

Receptor for advanced glycation end products and its ligand high-mobility group box-1 mediate allergic airway sensitization and airway inflammation

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Background: The receptor for advanced glycation end products (RAGE) shares common ligands and signaling pathways with TLR4, a key mediator of house dust mite (*Dermatophagoides pteronyssinus*) (HDM) sensitization. We hypothesized that RAGE and its ligand high-mobility group box-1 (HMGB1) cooperate with TLR4 to mediate HDM sensitization.

Objectives: To determine the requirement for HMGB1 and RAGE, and their relationship with TLR4, in airway sensitization.

Methods: TLR4^{-/-}, RAGE^{-/-}, and RAGE-TLR4^{-/-} mice were intranasally exposed to HDM or cockroach (*Blattella germanica*) extracts, and features of allergic inflammation were measured during the sensitization or challenge phase. Anti-HMGB1 antibody and the IL-1 receptor antagonist Anakinra were used to inhibit HMGB1 and the IL-1 receptor, respectively.

Results: The magnitude of allergic airway inflammation in response to either HDM or cockroach sensitization and/or challenge was significantly reduced in the absence of RAGE but not further diminished in the absence of both RAGE and TLR4. HDM sensitization induced the release of HMGB1 from the

airway epithelium in a biphasic manner, which corresponded to the sequential activation of TLR4 then RAGE. Release of HMGB1 in response to cockroach sensitization also was RAGE dependent. Significantly, HMGB1 release occurred downstream of TLR4-induced IL-1 α , and upstream of IL-25 and IL-33 production. Adoptive transfer of HDM-pulsed RAGE^{+/+} dendritic cells to RAGE^{-/-} mice recapitulated the allergic responses after HDM challenge. Immunoneutralization of HMGB1 attenuated HDM-induced allergic airway inflammation.

Conclusion: The HMGB1-RAGE axis mediates allergic airway sensitization and airway inflammation. Activation of this axis in response to different allergens acts to amplify the allergic inflammatory response, which exposes it as an attractive target for therapeutic intervention. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: Asthma, allergic sensitization, epithelium, TLR4, RAGE, IL-1 α , HMGB1, IL-25, IL-33, TSLP

Activation of pattern-recognition receptors (PRRs) at the airway mucosa is fundamental to the process of allergic sensitization, the strongest identifiable risk factor for the development of asthma.¹ Genetic analyses are revealing that single nucleotide polymorphisms in or near loci that encode for PRRs such as Toll-like receptor (TLR) family members and downstream signaling molecules are strongly associated with the etiology of asthma.^{1,2} We and other researchers have used experimental models to show that the absence of TLR4 is protective against house dust mite (*Dermatophagoides pteronyssinus*) (HDM) induced allergic airway inflammation.³⁻⁶ By using chimeric mice, the laboratory of Hammad and Lambrecht has shown that HDM-induced activation of TLR4 in airway epithelial cells (AEC), rather than immune cells, is sufficient to promote allergic sensitization via the release of innate cytokines such as IL-25, IL-33, and thymic stromal lymphopoietin, which are critical to the development of type 2 inflammation in asthma.^{3,7} Furthermore, they demonstrated that type 2 innate cytokines and chemokines are produced downstream of the endogenous damage-associated molecular patterns (DAMP), uric acid, and IL-1 α , the latter of which acts in an autocrine manner in AECs to amplify type 2 inflammation.^{7,8} However, it remains undetermined whether IL-1 α acts directly upstream of type 2 innate cytokines or additional DAMPs and/or other mediators are involved in this cascade.

Although there is consensus that TLR4 is important in the airway response to HDM, it is increasingly evident that different PRRs dominate in the response to different allergens.⁹ This calls for greater understanding of the mechanisms through which the

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This work was funded by a Deputy Vice Chancellor Bridging Grant (University of Sydney) and seed funding (University of Technology, Sydney) awarded to M.B.S., and an NHMRC Australia Project Grant (1023756) awarded to S.P.

Conflicts of interest: J. M. Hughes is employed by the National Health and Medical Research Council (NHMRC) of Australia Grant Review Panel Membership 2013; has received research support from the NHMRC of Australia (Project Grant APP 632830 2010-2012); and has received lecture fees from Learning Solutions, The University of Sydney Early Career Researcher Biomedical Program 2011-2012. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication August 2, 2013; revised November 23, 2013; accepted for publication December 3, 2013.

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0091-6749/\$36.00

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<http://dx.doi.org/10.1016/j.jaci.2013.12.1035>

Abbreviations used

AEC:	Airway epithelial cell
BALF:	Bronchoalveolar lavage fluid
CCL:	Chemokine ligand
CR:	Cockroach
DAMP:	Damage-associated molecular pattern
DC:	Dendritic cell
HMGB1:	High mobility group box-1
HDM:	House dust mite
IL-1R:	Interleukin-1 receptor
PRR:	Pattern-recognition receptor
RAGE:	Receptor for advanced glycation end products
TLR4:	Toll-like receptor 4
WT:	Wild type

activation of one or more PRRs are amplified, integrated, or coordinated in the immune response to allow strategic targeting of multiple PRRs or their common downstream pathways for therapeutic intervention. We hypothesized that the receptor for advanced glycation end products (RAGE) serves an integral role in the airway response to inhaled allergens via its remarkable capacity to interact with a diverse repertoire of DAMPs.¹⁰ RAGE shares common ligands with TLR4 and signals via intracellular adaptor proteins that also are used by TLR4.¹¹ There is some evidence that RAGE and TLR4 cooperate as essential partners to augment the inflammatory response.^{10,12-14} Recently, Milutinovic et al¹⁵ demonstrated that RAGE-deficient mice are protected against HDM-induced allergic airway inflammation in a mouse model of chronic asthma but did not investigate its role in the initiation of the allergic response (ie, sensitization phase). Thus, whether RAGE is involved in the process of allergic sensitization or cooperates with TLR4 to amplify the immune response during allergen sensitization is an important question that remains to be addressed. Because AECs respond to inhaled allergens through the release of DAMPs, it is conceivable that RAGE might also serve as a common mechanism for the recognition of multiple DAMPs released downstream of PRR activation.¹⁰ One such DAMP may be HMGB1, a well-characterized RAGE ligand reported to be associated with lung function impairment and more severe disease in subjects with asthma.¹⁶⁻²⁰ A role for HMGB1 in the effector phase of the allergic inflammatory response has recently been demonstrated,^{19,21} but whether it is involved in the process of allergic sensitization is unknown. The objectives of this study were to investigate whether RAGE is involved in the process of allergic airway sensitization, whether it collaborates with TLR4 in this response and whether its ligand HMGB1 is an effector molecule of allergic sensitization.

METHODS

For detailed methods including mice and reagents, experimental protocols and procedures and statistical analysis, please see this article's [Methods](#) section in the Online Repository available at www.jacionline.org.

RESULTS**RAGE does not synergize with TLR4 to augment the effector phase of allergic airway inflammation**

To investigate whether TLR4 and RAGE cooperate in the development of allergic airway inflammation, we performed an acute HDM model in wild type (WT), RAGE-deficient ($^{-/-}$),

TLR4 $^{-/-}$, and RAGE-TLR4 $^{-/-}$ mice as per the study design (Fig 1, A). TLR4 $^{-/-}$ mice were significantly protected against all features of allergic inflammation, including eosinophilia and neutrophilia, chemokine and T_H2 cytokine production, mucus production, increases in lung mast cell numbers, total serum IgE, and HDM-specific IgG1 (Fig 1, B-K). With the exception of neutrophilia and expression of the neutrophil-active chemokine CXCL1, RAGE $^{-/-}$ mice were significantly protected against all features of allergic inflammation measured. Importantly, RAGE $^{-/-}$ and TLR4 $^{-/-}$ mice were similarly protected, and, with the exception of IL-5 production, RAGE-TLR4 $^{-/-}$ mice were not afforded additional protection (Fig 1, B-K).

