces of connective tissues with the aid of a dissecting microscope. Single-cell suspensions were prepared from pieces of isolated epithelium. Cells were double-stained with anti-TLR2 and anti-CD45R or anti-TLR4 and anti-CD45R. We collected data on a FACS LSRII (Becton Dickinson) and analyzed it with FlowJo software (Treestar).

Colocalization studies within lipid rafts

To detect the TLR2 or TLR4 localization in lipid rafts in cells within airway epithelium, *in vivo*, mouse nasal and bronchial tissues were incubated with collagenase (0.14 mg/ml) for 15 minutes at 37 °C until the epithelium could be mechanically dislodged from

pieces of connective tissues with the aid of a dissecting microscope. Pieces of epithelium were isolated and cytopun onto slides. Cells on the slides were fixed with 4 % paraformaldehyde in PBS for 15 min at room temperature, permeabilized with 0.02 % Triton X-100 for 20 min and blocked with PBS containing 1 % BSA and 10 % normal goat serum. Then, we double stained with anti-TLR2 and anti-caveolin antibodies or anti-TLR4 and anti-caveolin antibodies in cells within nasal or bronchial epithelia, respectively. After extensive washing with PBS, cells were treated with Rhodamine (TRITC)-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG or Rhodamine-conjugated anti-mouse IgG and FITC-conjugated anti-goat IgG in nasal or bronchial epithelial cells, respectively. Localization of lipid rafts and TLR2 and TLR4 was visualized by confocal microscopy (Carl Zeiss; LSM 710).

Intracellular ROS assay by DCF-DA

ROS were measured by a method described previously⁹. After stimulating the confluent cells with HDM extract, the cells were washed with Hanks' balanced salt solution (HBSS) and incubated for 10 min in the dark in Krebs-Ringer solution containing 5 μ M 2',7'-dichlorofluorescein diacetate (DCF-DA) (Molecular Probes). Inside the cell, DCF-DA is converted into 2'7'-dichlorofluorescein (DCF) by intracellular esterases and then

oxidized to highly fluorescent 2'7'-DCF by reacting with ROS or reactive nitrogen species (RNS). The cells were washed with 1 ml of HBSS at one time to remove extracellular ROS. Culture dishes were examined with a Zeiss Axiovert 135 inverted confocal microscope equipped with an x20 Neofluor objective and a Zeiss LSM 410 confocal attachment. DCF fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 515-540 nm. Seven fields of each dish were randomly selected and the fluorescence intensity was measured with the Karl Zeiss vision system (KS400, version 3.0). The seven values were averaged to obtain the mean relative fluorescence intensity, and these means were used for comparison. All experiments were repeated at least 3 times.

Primary mouse nasal and bronchial epithelial cell cultures

Mouse nasal epithelial cell cultures were prepared by methods described previously¹⁰. Briefly, after the epithelia were removed from the nasal cavity, they were incubated in 1.5 mg/ml pronase and 0.1 µg/mL DNase in the 5 % CO₂ chamber at 37°C for 1 h. To stop the enzymatic dissociation, 5 mL of sterile 5% fetal bovine serum was added, followed by incubation for 2 min further. The epithelial cells were dissociated by gentle agitation of the sinonasal tissue, achieved by 12 inversions of the tube. The tissue was

removed from the suspension and transferred to 10 mL culture media consisting of 1:1 mixture media [Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12 (1:1 v/v), 15 mM HEPES, 3.6 mM sodium bicarbonate, 4 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml fungizone]. After incubating in tissue culture plates for 3–4 h at 37°C, floating epithelial cells were collected by centrifugation. Mouse nasal epithelial cells were seeded on a 12-mm diameter, polycarbonate semipermeable membrane with a pore size of 0.4 µm. Mouse bronchial epithelial cell cultures were prepared by methods described previously¹¹. Briefly, the isolated bronchi from mice were opened longitudinally, and were incubated in 1.5 mg/ml pronase at 4°C for 18h. Epithelial cells were dislodged from the bronchi, collected by centrifugation and resuspended in DNase solution to avoid clumping together. These cells were centrifuged and resuspended in basic media [Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12 (1:1 v/v), 15 mM HEPES, 3.6 mM sodium bicarbonate, 4mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml fungizone]. After incubating in tissue culture plates for 3–4 h at 37°C, floating epithelial cells were collected by centrifugation. Bronchial cells were seeded on a 12-mm diameter, polycarbonate semipermeable membrane with a pore size of 0.4 µm. The nasal and bronchial epithelial cells were cultured for 5 days with the culture media, which were

basic media supplemented with 10 $\mu\text{g/ml}$ insulin, 5 $\mu\text{g/ml}$ transferrin, 0.1 $\mu\text{g/ml}$ cholera toxin, 25 ng/ml epidermal growth factor, 30 $\mu\text{g/ml}$ bovine pituitary extract, 5 % fetal bovine serum (FBS) and 0.01 μM retinoic acid. The media were changed every 2 days until the transmembrane resistance (R_t) was $> 1000 \Omega \text{ cm}^2$, as measured by an epithelial Ohm-voltmeter. The media were removed from the upper chamber to establish an air–liquid interface (ALI), and the lower chambers only were provided with fresh media, which were basic media supplemented with 2% Nu Serum (Becton-Dickinson) and 0.01 μm retinoic acid every 2 days. All experiments used primary epithelial cells 3 days after the creation of the ALI.

Lentiviral shRNA and transduction to primary normal human nasal epithelial (NHNE) cells

For silencing of human TLR2, TLR4, Dectin-1, DUOX1, and DUOX2 in primary NHNE cells, control lentivirus (sc108080), TLR2 (sc-40256-V), TLR4 (sc-40260-V), Dectin-1 (sc-63276-V), DUOX1 (sc-60550-V), and DUOX2 (sc-60552-V) lentivirus were obtained from Santa Cruz. We treated the above lentivirus (MOI; 10) with NHNE cells for 1 day just before creating the airway liquid interface (ALI). No toxicity in NHNE cells by lentivirus treat was observed.

Real time-PCR

Total RNA was isolated from NHNE cells using TRIzol (Invitrogen). cDNA was synthesized from 1 µg RNA with random hexamer primers using Moloney murine leukemia virus reverse transcriptase (PerkinElmer Life Sciences and Roche Applied Science). To quantify gene expression, fluorescence real-time PCR was performed using the SYBR Green qPCR kit (Finnzymes, Finland). Primer pairs for real-time PCR were designed and manufactured by Bioneer (Bioneer, Korea). The unique primer numbers of the target genes were as follows: TLR2 (primer No. P104739), TLR4 (primer No. P181356), Dectin-1 (primer No. P119075), DUOX1 (primer No. P218762), DUOX2 (primer No. P294520), and GAPDH (sense: 5'-GAA GGT GAA GGT CGG TCG GAG TC-3', antisense: 5'-GAA GAT GGT GAT GGG ATT TC-3'). Real-time PCR was performed using the PE Biosystems ABI PRISM® 7700 sequence detection system. The thermocycling parameters were 50 °C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were performed in triplicate. The relative quantity of mRNAs was determined using the comparative threshold method, and the results were normalized against GAPDH as an endogenous control.

