Distinct Tlr4-expressing cell compartments control neutrophilic and eosinophilic airway inflammation

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Allergic asthma is a chronic, inflammatory lung disease. Some forms of allergic asthma are characterized by T helper type 2 (Th2)-driven eosinophilia, whereas others are distinguished by Th17-driven neutrophilia. Stimulation of Toll-like receptor 4 (TLR4) on hematopoietic and airway epithelial cells (AECs) contributes to the inflammatory response to lipopolysaccharide (LPS) and allergens, but the specific contribution of TLR4 in these cell compartments to airway inflammatory responses remains poorly understood. We used novel, conditionally mutant Tlr4fl/fl mice to define the relative contributions of AEC and hematopoietic cell Tlr4 expression to LPS- and allergen-induced airway inflammation. We found that Tlr4 expression by hematopoietic cells is critical for neutrophilic airway inflammation following LPS exposure and for Th17-driven neutrophilic responses to the house dust mite (HDM) lysates and ovalbumin (OVA). Conversely, Tlr4 expression by AECs was found to be important for robust eosinophilic airway inflammation following sensitization and challenge with these same allergens. Thus, Tlr4 expression by hematopoietic and airway epithelial cells controls distinct arms of the immune response to inhaled allergens.

INTRODUCTION

Allergic asthma is an increasingly prevalent disease that is characterized by chronic inflammation of the airway and reversible airway obstruction. Asthma is widely considered to stem from allergen-specific T helper type 2 (Th2) responses that result in eosinophilic inflammation but, in many individuals, neutrophils are the predominant leukocytes in the airway. Several studies have suggested that neutrophilic forms of asthma arise from allergen-specific Th17 responses, and are characterized by airway hyperresponsiveness and resistance to glucocorticoid therapy. These findings have been replicated in mouse models of experimental allergic asthma. Defining the cellular and molecular events that promote Th2 and Th17 responses to inhaled allergens will likely be critical for developing novel, effective strategies targeting specific subtypes of asthma.

A large body of evidence suggests that many allergens trigger maladaptive immune responses by direct interactions with innate immune receptors such as Toll-like receptors (TLRs). The best-characterized TLR in this regard is TLR4 that signals in response to lipopolysaccharide (LPS), a membrane component of Gram-negative bacteria. However, the relationship between environmental LPS and asthma is complex. Whereas some epidemiologic studies have linked LPS exposure to an increased prevalence of asthma, other studies have suggested that exposure to LPS decreases the risk of developing allergic asthma. Mouse models of asthma have confirmed a role for LPS during allergic sensitization to experimental allergens, with very high doses of inhaled LPS triggering Th1 responses to ovalbumin (OVA), and lower doses promoting Th2 and Th17 responses. In addition to LPS, some bona fide allergens display structural and functional homology to components of the TLR4 receptor complex, and can directly trigger TLR4 signaling and consequent allergic sensitization. Despite this wealth of evidence supporting an important role for TLR4 in the development of allergic responses, the specific function of this receptor during allergic sensitization remains unclear. In particular, studies employing bone marrow chimera
techniques have led to diverse conclusions regarding the relative contribution of different Tlr4-expressing cell populations to LPS or allergen responsiveness.16–18 Unfortunately, experiments of this type are confounded by the persistence of large numbers of host macrophages and dendritic cells after irradiation.19 Furthermore, although airway epithelial cells (AECs) are widely regarded as radioresistant, radiation induces profound transcriptional changes in these cells that have unknown effects on their function.20 Thus, although studies of irradiated bone marrow chimeric mice are useful, they have inherent limitations.

Here, we describe the creation of conditionally mutant Tlr4 mice that can be used to delete Tlr4 in distinct cell compartments without the complications associated with irradiation. Our studies revealed that Tlr4 expression by hematopoietic cells controls neutrophilic responses to inhaled LPS and allergens, whereas expression of this receptor by AECs is important for eosinophilic responses to inhaled allergens.

RESULTS
Generation of mice lacking Tlr4 expression by AECs or hematopoietic cells
We generated conditionally mutant Tlr4 mice by flanking the third exon of Tlr4 with LoxP sites (Tlr4fl/fl) (Figure 1a). This strategy was chosen because germline mutant mice lacking the third exon are unresponsive to LPS.9 To inactivate Tlr4 in the airway epithelium, Tlr4fl/fl mice were bred to animals in which expression of Cre recombinase is controlled by the Shh locus (Shh-Cre mice).21 Shh expression by the lung epithelium arises at E9.5 with full expression throughout the lung epithelium by E12.5.22,23 Thus, Cre-mediated genetic excisions are maintained in the progeny of these cells, including all pulmonary epithelial cell lineages.24 Furthermore, to inactivate TLR4 in the hematopoietic compartment, Tlr4fl/fl mice were bred to transgenic animals expressing Cre under control of the HS2145-vav1 oncogene promoter (Vav1-Cre mice).25