The lack of additional protection against HDM-induced inflammation in the RAGE-TLR4 $^{-/-}$ mice may reflect the dominance of TLR4 in the response to this allergen.³⁻⁶ Thus, we next performed an acute model of cockroach (*Blattella germanica*) (CR) induced allergic airway inflammation in which TLR4 plays a less important role²² (study design, same as Fig 1, A). Interestingly, TLR4 $^{-/-}$ mice were significantly protected against neutrophilia but not eosinophilia, whereas RAGE $^{-/-}$ mice were protected against eosinophilia but not neutrophilia (Fig 2, A and B). Further, although both TLR4 $^{-/-}$ and RAGE $^{-/-}$ mice were similarly protected against IL-5 production; TLR4 $^{-/-}$ mice were not protected against increases in total serum IgE and CR-specific IgG1 and mucus production, whereas RAGE $^{-/-}$ mice were protected against all these aspects of the inflammatory response (Fig 2, C-F). Similar to the findings with HDM, the absence of both RAGE and TLR4 did not further diminish the inflammatory-immune response (Fig 2, A-F).

RAGE contributes to airway sensitization but does not synergize with TLR4

Next, we determined whether RAGE is involved, initially, in the process of allergic sensitization and whether it cooperates with TLR4 in this response. We isolated lymph node cells 5 days after HDM sensitization in WT, TLR4 $^{-/-}$, RAGE $^{-/-}$, and RAGE-TLR4 $^{-/-}$ mice to study T_H2 cell priming *ex vivo* (study design, Fig 3, A). HDM-stimulated IL-13 production was significantly reduced in lymph node cells isolated from TLR4 $^{-/-}$ and RAGE $^{-/-}$ mice, with no further reduction in RAGE-TLR4 $^{-/-}$ mice (Fig 3, B).

Because dendritic cells (DCs) play a critical role in allergic sensitization and adaptive immune responses to inhaled allergens,²³ we determined whether protection against allergic sensitization in RAGE $^{-/-}$ mice is due to a defect in DC function (study design, Fig 3, A). Compared with WT controls, RAGE $^{-/-}$ mice had significantly reduced numbers of DCs in the lung and draining lymph nodes at day 3 after HDM sensitization (Fig 3, C and D). In view of these results, we investigated whether reconstitution of RAGE $^{-/-}$ mice with HDM-pulsed RAGE $^{+/+}$ DCs (ie, replacing the HDM exposure during sensitization) was sufficient to promote allergic airway inflammation after HDM challenge (study design, Fig 4, A). Importantly, the magnitude of allergic airway inflammation was the same after the adoptive transfer of HDM-pulsed RAGE $^{+/+}$ DCs, irrespective of whether the recipient mouse was RAGE $^{-/-}$ or RAGE $^{+/+}$ (Fig 4, B-G).

HMGB1 is secreted by AECs during allergen sensitization

Because we demonstrated that RAGE signaling in DCs is important for the process of allergic sensitization and the later

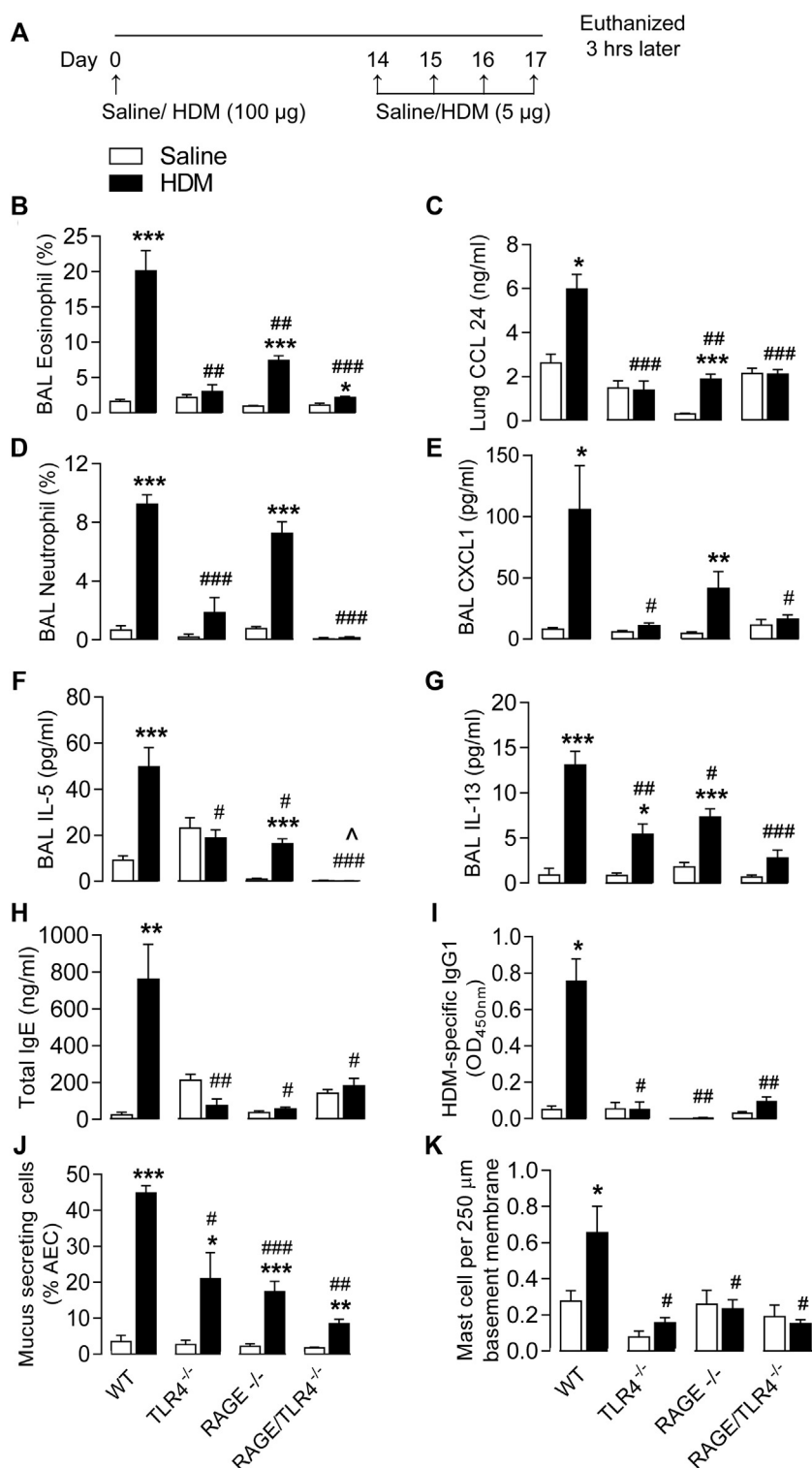


FIG 1. HDM-induced allergic airway inflammation. **A**, Study design. **B-K**, Allergic inflammation in saline solution or HDM sensitized and challenged mice. Bars: mean ($\pm$ SEM), $n = 4-6$ mice per group. *** $P < .001$, ** $P < .005$, * $P < .05$ vs saline solution control. ### $P < .001$, ## $P < .005$, # $P < .05$ vs HDM-exposed WT controls. $P < .05$ vs HDM-exposed TLR4^{-/-} and RAGE^{-/-} mice.

development of allergic airway inflammation, we next investigated whether the RAGE ligand HMGB1 also is involved in the sensitization process. HMGB1 is a nuclear protein but also functions as an immunostimulatory molecule after its

translocation to the cytoplasm and extracellular release.¹⁶ To address whether HMGB1 expression was altered during allergic sensitization and regulated by TLR4 and RAGE, we measured HMGB1 in the bronchoalveolar lavage fluid (BALF) at day 1,