Surface biotinylation and Western blot analysis

Mouse nasal and bronchial tissues were incubated with collagenase (0.14 mg/ml) for 15 minutes at 37 °C until the epithelium was able to be mechanically dislodged from pieces of connective tissues with the aid of a dissecting microscope. The plasma membrane proteins in the isolated epithelium were then biotinylated by gently shaking the pieces of epithelium in a borate buffer containing sulfo-NHS-SS-biotin (Pierce, Rockford, IL) for 30 min. After biotinylation, the epithelia were washed extensively with quenching buffer to remove excess biotin and then washed twice with PBS. The epithelia were then homogenized in a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 % (v/v) Nonidet P-40, 0.25 % (v/v) sodium deoxycholate, and complete protease inhibitor mixture (Roche Applied Science) and incubated overnight at 4 °C with avidin solution (UltraLink Immobilized NeutrAvidin Beads 10%, Pierce). Avidin-bound complexes were pelleted (13,000 rpm) and washed three times. Biotinylated proteins were eluted in a 2 X sample buffer and separated by 10 % SDS-PAGE and transferred to PVDF (Bio-Rad). Membranes were blocked with 5% skim milk in TTBS (0.5% Tween-20), and then incubated overnight with mouse anti-TLR2 at 1:1000 and mouse anti-TLR4 at 1:500 with 5 % skim milk in TTBS. After washing with TTBS, the blot was incubated with HRP-conjugated anti-rabbit with 5% skim milk in

TTBS for 2 hrs at room temperature. After washing with TTBS, the blot was visualized using the ECL system.

Immunoprecipitation

For immunoprecipitation, 200 µg proteins purified from NHNE cell lysates were incubated with DUOX2 antibody overnight at 4 °C and then incubated with Protein A-sepharose bead for 1h at 4 °C. Immunoprecipitates were collected and washed three times with lysis buffer, then separated by SDS-PAGE and analyzed by immunoblot.

Cytokine and chemokine analysis

We performed ELISAs for CCL20, IL-33, GM-CSF, TSLP, IL-4, IL-5, IL-13, and IFN-γ with kits from R&D systems according to the manufacturer's instructions. For CCL20, IL-33, GM-CSF and TSLP, NAL fluid or BAL fluid was collected from nasal or lung mucosa, respectively, and measured. For IL-4, IL-5, IL-13, and IFN-γ, the cervical lymph nodes (CLN) and mediastinal lymph nodes (MLN) were removed and cells were dissociated by syringe plunger over a cell strainer. Red blood cells were depleted by NH₄Cl lysis. Single cell suspensions were seeded into a flat-bottomed 96-well plate at 0.5×10^6 per well and treated with HDM extract. After a 4-day incubation, cell-free

supernatants were harvested and measured by ELISA.

Histology in the AA mouse model

Periodic acid-Schiff (PAS) staining was performed as corresponding levels of goblet cell metaplasia in a AA mouse model. Briefly, the lungs were fixed for 24 hours in 10% neutral-buffered formalin, embedded, and serially sectioned (5 μ m). Then the sections were mounted on poly-L-lysine-coated slides. Paraffin sections, after deparaffination and rehydration, were incubated with 0.5 % periodic acid solution for 5 minutes at room temperature and rinsed with distilled water. The sections were incubated with Schiff reagent for 15 minutes at room temperature and washed with lukewarm tap water for 5 minutes. Finally, the sections were counterstained with hematoxylin and cover-slipped with mounting media.

Measurement of AHR

Mice were mechanically ventilated while AHR to inhalation of β -methacholine (determined by alterations in resistance) was measured as previously described¹². Baseline lung resistance and responses to saline were also measured, followed by measurement of responses to increasing doses of aerosolized β -methacholine (3.12-50

mg/ml). The three highest values of lung resistance were obtained for each dose of methacholine, which were averaged to obtain final values for each dose.

Evaluation of allergic symptoms in AR mice

We counted the number of times in which AR mice scratched their noses for 15 min after the last intranasal challenge.

Statistical analyses

Results are presented as the mean of three determinations $\pm$ s.e.m. One-way ANOVA was performed to determine differences between groups. *P*-values less than 0.05 were considered statistically significant

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Fig E1. β -glucans and LPS within HDM are responsible for inducing DC-activating cytokines from the nasal and lung mucosae, respectively. **(A)** Quantification of IL-33 from NAL fluid of WT mice by challenging (4 hr) with HDM, HDM $\Delta\beta$ -glucan, and HDM Δ LPS. **(B)** Quantification of GM-CSF, TSLP, and IL-33 from BAL fluid of WT mice challenged with HDM, HDM $\Delta\beta$ -glucan, and HDM Δ LPS. * $P < 0.05$. ** $P < 0.01$. All results are shown as means $\pm$ s.e.m. and are representative of four independent experiments. Three to five mice were used per group.

Fig E2. TLR2 is the main PRR responsible for HDM-induced CCL20 secretion in primary normal human nasal epithelial (NHNE) cells. **(A)** Relative gene expression of Dectin-1, TLR2, and TLR4 in NHNE cells treated with Dectin-1, TLR2, and TLR4 shRNA-containing lentivirus particles, respectively. The Dectin-1, TLR2, and TLR4 expression levels of NHNE cells treated with lentivirus expressing control shRNA was arbitrarily set as 1. **(B)** Quantification of CCL20 secretion after HDM treatment (24 hr) to NHNE cells treated with Dectin-1, TLR2, and TLR4 shRNA-containing lentivirus particles. * $P < 0.05$, ** $P < 0.01$. All results are shown as means $\pm$ s.e.m. These results are representative of four independent experiments.

Fig E3. The intracellular proteins of TLR2 and TLR4 in the nasal and bronchial epithelia are not increased by HDM challenge. Nasal epithelium and bronchial

epithelium isolated from the murine airway mucosa were taken and analyzed. The level of intracellular proteins of TLR2 and TLR4 were measured after HDM challenge by Western blot analysis.

Fig E4. HDM do not induce Dectin-1 surface expressions in nasal and bronchial epithelial cells. Nasal and bronchial epithelium isolated from the murine airway mucosa was taken and analyzed. Dectin-1 surface expressions in nasal and bronchial epithelia after HDM challenge by FACS analysis. All results are shown as means $\pm$ s.e.m. These results are representative of four independent experiments.

Fig E5. HDM induce TLR2 and TLR4 surface expressions in primary nasal and bronchial epithelial cells, respectively. (A) TLR2 and TLR4 surface expressions induced by HDM treatment (1hr) in primary nasal or bronchial epithelial cells cultured from the murine airway epithelium. (B) Mean intensity of TLR2 and TLR4 surface expressions in (A). * $P < 0.05$, All results are shown as means $\pm$ s.e.m. These results are representative of four independent experiments.