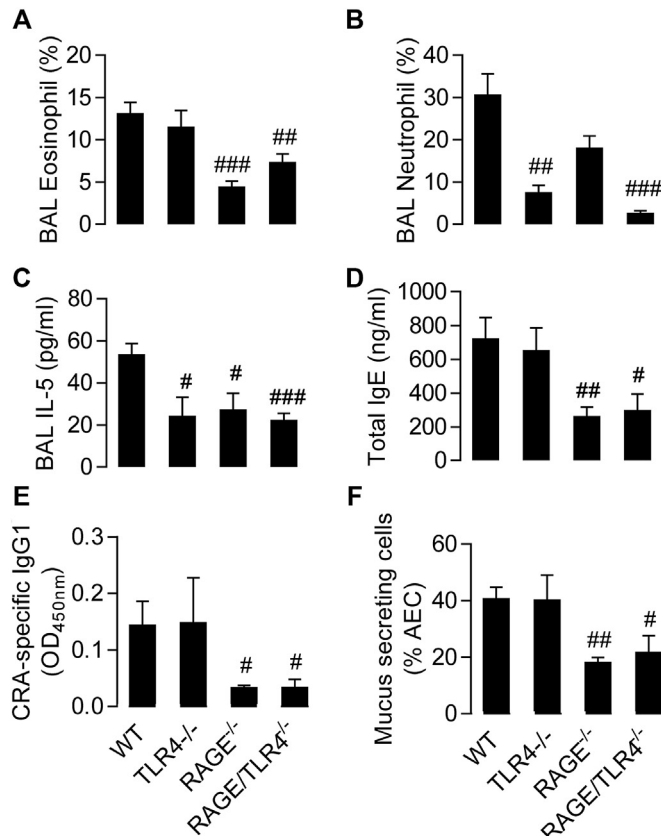


FIG 2. CR-induced allergic airway inflammation. **A-F**, Allergic inflammation in CR sensitized and challenged mice. Bars: mean ($\pm$ SEM), $n = 5-6$ mice per group. ### $P < .001$, ## $P < .005$, # $P < .05$ vs CR-exposed WT controls.

2, and 3 after HDM exposure (study design, Fig 5, A). In WT mice, HMGB1 levels in BALF were elevated at day 1, peaked at day 2, and waned at day 3. In TLR4^{-/-} mice, basal levels of HMGB1 were high, although, notably, there was no increase in HMGB1 expression in response to HDM treatment. In contrast, the profile of HMGB1 release in RAGE^{-/-} mice was similar to that observed in the WT controls, except at day 3, when it was significantly lower. Negligible HMGB1 protein was detected in the BALF of RAGE-TLR4^{-/-} mice (Fig 5, B). Thus, although TLR4 is critical for HMGB1 release, activation of RAGE secondary to TLR4 augments HMGB1 release.

To determine whether HMGB1 release is a general feature of airway sensitization and not specific to HDM, we measured HMGB1 protein levels in BALF at day 3 after CR sensitization (study design, Fig 5, A). Similar to the findings with HDM, CR-induced HMGB1 expression in BALF was diminished in the absence of RAGE (Fig 5, C). Having demonstrated that HMGB1 release was TLR4 dependent and given that epithelial TLR4 is necessary for HDM sensitization, we next investigated whether HDM exposure promoted HMGB1 translocation in AECs during the sensitization phase using immunohistochemistry (study design, Fig 5, A). In saline solution-exposed WT mice, HMGB1 expression was primarily localized to the nuclei of AECs, consistent with its expected location under physiological conditions (Fig 5, D). However, at day 3 after HDM sensitization, HMGB1 expression was predominantly cytoplasmic, which provided evidence of active HMGB1 nuclear

to cytoplasmic translocation during the sensitization phase (Fig 5, E). Interestingly, in WT mice, cytoplasmic expression was biphasic, with an initial increase at day 1, followed by a second increase at day 3 after HDM exposure. Notably, the first phase of HMGB1 translocation was TLR4 dependent and RAGE independent, whereas the second phase was RAGE dependent and TLR4 independent. As expected, both phases were absent in RAGE-TLR4^{-/-} mice, although, unexpectedly, there was an increase in HMGB1 cytoplasmic expression at day 2, which may reflect the activation of other pathways in the absence of TLR4 and RAGE signaling (Fig 5, F). This phasic release of HMGB1 in AECs, but not in the airway lumen (Fig 5, B), may be evidence of other cell types that contribute to the increase in HMGB1 during the process of allergic sensitization. However, it is important to emphasize that, in both the airway lumen and in AECs, early (day 1) release of HMGB1 is TLR4 dependent, whereas its later (day 3) release is RAGE dependent.

HMGB1 is released downstream of IL-1 α , and IL-1 α -HMGB1 complexes are present in BALF during allergic sensitization

Because both HMGB1 translocation in AECs, and HMGB1 release into the BALF was TLR4 dependent at day 1 after HDM sensitization, we determined whether the HMGB1-RAGE signaling pathway contributes to the TLR4-uric acid-IL-1 α cascade that leads to the production of type 2 innate cytokines.⁷ To do this, we first examined the time course of IL-1 α expression in WT, TLR4^{-/-}, RAGE^{-/-}, and RAGE-TLR4^{-/-} mice after HDM sensitization (study design, Fig 5, A). Although IL-1 α release was regulated by TLR4 and RAGE in a manner similar to HMGB1, IL-1 α levels peaked at day 1 (Fig 6, A) before the peak of HMGB1 release (Fig 5, B), which suggests that IL-1 α production was upstream of HMGB1 release. Treatment of WT mice with an IL-1R antagonist (Anakinra) significantly decreased HMGB1 production at day 1 after HDM exposure, which indicated that HMGB1 release is downstream of IL-1 α (Fig 6, A).

Next, we measured the temporal production of IL-25, IL-33, and TSLP in the sensitization phase. In contrast to IL-1 α and HMGB1, expression of IL-25, IL-33, and TSLP peaked at day 3 in WT mice, which suggests that these cytokines are produced downstream of IL-1 α and HMGB1. Similar to HMGB1, basal levels of IL-25 and IL-33 and TSLP were elevated in TLR4^{-/-} mice, but levels of all 3 cytokines were significantly attenuated by day 3 after HDM exposure (the time point of their maximal expression). The expression of all 3 cytokines also was significantly diminished at day 3 in RAGE^{-/-} mice. Although regulation of IL-25, IL-33, and TSLP before day 3 (ie, on day 1 and day 2 after HDM exposure) was variable in TLR4^{-/-} mice, RAGE deletion had a negligible influence at days 1 and 2. IL-25 and IL-33 levels were significantly attenuated at all time points in TLR4-RAGE^{-/-} mice. Interestingly, although TSLP levels also were significantly attenuated at day 3 in TLR4-RAGE^{-/-} mice, TSLP expression at day 2 was significantly increased, again consistent with activation of other pathways in the absence of TLR4 and RAGE signaling (Fig 6, B). To determine whether IL-25, IL-33, and TSLP release occurred downstream of HMGB1, mice were treated with anti-HMGB1 neutralizing antibody (30 minutes before HDM sensitization, on day 1, and on day 2). Immunoneutralization of HMGB1 successfully reduced

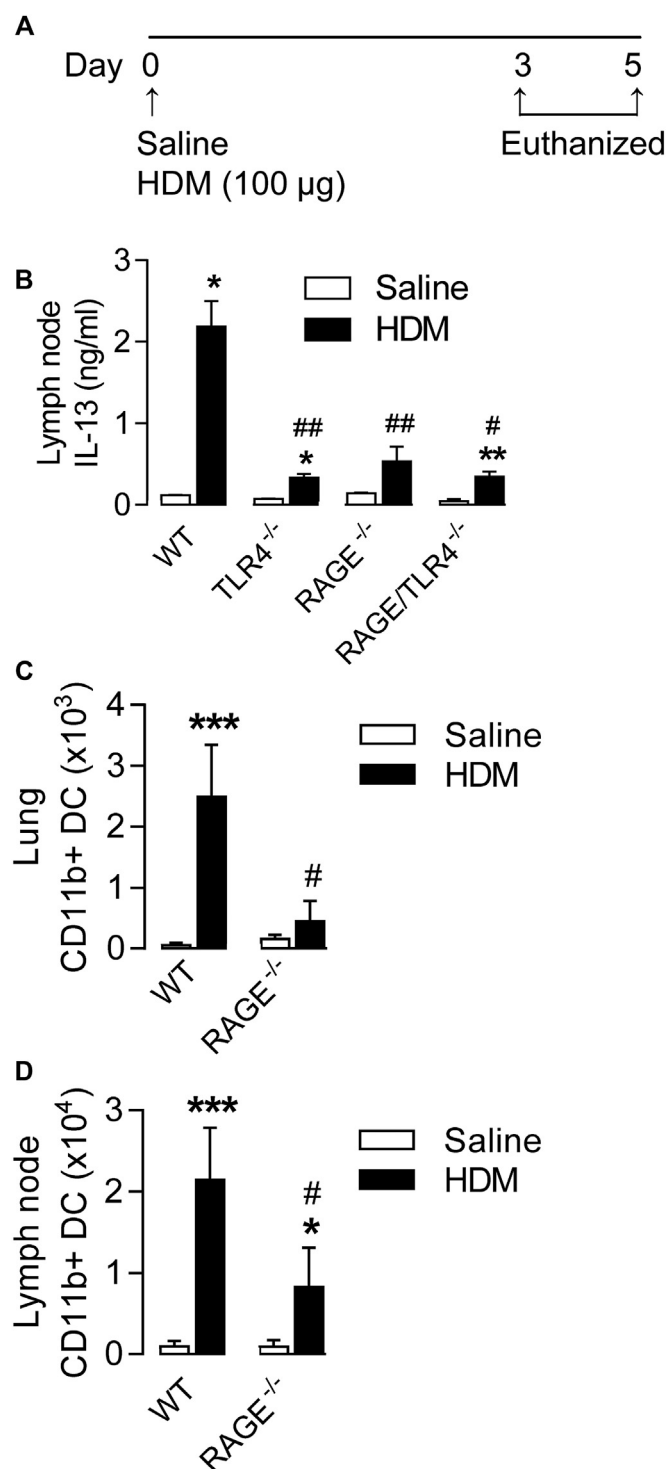


FIG 3. RAGE is required for T_H2 -cell priming and DC migration. **A**, Study design. **B**, Lymph node cells from saline solution or HDM sensitized mice were restimulated with HDM and IL-13 release was measured. **C** and **D**, DC numbers 3 days after saline solution or HDM sensitization. Bars: mean ($\pm$ SEM), $n = 4$ mice per group. *** $P < .001$, ** $P < .005$, * $P < .05$ vs saline solution control. ## $P < .005$, # $P < .05$ vs HDM-exposed WT controls.

BALF HMGB1 levels at day 3 and also significantly diminished the production of IL-25 and IL-33 but not TSLP (Fig 6, C).

HMGB1 binds to a variety of mediators, including IL-1 α to form biologically active complexes that can augment the

inflammatory response.²⁴ To determine whether IL-1 α -HMGB1 complexes are formed and present in the BALF during the sensitization phase, we developed a hybrid ELISA. Intriguingly, IL-1 α -HMGB1 complexes were detectable in BALF at day 1 and persisted to day 3 after HDM exposure (Fig 6, D).

HMGB1 is an effector molecule of allergic airway sensitization and inflammation

Because HMGB1 was released downstream of TLR4-RAGE ligation in AECs and functionally regulated the release of the T_H2 -instructive cytokines, IL-25 and IL-33, we investigated whether neutralization of HMGB1 would reduce HDM-induced allergic airway inflammation. Anti-HMGB1 antibody treatment during the HDM sensitization and challenge phase significantly inhibited all features of the allergic inflammatory response, including airway eosinophilia and neutrophilia, CXCL1, and CCL24 expression, mucus-positive cells, mast cell numbers, T_H2 cytokine expression, total IgE, and HDM-specific IgG1 production (Fig 7, A-I).

DISCUSSION

We confirmed and extended a primary role for TLR4 in the airway inflammatory response to HDM extracts and established for the first time, to our knowledge, that the HMGB1-RAGE axis plays a critical role in the development of allergic sensitization. Although TLR4 and RAGE share common ligands and signal transduction pathways, we established that these receptors do not functionally interact to augment the allergic inflammatory response but rather appear to be sequential components in a molecular cascade that underlies allergic sensitization. We found clear evidence that RAGE is engaged secondary to TLR4 after allergen inhalation and acts to amplify the expression of HMGB1 and downstream innate cytokines involved in the activation of type 2 inflammation (see schematic, Fig E1 in this article's Online Repository at www.jacionline.org).

The elucidation of the critical role of PRRs in the airway response to inhaled allergens has significantly improved our understanding of the process of allergic sensitization in asthma. Recent efforts have focused on delineating the specific PRRs involved, the mechanisms responsible for their activation, and the downstream pathways that lead to the priming of adaptive immunity.¹ As a result, it is now evident that numerous PRRs mediate allergen recognition and that different PRRs dominate in the response to different allergens.^{3-6,22,25} So that tractable targets for therapeutic intervention can be identified, it is important to understand the mechanisms of PRR collaboration and to delineate common pathways across multiple allergens. Because RAGE has been shown to synergize with TLR4 in the induction of nonallergic immune and inflammatory responses,^{12,13} we hypothesized that RAGE might amplify TLR4-mediated HDM sensitization.¹⁰ Compared with the absence of either TLR4 or RAGE, the absence of both receptors did not afford additional protection against HDM nor CR induced allergic inflammation, suggesting that RAGE and TLR4 do not functionally interact to augment the effector phase of the allergic inflammatory response. Because TLR4 and RAGE share common ligands and signaling pathways, these findings point toward possible redundancy. However, as discussed below, we believe that this is unlikely.

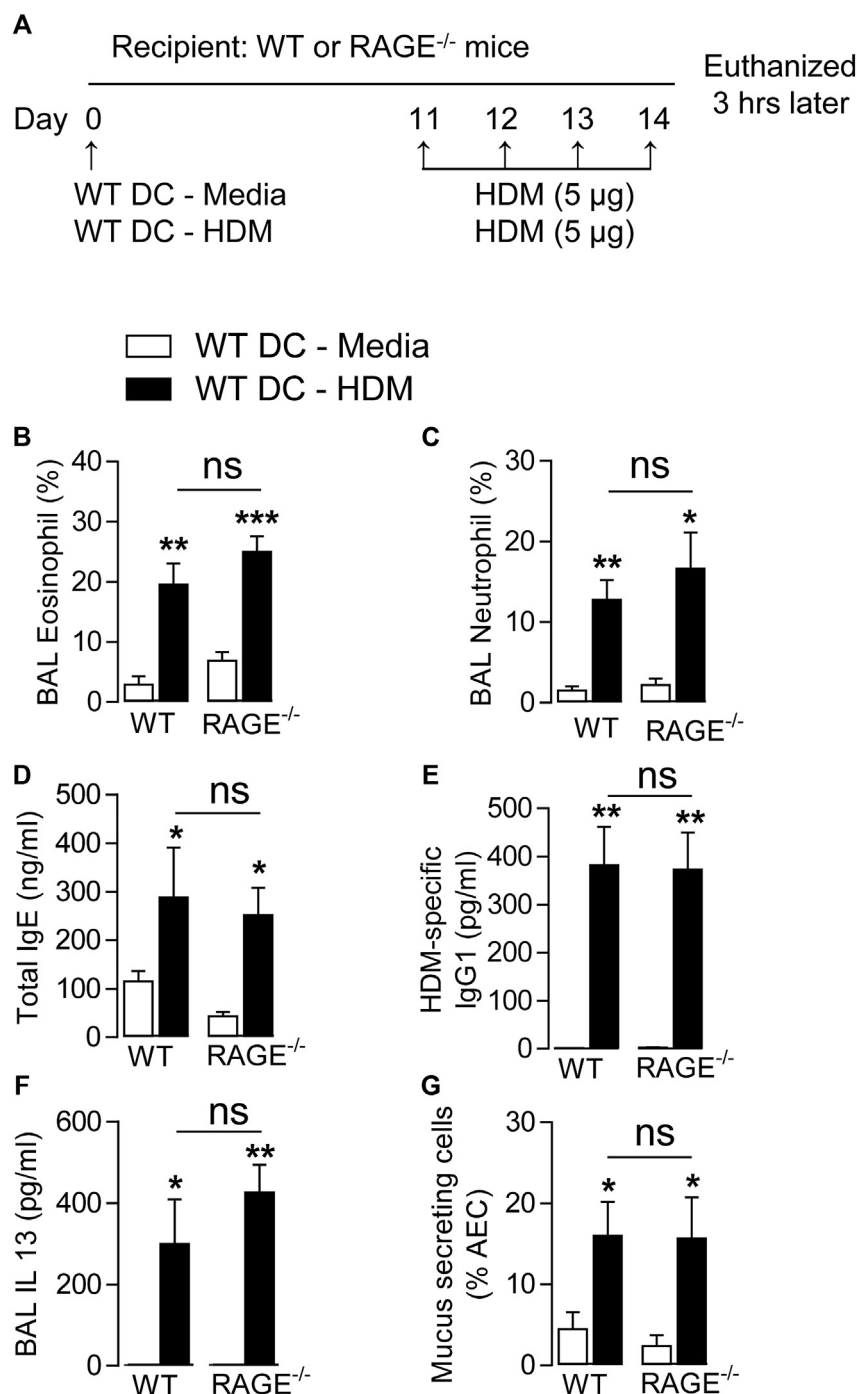


FIG 4. RAGE ligation in DCs is sufficient for the development of allergic airway inflammation. **A**, Study design. **B-G**, Allergic inflammation in mice administered media alone or HDM-pulsed WT-DC and then challenged with HDM. Bars: mean ($\pm$ SEM), $n = 4-6$ mice per group. *** $P < .001$, ** $P < .005$, * $P < .05$ vs saline solution control. NS, Not significant vs WT mice.