Fig E6. β -glucans and LPS within HDM are responsible for TLR2 and TLR4 surface expressions of primary nasal and bronchial epithelial cells, respectively. Mean intensity of TLR2 and TLR4 surface expressions by challenging (1 hr) with HDM, HDM $\Delta\beta$ -glucan, and HDM Δ LPS in nasal and bronchial epithelial cells cultured from the WT

mice airway mucosa. $^*P < 0.05$, $^{**}P < 0.01$. All results are shown as means $\pm$ s.e.m.

These results are representative of four independent experiments.

Fig E7. HDM induce TLR2 and TLR4 translocation to lipid rafts in cells of nasal and bronchial epithelia, respectively. Nasal and bronchial epithelium obtained from murine airway mucosa was taken and analyzed. TLR2 (green) and TLR4 (green) translocation to lipid rafts (caveoline: red) in cells of nasal and bronchial epithelia, respectively, after challenge with HDM (0, 15, 30, 60 min).

Fig E8. HDM induce ROS production in primary bronchial epithelial cells. **(A and B)** primary mouse bronchial epithelial cells were treated with HDM extract for 5 min in the indicated concentrations **(A)** or with 50 μ g/ml in the indicated times **(B)**, followed by 10 min incubation of 2',7'-dichlorofluorescein diacetate (DCF-DA). Green fluorescence protein (GFP) intensity was observed by confocal microscope or quantified by densitometric analysis. The value of ROS production in a PBS-treated cell was set arbitrarily as 1. $^*P < 0.05$, $^{**}P < 0.01$. All results are shown as means $\pm$ s.e.m. These results are representative of four independent experiments.

Fig E9. DUOX2 is responsible for HDM-induced ROS production in NHNE cells. **(A)** Relative gene expression of DOUX1 and DUOX2 in NHNE cells treated with DOUX1 and DUOX2 shRNA-containing lentivirus particles, respectively. The DOUX1 and

DUOX2 expression levels of NHNE cells treated with retrovirus expressing control shRNA were arbitrarily set as 1. **(B)** Quantification of ROS production by HDM treatment to NHNE cells treated with DUOX1 or DUOX2 shRNA-containing lentivirus particles. The value of ROS production by PBS treatment to NHNE cells treated with retroviruses expressing Cont shRNA was arbitrarily set as 1. $^*P < 0.05$, $^{**}P < 0.01$. All results are shown as means $\pm$ s.e.m. These results are representative of four independent experiments.

Fig E10. DUOX2 is responsible for HDM-induced TLR2 surface expression in NHNE cells. Mean intensity of TLR2 or TLR4 surface expression after HDM treatment in NHNE cells pre-treated with DUOX2 shRNA-containing lentivirus particles. $^*P < 0.05$, $^{**}P < 0.01$. All results are shown as means $\pm$ s.e.m. These results are representative of four independent experiments.

Fig E11. DUOX2 interact with endogenous TLR2 or TLR4 in NHNE cells. Immunoprecipitation with DUOX2 antibody and immunoblot analysis of the resulting precipitates or cell lysate with TLR2, TLR4 or DUOX2 antibodies.

Fig E12. Protocol diagrams for the induction of AR and AA mouse models.

Fig E13. Allergic features of AA in the AR mouse model are not detected. **(A-C)** Allergic features of AA in HDM/PBS and HDM/HDM AR mice were measured. **(A)**

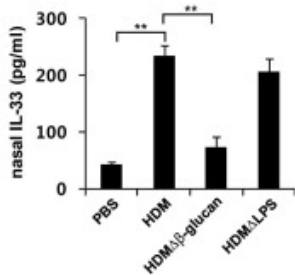
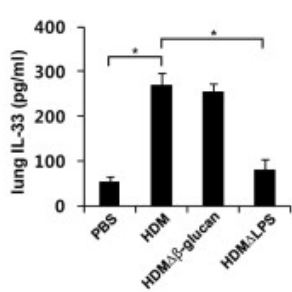
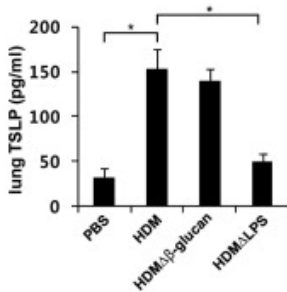
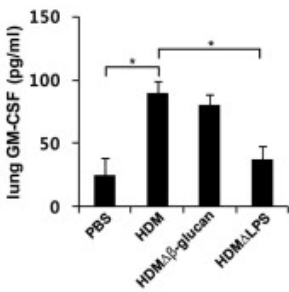
Periodic acid-Schiff (PAS) staining of lung tissue. **(B)** Percentage of CCR3⁺CD3⁻ eosinophils in BAL fluid. **(C)** Quantification of cytokine production in MLN cells. All results are shown as means $\pm$ s.e.m. and are representative of four independent experiments. Three to five mice were used per group.

Fig E14. Airway hyper-responsiveness (AHR) to the bronchoconstrictor methacholine in the AR mouse model is not observed. Lung function, as determined by invasive measurement of airway resistance (R) in response to increasing concentrations of metacholine. AHR analysis of AR HDM/HDM compared with AR HDM/PBS or AA HDM/HDM compared with AA HDM/PBS. * $P < 0.05$, ** $P < 0.01$. All results are shown as means $\pm$ s.e.m. and are representative of four independent experiments. Three to five mice were used per group.

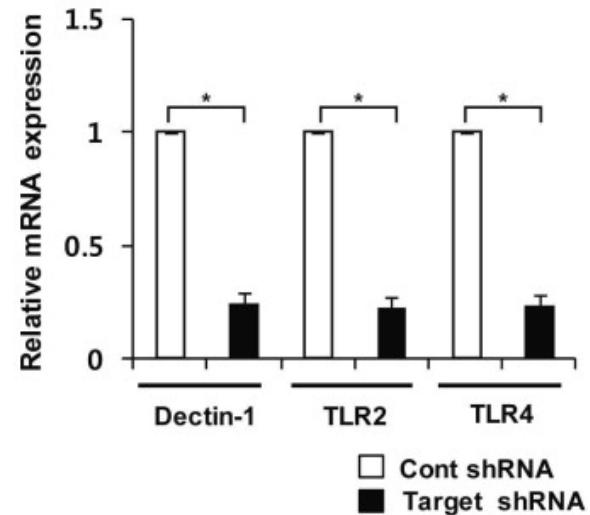
Fig E15. HDM-derived protease are dispensable for the development of AR under HDM-sensitized conditions **(A-C)** Allergic features of AR in HDM/PBS, HDM/HDM, and HDM/ HDM Δ proteases AR mice were measured. **(A)** Frequencies of nose scratching. **(B)** CCR3⁺CD3⁻ eosinophils in the nasal mucosa. **(C)** Cytokine production in cervical lymph node (CLN) cells restimulated *ex vivo* for 4 days with PBS (HDM/PBS), HDM (HDM/HDM), and HDM Δ proteases (HDM/HDM Δ proteases).. * $P < 0.05$, All results are shown as means $\pm$ s.e.m, and are representative of four independent

experiments. Three to five mice were used per group.