Previous studies have shown that HDM-induced TLR4 ligation in AECs triggers the release of uric acid and that uric acid triggers IL-1 α release, which acts to amplify type 2 inflammation via autocrine activation of IL-1R signaling.^{7,8} Our studies extend these findings to show that HMGB1 is released downstream of IL-1R signaling and is a third DAMP in the cascade upstream of type 2 cytokine production (IL-25 and IL-33). Although we did not measure uric acid production, uric acid has been shown

to induce HMGB1 secretion in endothelial cells,²⁶ which lends further support to the existence of an uric acid-IL-1 α -HMGB1 cascade. Importantly, we showed that, after HDM exposure, peak expression of IL-1 α (day 1) and HMGB1 (day 2) in BALF is TLR4 but not RAGE dependent. Moreover, BALF levels of IL-1 α and HMGB1 were significantly attenuated at day 3 after HDM exposure in RAGE^{-/-} mice, which suggests that activation of RAGE secondary to TLR4 acts to sustain and/or augment the

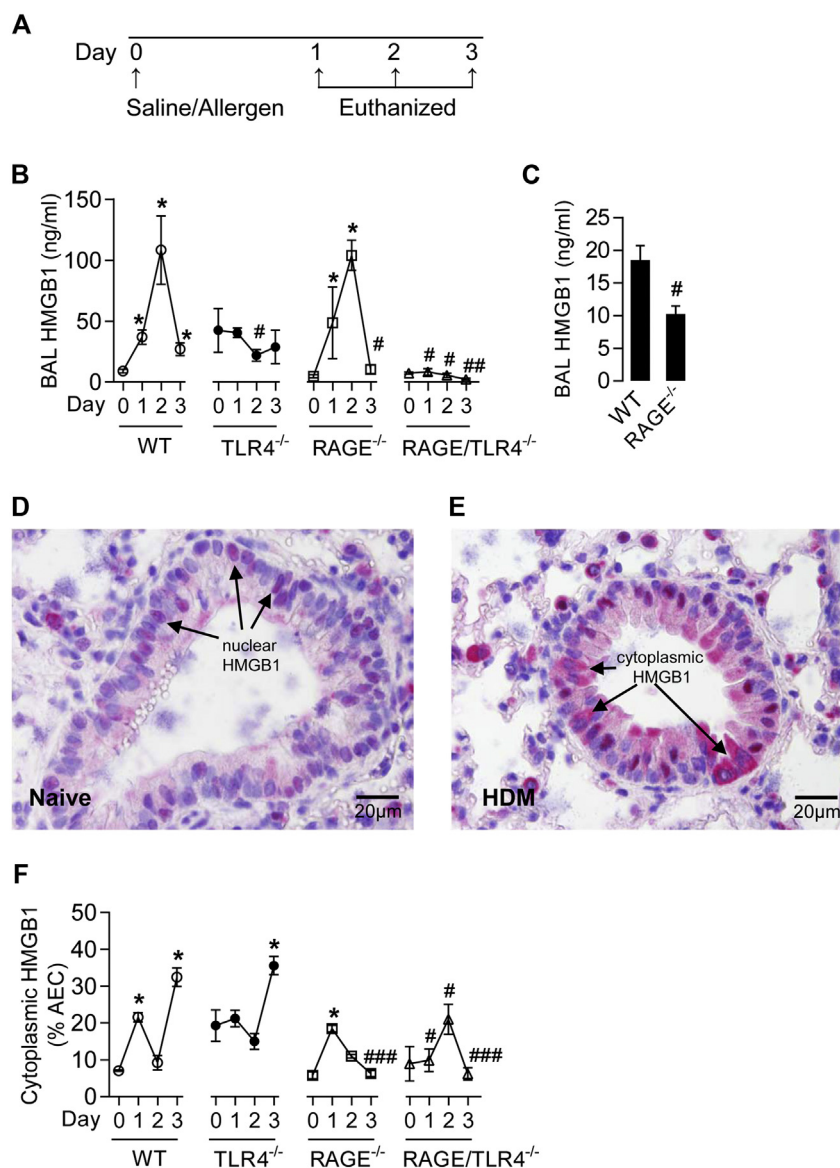


FIG 5. Expression and regulation of HMGB1 in AECs during allergen sensitization. **A**, Study design. HMGB1 expression during HDM sensitization (**B**) and day 3 after CR sensitization (**C**). **D** and **E**, Lung HMGB1 immunostaining ($\times 400$ magnification). **F**, HMGB1 cytoplasmic expression in AECs. Data: mean ($\pm$ SEM), $n = 3-9$ mice per group. * $P < .05$ vs time 0. ### $P < .001$, ## $P < .005$, # $P < .05$ vs WT controls at respective time points.

expression of these early response DAMPs. These data, together with evidence that the first and second wave of HMGB1 cytoplasmic expression in AECs appears to be regulated by the sequential activation of TLR4 and RAGE, respectively, strongly supports the notion that TLR4 and RAGE are not redundant but rather act at critical but sequential points in the process of allergic sensitization, with interception of either point being sufficient to retard the progression of the allergic response.

Our finding that RAGE is engaged secondary to TLR4 during allergen sensitization is significant for 2 reasons. First, it suggests that RAGE serves as a common mechanism downstream of various allergen-activated PRR pathways. This point is best illustrated by our demonstration that HMGB1 release occurred in response to 2 different extracts, HDM and CR, before the apparent onset of RAGE signaling. Because HDM and CR are

“sensed” by distinct PRRs, primarily TLR4 and PAR-2,^{22,27} respectively, and HMGB1 is a well-characterized RAGE ligand,^{14,16} it is conceivable that HMGB1 secretion downstream of initial “sensing” PRRs converges on RAGE signaling to propagate the allergic inflammatory response to multiple allergens. Second, we observed that RAGE activation regulates HMGB1 secretion and sustains its expression at the level of the airway epithelium. Since we showed that HMGB1 mediates the release of the T_H2 -instructive cytokines IL-25 and IL-33, it appears that RAGE, by mediating the recognition and amplification of DAMP signals required for the development of type 2 inflammation, also is an important mechanism for the amplification of the allergic inflammatory response. Interestingly, although we found that RAGE mediated the expression of all 3 type 2 cytokines studied (IL-25, IL-33, and TSLP), this was not