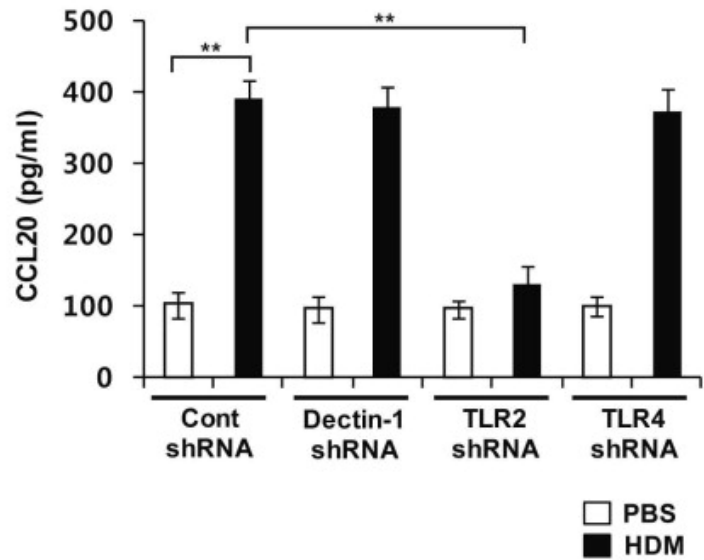
Fig E16 A schematic model representing the distinctive mechanisms of innate immunity of the upper and lower airways activated by HDM, and their role in triggering AR and AA, respectively. β -Glucans/TLR2 and LPS/TLR4 signaling axes are required for HDM-induced ROS generation via DUOX2, and increased DUOX2-mediated ROS positively regulates TLR2 and TLR4 surface expression and translocation to lipid rafts to the nasal and bronchial epithelial cells, respectively. This activation of innate immunity in nasal and bronchial epithelial cells triggers HDM-induced AR and AA, respectively through CCL20 secretion, DCs recruitment, and T_H2 -mediated allergic inflammation.

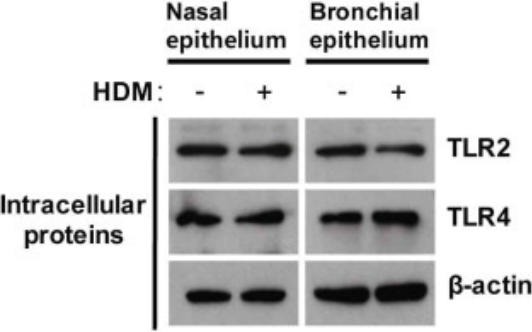
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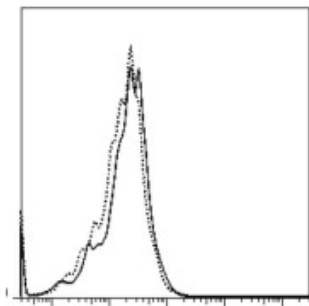


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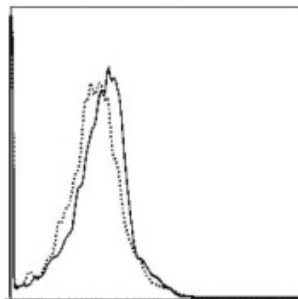




Nasal
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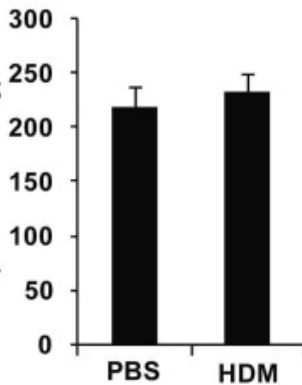


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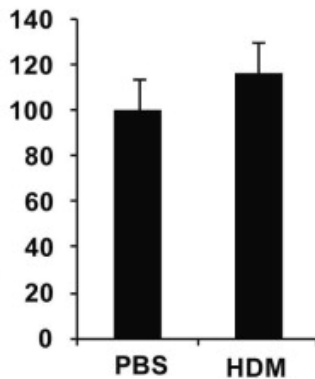


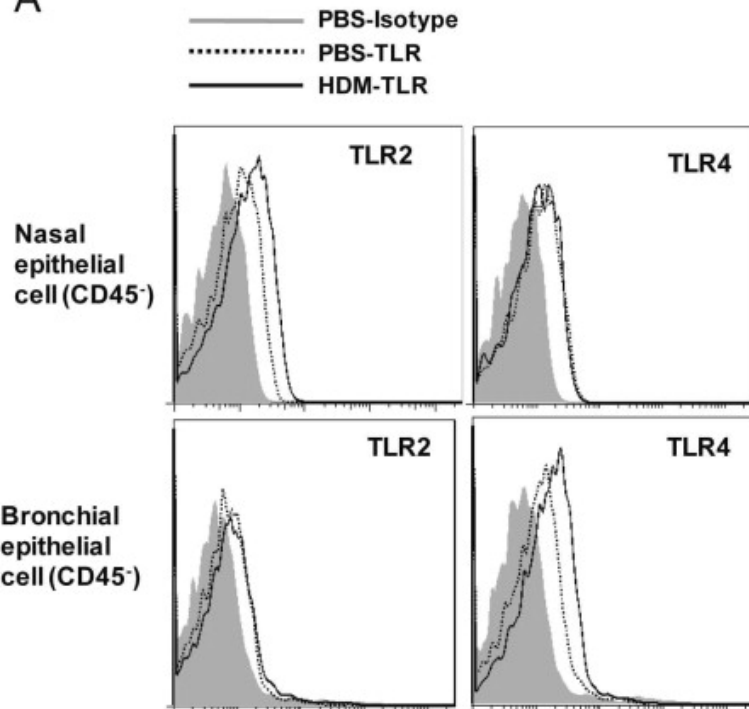
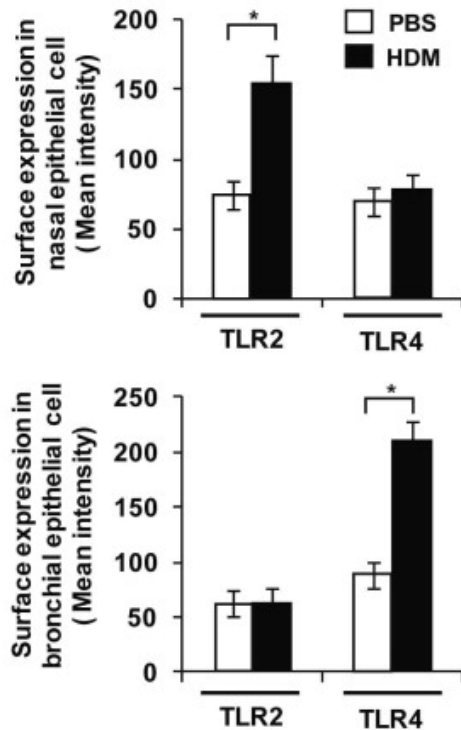
..... PBS
———— HDM

Dectin-1 surface expression
in nasal epithelium
(Mean intensity)

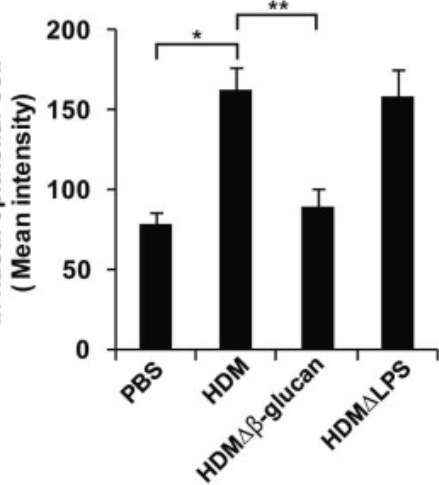


Dectin-1 surface expression
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(Mean intensity)

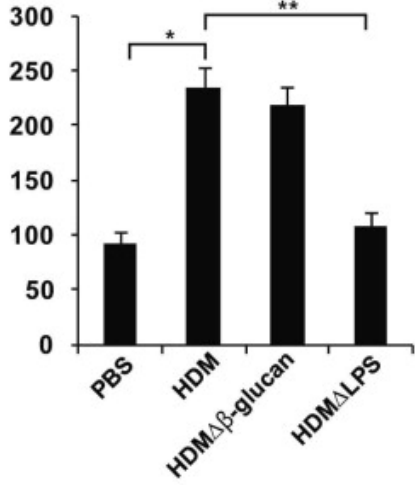


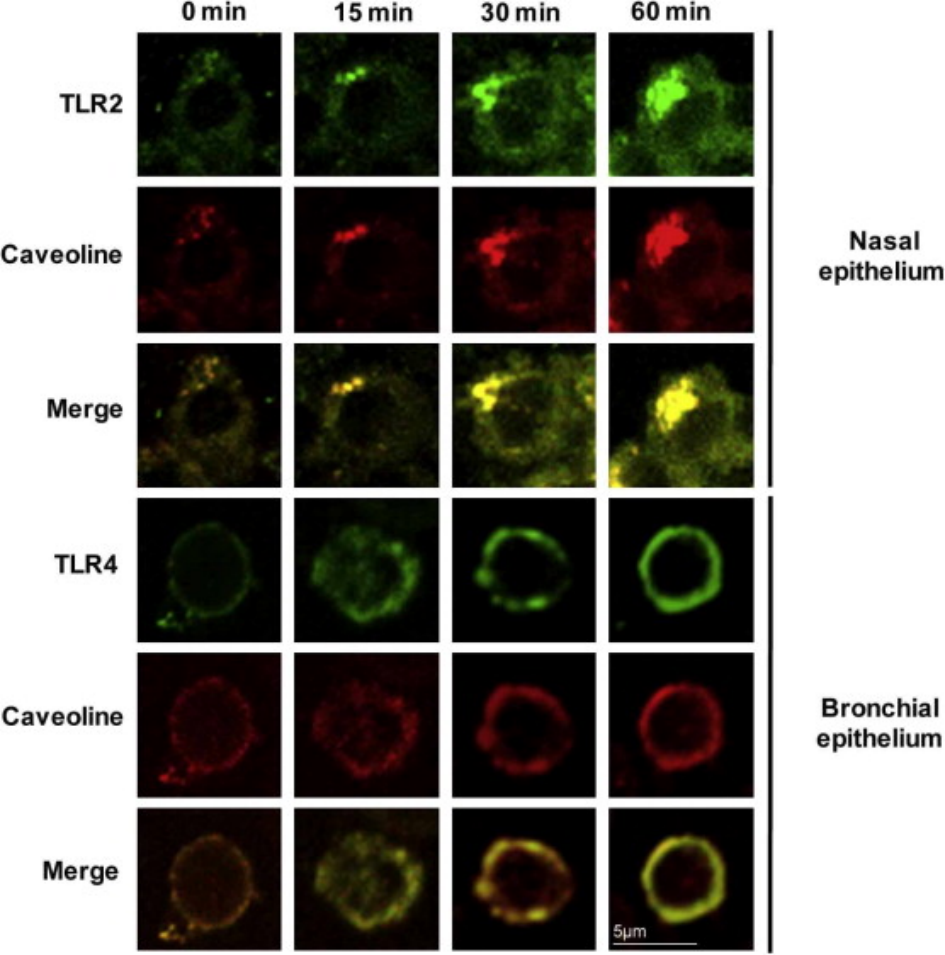
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TLR2 Surface expression
in nasal epithelial cell