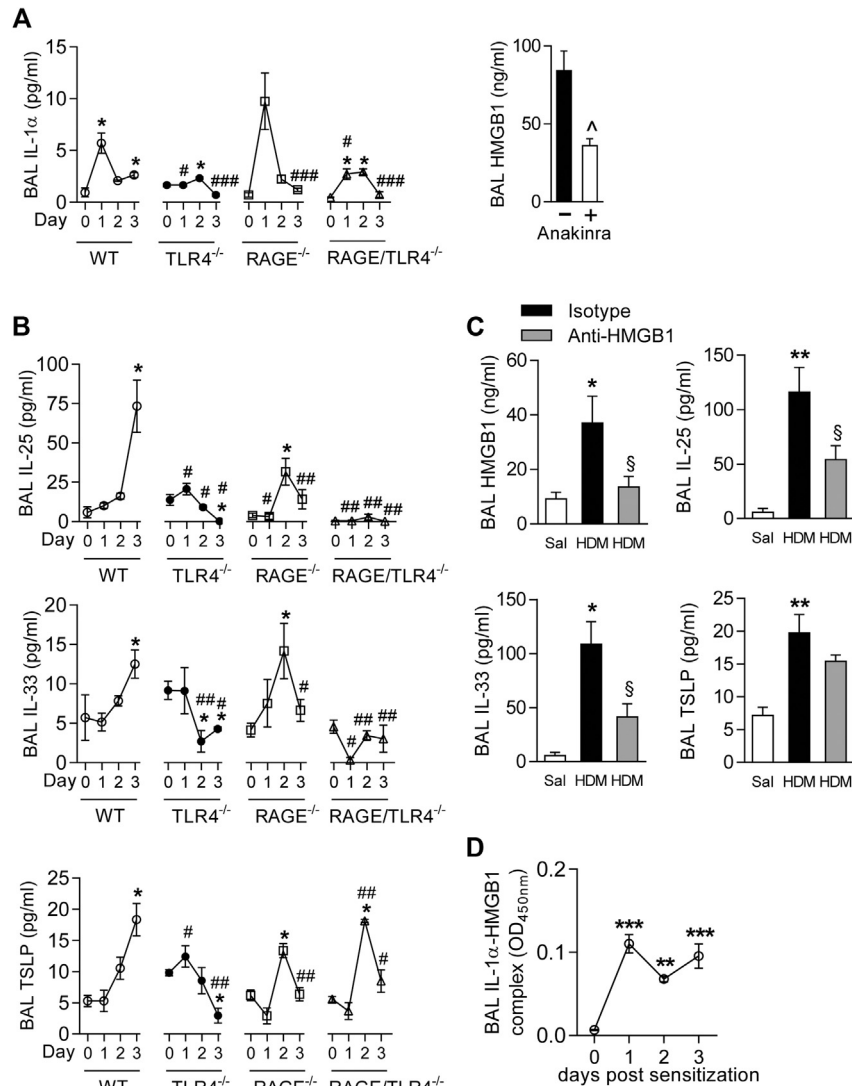


FIG 6. Relationship among HMGB1, IL-1 α and type 2 cytokine release during allergen sensitization. **A**, IL-1 α expression during HDM sensitization and HMGB1 expression day 1 after HDM sensitization. **B**, Type 2 cytokine expression during HDM sensitization. **C**, HMGB1 and type 2 cytokine expression day 3 after sensitization with saline (Sal) solution or HDM. **D**, IL-1 α -HMGB1 complexes in BALF during HDM sensitization. Data: mean ($\pm$ SEM), $n = 3$ -8 mice per group; *** $P < .001$, ** $P < .005$, * $P < .05$ vs time 0 (**A**, **B**, **D**) or saline solution control (**C**). ### $P < .001$, ## $P < .005$, # $P < .05$ vs WT controls at respective time points. * $P < .05$ vs mice exposed to HDM. § $P < .05$ vs mice exposed to HDM and isotype control antibody.

the case for HMGB1 because it did not appear to be an important determinant of TSLP expression. This, together with the fact that HMGB1 immunoneutralization only partially inhibited IL-25 and IL-33 release, suggests that other RAGE ligands such as serum amyloid A and DNA may also play a role.^{10,28,29}

Upon allergen exposure, AECs release innate cytokines and chemokines, which promote the recruitment of DCs to the airway mucosal surface where they take up antigens.¹ Antigen-loaded DCs then process the antigen and migrate to the draining lymph nodes where they activate T_H2 adaptive immune responses.²³ Our studies indicate that RAGE ligation in DCs is critical for T_H2 priming and the development of the allergic inflammatory response. Because we demonstrated that AECs secrete HMGB1 in response to allergen exposure and other researchers showed that HMGB1-RAGE signaling is required for DC migration and activation,^{30,31} we postulate that epithelial-derived HMGB1

directly contributes to the activation of T_H2 responses via engaging RAGE in DCs.

Interestingly, we observed 2 distinct phases of HMGB1 expression in AECs after allergen exposure. Because each phase of HMGB1 secretion was differentially regulated (the first being TLR4 dependent, and the second being RAGE dependent), its secretion may be in response to different factors. Given that TLR4 plays an important role in the recognition of HDM extracts,^{3,6} it is most likely that the first phase was due to HDM-induced TLR4 ligation in AECs. However, it is not clear whether the second phase is due to a feed-forward loop driven by epithelial-derived HMGB1 or by HMGB1 secretion from activated immune cells (eg, DCs). Also, it is not clear whether this is due to RAGE ligation in AECs or a consequence of RAGE ligation in immune cells and the generation of an additional factor that signals the airway epithelium to release HMGB1 (Fig E1).

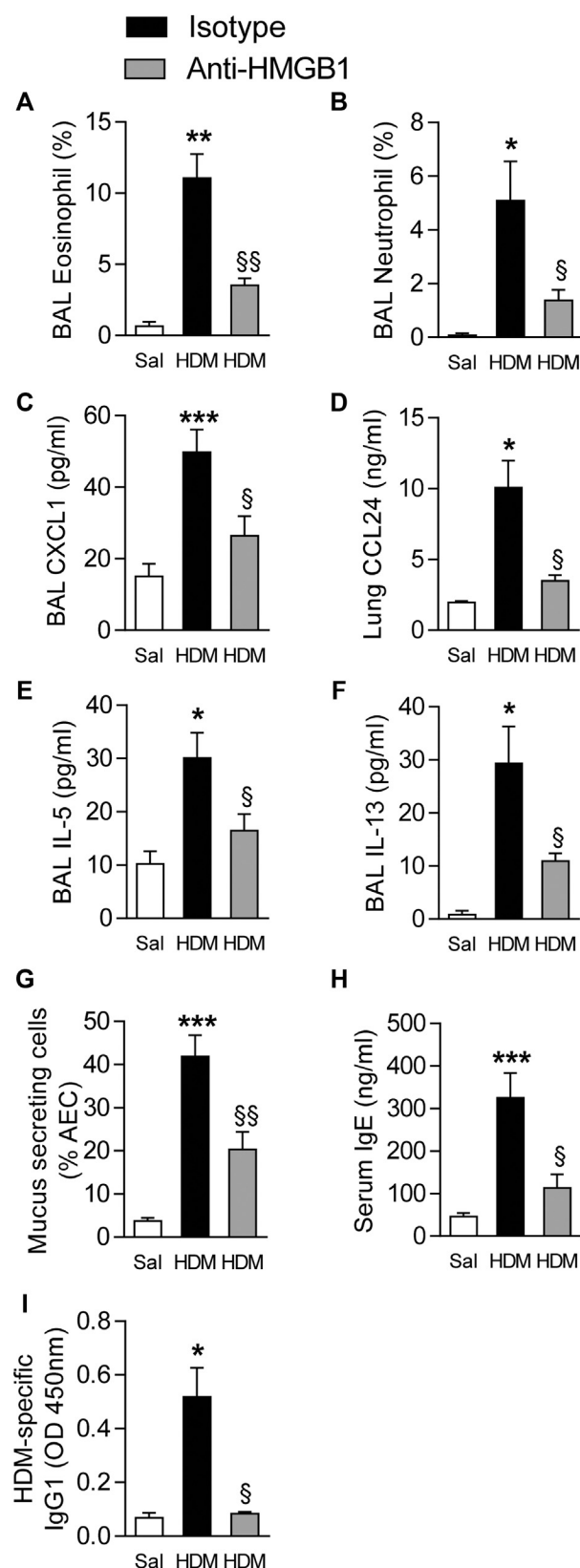


FIG 7. HMGB1 is an effector molecule of allergic airway inflammation. **A-I**, Allergic inflammation in saline (Sal) solution or HDM sensitized and challenged mice. Bars: mean ($\pm$ SEM), $n = 4-6$ mice per group. *** $P < .001$, ** $P < .005$, * $P < .05$ vs saline solution control. \$\$\$ $P < .005$, \$\$ $P < .01$, \$ $P < .05$ vs mice exposed to HDM and isotype control antibody.

Given that we demonstrated an important role for RAGE ligation in DCs in the development of the allergic inflammatory response, together with evidence of bidirectional cross-talk between DCs and AECs,³² it is possible that the second phase of HMGB1 secretion may be due to RAGE ligation in DCs. The finding that the second phase of HMGB1 secretion was unaffected in TLR4-deficient mice (in which RAGE is still functional) supports this idea. Further studies are required to delineate the mediators that drive epithelial HMGB1 release, the site(s) of RAGE activation, and whether bidirectional cross-talk between DCs or other innate immune cells and AECs contributes to feed-forward loops that propagate HMGB1-RAGE signaling (Fig E1).

Lipopolysaccharide is a well-established TLR4 ligand, and HDM-induced TLR4 ligation in AECs is partly attributed to low levels of contaminating microbial LPS.⁶ We previously showed that LPS also binds RAGE and is an effective mediator of RAGE signaling.³³ This raised the possibility that RAGE ligation occurs directly in response to LPS and perhaps at the same time as TLR4 rather than secondary to the activation of TLR4. However, this is unlikely because we did not detect any significant binding interaction between RAGE and the HDM allergen extracts used in our studies when using surface plasmon resonance assays (unpublished data), which suggests that, and consistent with our observations, RAGE ligation occurs secondary to activation of TLR4 and the release of RAGE ligands, for example, HMGB1.

Although numerous studies have demonstrated the adjuvant properties of HMGB1-ligand complexes *in vitro*, few have examined their presence or function *in vivo*.¹⁶ Our finding that IL-1 α -HMGB1 complexes are formed during the allergen sensitization phase raises the possibility that HMGB1 facilitates the process of allergic sensitization by augmenting IL-1 α -dependent inflammatory responses,²⁴ and, certainly, this is an important area for further investigation. Further studies are also required to establish whether the IL-1 α -HMGB1 cascade is common to all aeroallergens. Our finding that HMGB1 is involved in the airway response to at least 2 allergens that use distinct PRR pathways suggests that this cascade is unlikely to be determined by the nature of the allergen or the specific PRR engaged. Rather, because reactive oxygen species play an important role in allergic sensitization^{1,6,34-37} and also induce the release of various DAMPs, including IL-1 α and HMGB1,^{38,39} we speculate that activation of DAMP pathways is downstream of allergen-induced reactive oxygen species production in AECs.

Finally, we observed that neutrophilic inflammation was mediated by TLR4 but not by RAGE, in both HDM and CR models of allergic inflammation. This was surprising because a RAGE involvement in neutrophil recruitment is well documented, and we and other researchers have suggested that this might be relevant to neutrophilic inflammation in certain phenotypes of asthma.^{10,40,41} However, several groups have identified a critical role for IL-1 α in neutrophil recruitment.⁴²⁻⁴⁴ Thus, the differential regulation of IL-1 α during the allergen sensitization phase in TLR4^{-/-} mice, in which IL-1 α is completely abrogated, in contrast to RAGE^{-/-} mice, in which peak expression levels of IL-1 α are not affected (Fig 6, A), might be an explanation. These observations deserve further study as new mechanisms underlying neutrophilic inflammation in distinct phenotypes of asthma may be discovered.

In conclusion, we uncovered previously unknown roles for RAGE and its ligand HMGB1 in allergic airway sensitization and the progression of the allergic airway inflammatory response. Combined with previous observations that inhibition of HMGB1 during allergen challenge (but not during sensitization) significantly inhibits allergic airway inflammation in an ovalbumin mouse model of asthma,^{19,21} there is growing evidence that inhibition of the HMGB1-RAGE axis may have therapeutic benefit in asthma, both in primary prevention and in established disease.⁴⁵

We thank Associate Professor Matthew J. Sweet, PhD, for providing TLR4-deficient mice for use in these studies. We thank Professor Kevin J. Tracey, MD, for his advice and support, and we also thank Professor Dirkje S. Postma, MD, PhD, for her review of this manuscript.

Key messages

- Allergen-dependent activation of TLR4 induces secretion of the RAGE ligand HMGB1, a danger-associated molecular pattern critical to the development of allergic inflammation.
- HMGB1 release and RAGE activation is upstream of IL-25 and IL-33, and contributes to allergic sensitization.
- Activation of the HMGB1-RAGE pathway occurs in response to more than 1 allergen, which suggests that it may be a useful therapeutic target.

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METHODS

Mice

All mice strains were on C57Bl/6 background, and all studies were performed in 8- to 12-week-old males. WT control mice were purchased from the University of Queensland Biological Resources (Brisbane, Australia). TLR4^{-/-} mice were provided by Matthew J. Sweet, PhD, (Institute for Molecular Bioscience, University of Queensland, Australia), and RAGE^{-/-} mice were provided by Ann Marie Schmidt, PhD (NYU Langone Medical Centre and School of Medicine, NY). TLR4^{-/-} mice were cross-bred with RAGE^{-/-} mice to generate RAGE-TLR4^{-/-} mice. All experimental procedures and protocols were approved by the animal care and ethics committees of the University of Queensland (Brisbane, Australia).

Allergen extracts

HDM and CR extracts were purchased from Greer Laboratories, Lenoir, NC. Extracts were prepared from HDMs and CRs that were grown under controlled conditions. Extracts were prepared from the crushed bodies of whole HDM and from CRs that were defatted, powdered, and dried. In each case, the raw material was subjected to bilevel extraction in ammonium bicarbonate solution and then dialyzed against distilled water. The endotoxin level for HDM and CR extracts used were 3.5 EU and 395 EU per 100 µg protein, respectively. Endotoxin concentration for HDM extracts was supplied by the manufacturer, whereas endotoxin concentration for CR extracts was determined by using Endosafe-PTS Limulus amoebocyte lysate (Charles River Laboratory, Charleston, SC) at the Protein Expression Facility (Australian Institute of Bioengineering and Nanotechnology, The University of Queensland).

Induction of allergic sensitization and airway inflammation

On day 0, the mice were anesthetized with isoflurane and sensitized via nasal instillation with either saline solution or 100 µg of HDM or CR extract. At day 14, 15, 16, and 17, mice were challenged intranasally with either saline solution or 5 µg of HDM or CR, and were euthanized 3 hours after the last challenge (Fig 1, A). In experiments in which only the sensitization phase was studied, the mice were euthanized at different time points from 1 to 5 days after allergen sensitization (Figs 3, A and 5, A). In studies that examined the role of HMGB1, 50 µg of HMGB1 neutralizing antibody (clone 2G7, a kind gift from Kevin J. Tracey, MD, Feinstein Institute of Medical Research, New York, NY) was administered intranasally during the sensitization phase (30 minutes before HDM administration and on days 1 and 2) and/or HDM challenge phase (30 minutes before HDM challenge on days 14, 15, and 16). To study the role of IL-1α signaling, 1 mg of an IL-1 receptor antagonist, Anakinra (Amgen, Sydney, Australia, Pty Ltd; a kind gift from Mark R. Hutchinson, PhD, University of Adelaide, Australia) was administered intranasally 0.5, 6, and 12 hours after HDM sensitization.

DC generation and adoptive transfer studies

Bone-marrow-derived DCs were generated from WT naive mice by following the method of Lutz et al^{E1} with minor modification. Briefly, femurs and tibias of WT mice were dissected, and bone marrow was flushed out with RPMI-1640 growth medium (which contained 1 mM sodium pyruvate, 10% FBS, 2 mM L-glutamine, 20 mM HEPES, 100 U/mL penicillin/streptomycin, and 50 µM 2-mercaptoethanol). After red blood cell lysis, the cells were cultured in growth media supplemented with 20 ng/mL of GM-CSF (R & D Systems, Minneapolis, Minn) for 8 days (media was replenished on day 4 of culture). At the end of the culture period, nonadherent cells were harvested, and CD11c positive selection was performed by using biotin-conjugated anti-mouse CD11c, followed by streptavidin-conjugated magnetic beads. The resulting CD11c⁺CD11b⁺ bone-marrow-derived DC population was 85%-90% pure. The cells then were either left in media alone or pulsed with HDM extract (100 µg/mL) for 24 hours, before they were harvested and washed twice with PBS; 1 × 10⁶ unstimulated or HDM treated bone-marrow-derived DCs then were administered via the intranasal route to WT or RAGE^{-/-} mice at day 0. At day 11, 12, 13, and 14, mice were challenged

with 5 µg of HDM and euthanized 3 hours after the last challenge (Fig 4, A). This protocol did not induce significant lung inflammation in mice that received unstimulated bone-marrow-derived DCs.