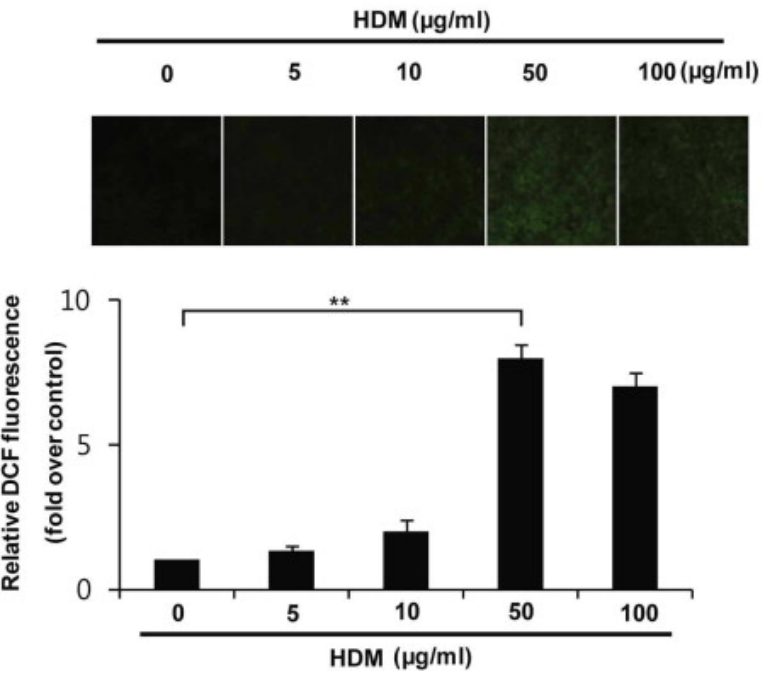


TLR4 Surface expression
in bronchial epithelial cell

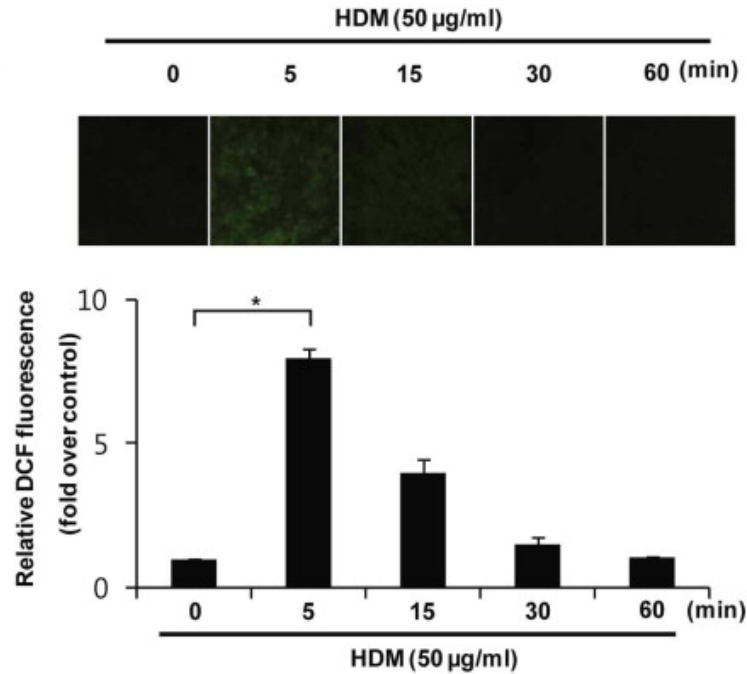


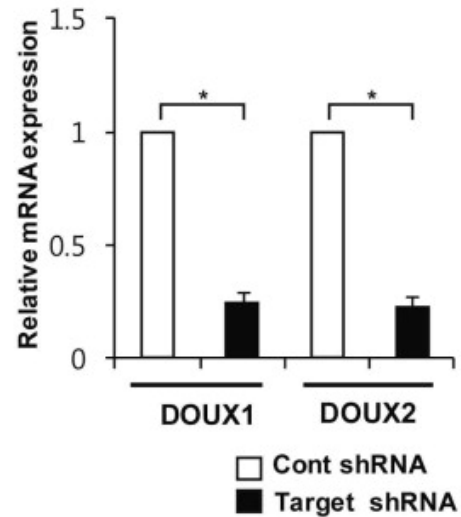
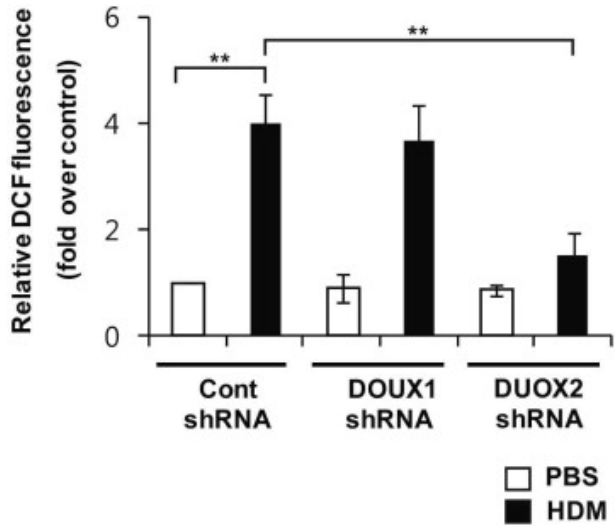


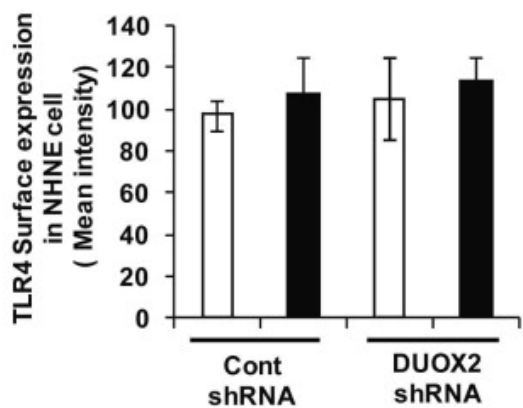
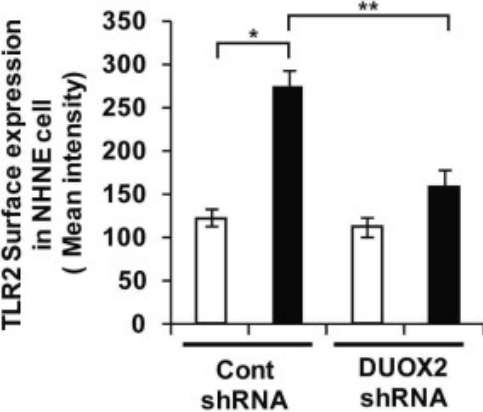
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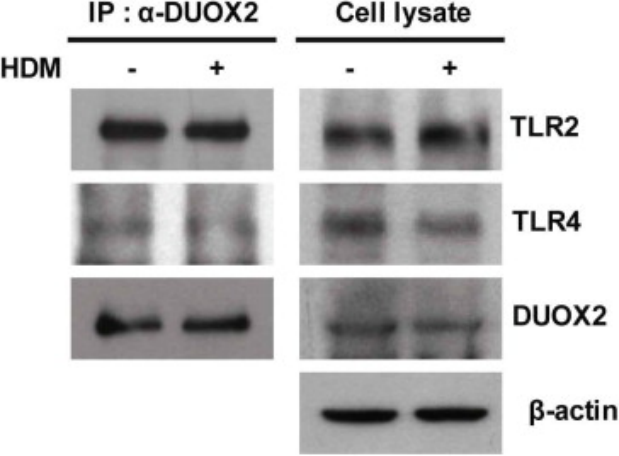
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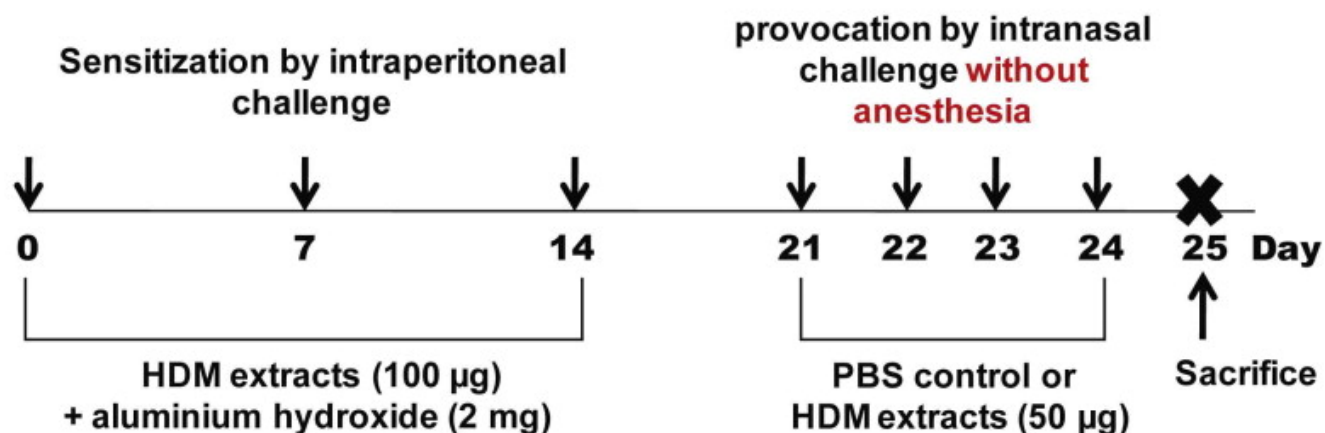
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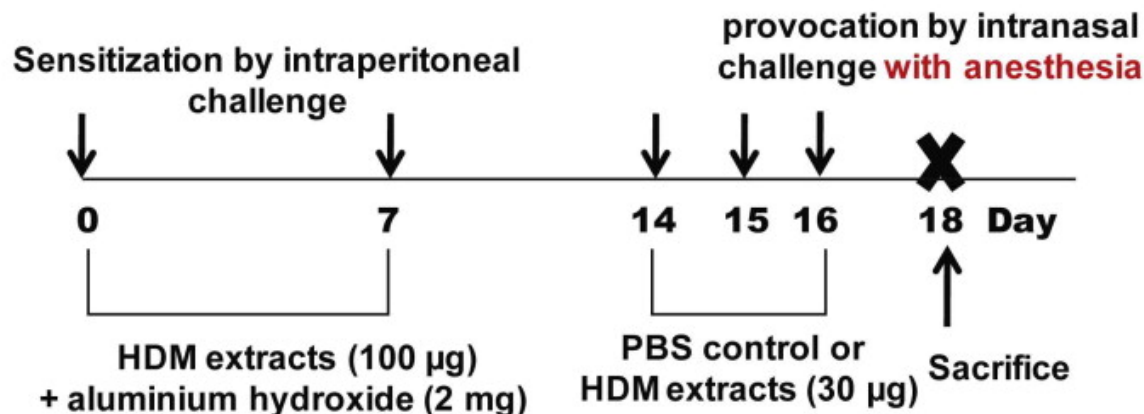
□ PBS
■ HDM