Analysis of allergic airway inflammation

The lungs were lavaged with 600 µL of ice cold PBS, and the recovery was consistently between 400 and 500 µL. To determine differential cell counts, BAL fluids were spun down to separate cells. Red blood cells were then lysed, and total blood cell counts were performed. Cytospins were prepared and stained with May-Grunwald Giemsa solution (Sigma-Aldrich, St Louis, Mo). A total of 400 cells were counted to enumerate differential cell counts. BALF and/or cell lysates prepared from lung homogenates were assayed for cytokine expression by ELISA. To prepare lung homogenates, lung lobes were homogenized in radioimmunoprecipitation assay buffer (Sigma-Aldrich) by using a Tissue Tearor homogenizer (Model 985-370; BioSpec Products Bartlesville, Okla). Lung lobes were processed, and sections were prepared to enumerate mucus-producing goblet cells or mast cells. To examine T_H2-cell priming during the sensitization phase, draining lymph nodes were excised 5 days after HDM sensitization, and a single-cell suspension was prepared and cultured with HDM 20 µg/mL for 4 days, as previously described.^{E2}

Histologic analysis of mucus-secreting cells and mast cells

Paraffin-embedded lung biopsy specimens were stained with periodic acid-Schiff reagents to enumerate mucus-secreting cells. The number of mucus-secreting cells among the AECs were counted (4-5 airways per mouse) and expressed as the percentage of total AECs in the respective airways. For mast cell analysis, lung biopsy specimens were stained with toluidine-blue reagent, and the number of mast cells around the airways were counted and described as the number of cells per 250 µm of airway epithelium basement membrane.

Immunohistochemical detection of HMGB1

Paraffin-embedded lung biopsy specimens were rehydrated and then antigen retrieval was performed by incubating sections in citrate buffer and heating in a pressure cooker microwave. After washing the sections, goat serum was added to block nonspecific binding. Sections were incubated at 4°C with rabbit anti-mouse HMGB1 antibody (Abcam plc, Cambridge, Mass) overnight, followed by anti-rabbit alkaline phosphatase secondary antibody. Sections then were incubated in Fast Red solution, counterstained with hematoxylin and mounted with glycerol. The number of AECs positive for cytoplasmic HMGB1 was quantified and expressed as the percentage of total AECs. A total of 4-5 airways per mouse were used for quantification.

Enumeration of DCs by flow cytometry

Lung lobes and thoracic draining lymph nodes were excised 3 days after HDM sensitization, and single-cell suspensions were prepared by gentle pressure with a syringe plunger over a cell strainer. Red blood cells were lysed by using Gey lysis buffer. Cells then were incubated with FC receptor blocker antibody and stained for DCs by using PerCP-cy5.5 conjugated anti-CD11b (BD Biosciences, San Jose, Calif) and brilliant violet 785 conjugated CD11c (Biolegend Inc, San Diego, Calif). BD LSRII was used for sample acquisition, and BD FACS DIVA software was used for data analysis.

Measurement of serum IgE and IgG1

Ninety-six-well plates were coated overnight with either anti-IgE antibody, HDM extract (50 µg/mL), or CRA extract (100 µg/mL). To block nonspecific binding, wells were coated with 1% bovine serum albumin (BSA) in PBS before adding serum or standards. Plates then were incubated with biotinylated anti-IgE or anti-IgG1 followed by streptavidin-horseradish peroxidase. Tetramethylbenzidine substrate was added, and the reaction was stopped with 1 N H₂SO₄. Optical density was determined at 450 nm by using a microplate reader (Bio-Rad, Hercules, Calif).

ELISA

All cytokine measurements were determined by ELISA according to the manufacturer instructions. The detection limits of the assays were 15.6 pg/mL for CCL24, 7.8 pg/mL for CXCL1, 7 pg/mL for IL-5, 1.5 pg/mL for IL-13, 3.5 pg/mL for IL-33 (R & D Systems); 1 pg/mL for IL-1 α , 7.5 pg/mL for IL-25 (Biolegend Inc) and 1.6 ng/mL for HMGB1 (Chondrex Inc, Redmond, Wash). To determine the presence of HMGB1-IL-1 α complexes in BAL fluid, a hybrid ELISA was performed according to the method of Venereau et al^{E3} with minor modification. Briefly, 96-well plates were coated with an antimouse HMGB1 antibody (2 μ g/mL) (Abcam plc) overnight at room temperature. Wells then were incubated with 1% BSA in PBS to block nonspecific binding. BALF samples were then added to the wells and incubated for 2 hours at room temperature before addition of a biotinylated anti-IL-1 α antibody and streptavidin-horseradish peroxidase. Washing steps were performed with 0.05% Tween-80/PBS solution. Tetramethylbenzidine substrate was added, and the reaction was stopped with 1 N H₂SO₄. Optical density was determined at 450 nm by using a microplate reader (Bio-Rad, Hercules, Calif).

Statistical analysis

All the data are presented as mean ($\pm$ SEM). Data sets were analyzed by unpaired Student *t* test or 1-way ANOVA by using the software GraphPad Prism version 5.0 (La Jolla, Calif). A *P* value $\leq$ 0.05 was considered to be statistically significant.

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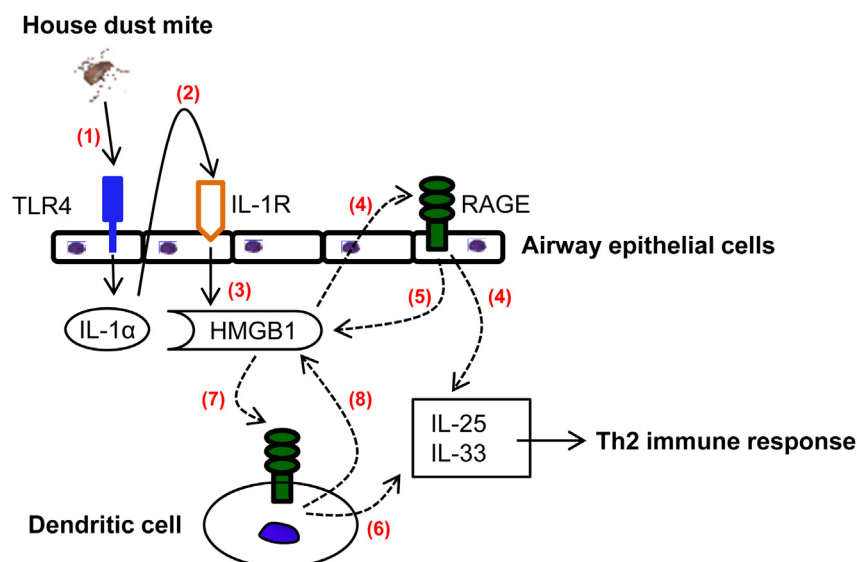


FIG E1. Schematic representation of HMGB1-RAGE signaling during allergic sensitization. HDM-induced activation of TLR4 in AECs (1) triggers IL-1 α release, activation of IL-1R signaling (2), and HMGB1 release (3). HMGB1 induces the expression of the epithelial-derived Th2 instructive cytokines IL-25 and IL-33. It remains to be determined whether this occurs via activation of RAGE on AECs (4). Activation of RAGE signaling acts to sustain and/or amplify HMGB1 expression at the level of the airway epithelium (5) and also is required for DC activation and orchestration of Th2 immune responses (6). It remains to be determined whether epithelial-derived HMGB1 engages RAGE signaling in DCs (7). We speculate that bidirectional cross-talk between epithelial cells and DCs drives HMGB1-RAGE feed-forward loops that contribute to the development and propagation of the allergic inflammatory response (8). We also speculate that the formation of IL-1 α -HMGB1 complexes contributes to the process of allergic sensitization.