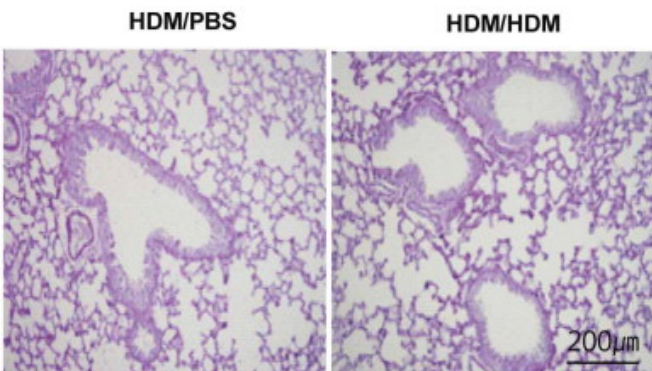
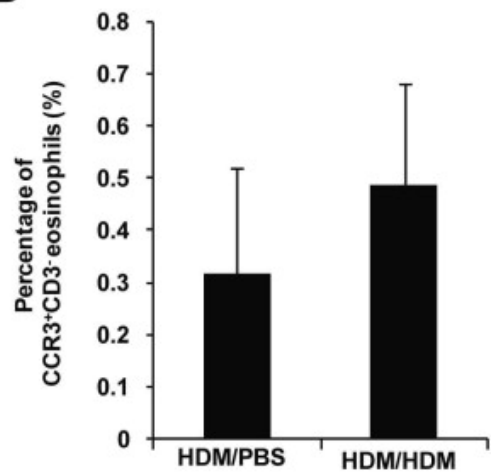
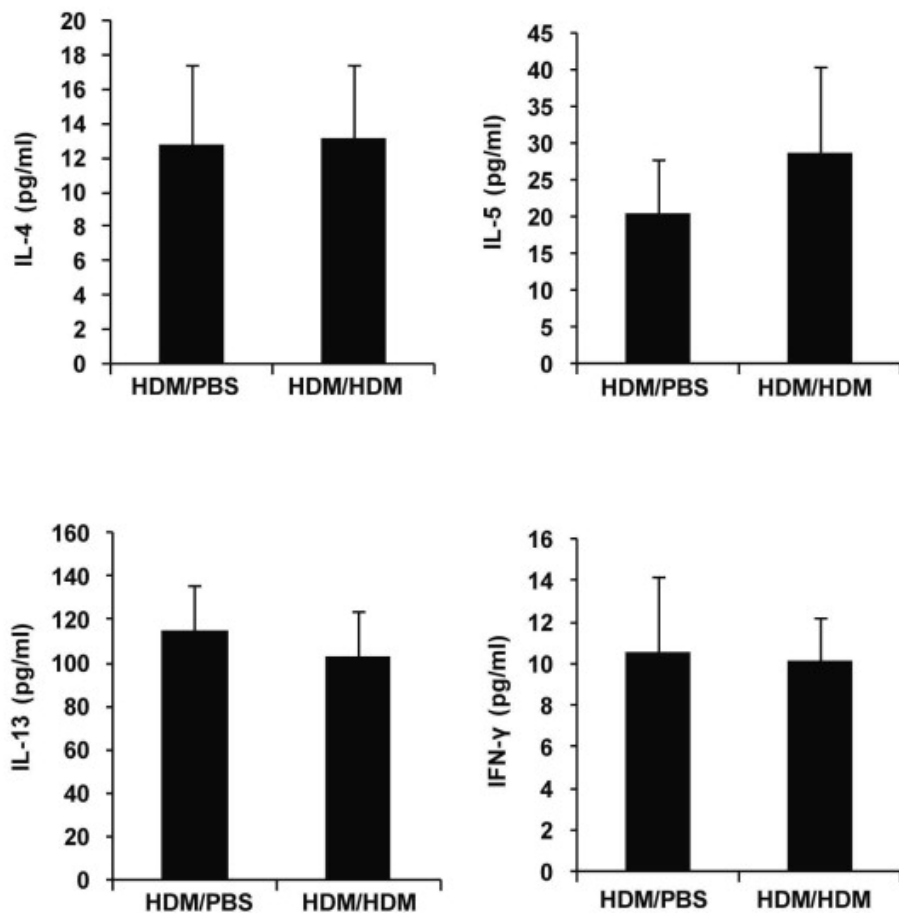


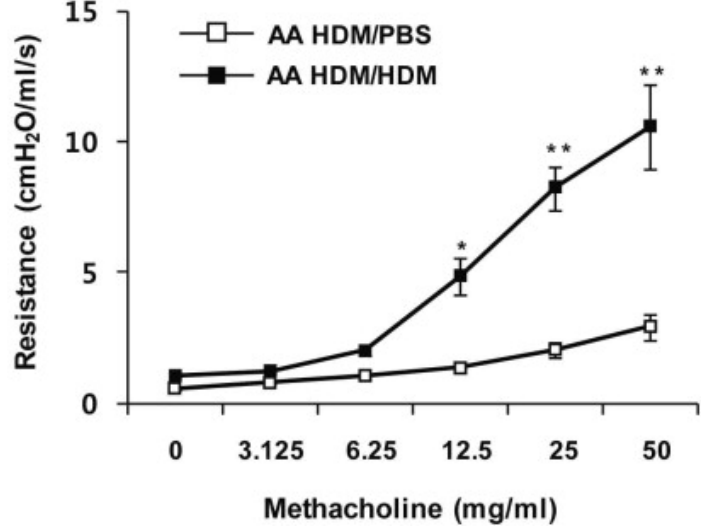
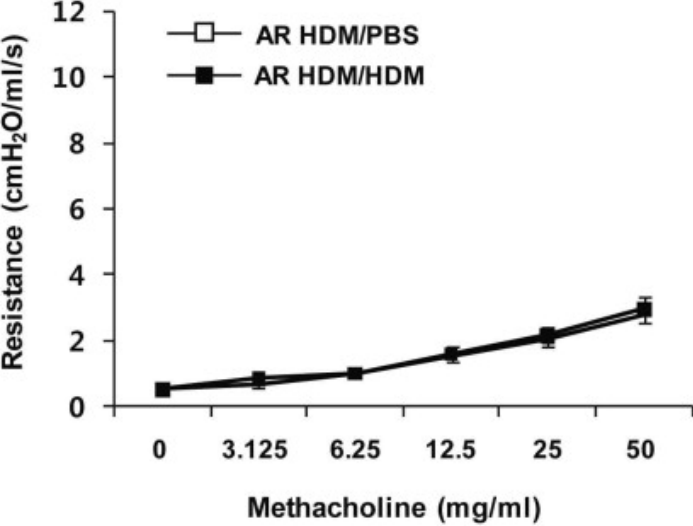
Allergic rhinitis mouse model

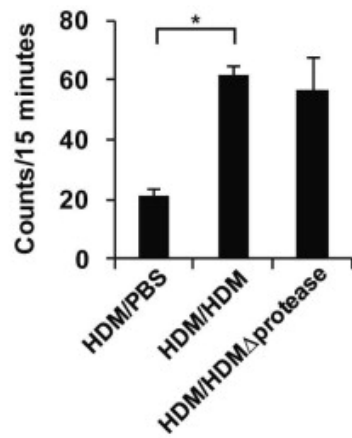
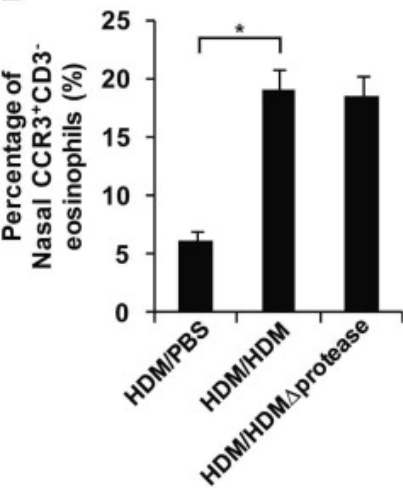
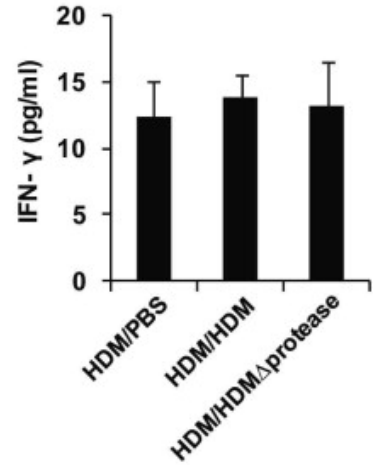
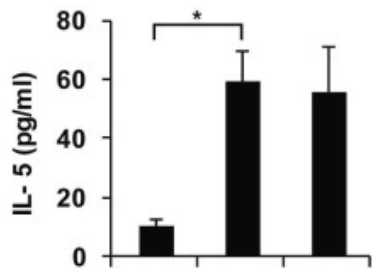
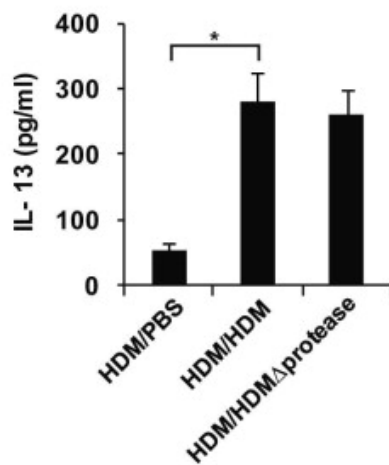
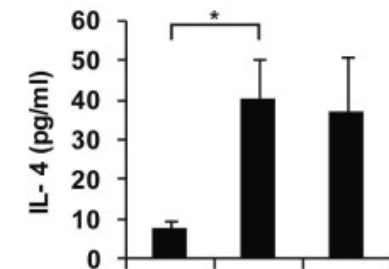


Allergic asthma mouse model

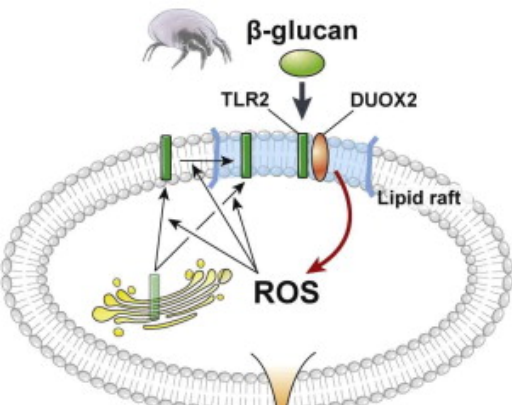


A**B****C**



A**B****C**

Nasal epithelial cell



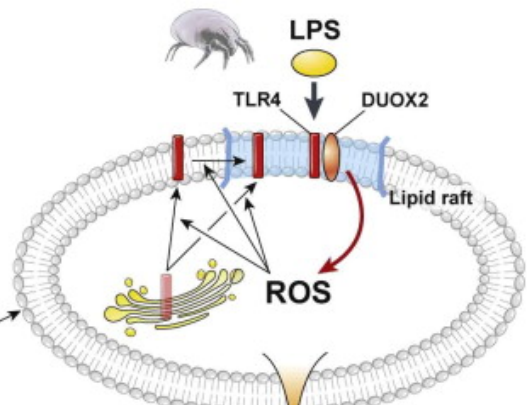
CCL20 secretion

DCs recruitment

T_H2 cell activation

Allergic rhinitis

Bronchial epithelial cell



CCL20 secretion

DCs recruitment

T_H2 cell activation

Allergic asthma