Selective Tlr4 deletion in AECs of Tlr4fl/flShh-Cre mice, and in hematopoietic cells of Tlr4fl/flVav1-Cre mice, was confirmed by PCR analysis of genomic DNA (Figure 1b) and by analysis of Tlr4-specific RNA prepared from AECs (Figure 1c) and total splenocytes (Figure 1d). Inactivation of Tlr4 was also confirmed by in vitro and in vivo functional studies. Bone marrow-derived dendritic cells from Tlr4fl/flShh-Cre mice and their Cre-negative Tlr4fl/fl littermates produced comparable amounts of tumor necrosis factor-α after treatment with LPS, whereas bone marrow-derived dendritic cells from Tlr4fl/flVav1-Cre mice failed to produce this cytokine following LPS stimulation, although they responded strongly to the

Figure 1  Generation of a Tlr4fl/fl mouse. (a) Structure of the targeting vector and strategy used to create Tlr4fl/fl mice. (b) PCR-based assay confirming selective deletion of Tlr4 from genomic DNA from the indicated cell types. AEC, airway epithelial cell; BM, bone marrow; Spl, total splenocyte; TLR4, Toll-like receptor 4. Section 1, Tlr4fl/fl; section 2, Tlr4fl/flVav1-Cre; section 3, Tlr4fl/fl; section 4, Tlr4fl/flShh-Cre. Tlr4 mRNA levels in (c) AECs and (d) total splenocytes. Data shown represent mean ± s.e.m., and are from one experiment representative of two–three experiments. N = 4–6 mice per group. *P < 0.05; ***P < 0.001.
TLR2 ligand, Pam3Cys (Figure 2a). Conversely, AECs from Tlr4fl/fl Vav1-Cre and Tlr4fl/fl mice produced comparable levels of the epithelium-derived chemokine, CCL20, in response to LPS or house dust mite (HDM) challenge, whereas AECs from Tlr4fl/fl Shh-Cre mice had dramatically reduced responses to these stimuli (Figure 2b). Finally, Tlr4fl/fl Shh-Cre mice, but not Tlr4fl/fl Vav1-Cre, exhibited high levels of serum tumor necrosis factor-α after intraperitoneal (IP) injection of LPS (n = 6–8 mice/group). Data represent mean ± s.e.m. from one of two–three experiments yielding similar results. *P < 0.05; **P < 0.01; ***P < 0.001.

TLR4 expression by hematopoietic cells promotes LPS-mediated airway inflammation

Previous studies employing bone marrow chimeric mice to address the relative contribution of different Tlr4-expressing cell populations to LPS responsiveness have led to diverse conclusions.16–18 To address this issue without the complications associated with irradiation, we administered ultrapure LPS to Tlr4fl/fl Shh-Cre mice and Tlr4fl/fl Vav1-Cre mice by intratracheal aspiration. Two peaks of neutrophil accumulation were seen in the airways of Tlr4fl/fl and Tlr4fl/fl Shh-Cre mice; at 4 and 24 h after LPS treatment (Figure 3a). However, at both of these time points, Tlr4fl/fl Vav1-Cre mice had significantly reduced numbers of these neutrophils, regardless of the amount of LPS used (Figure 3b). Thus, in nonirradiated mice, hematopoietic cell-specific Tlr4 expression is critical to LPS-mediated neutrophil recruitment to the airway and AEC-specific expression of this gene is dispensable. Tlr4fl/fl Shh- and Vav1-Cre mice also had a trend toward reduced neutrophil recruitment to the lung following phosphate-buffered saline treatment when compared with phosphate-buffered saline alone.
saline-treated littermate controls, although these differences did not reach statistical significance. This trend might have been because of the role of Tlr4 in neutrophilic responses to minor injury caused by repeated fluid installation into the lungs.26

The markedly reduced neutrophil accumulation seen in Tlr4fl/fl Vav1-Cre mice following LPS instillation suggested that neutrophil-attracting chemokines might also be reduced in these animals. To test this, we instilled LPS into the airways of mice and assayed bronchoalveolar lavage (BAL) fluid for CXCL1, CXCL2, and CXCL5. Each of these chemokines were significantly reduced in the airways of Tlr4fl/fl Vav1-Cre mice compared with Tlr4fl/fl littermates, whereas concentrations of these chemokines in Tlr4fl/fl Shh-Cre mice were generally similar to their nontransgenic littermates (Figure 3c). An exception was the reduction in CXCL1 seen in Tlr4fl/fl Shh-Cre mice, although this reduction was apparently insufficient to significantly affect the number of neutrophils recruited to the airways. In addition to the reduction in amounts of neutrophil-attracting chemokines in the airway, Tlr4fl/fl Vav1-Cre mice also had significantly reduced amounts of interferon-γ, tumor necrosis factor-α, and interleukin (IL)-6 in BAL fluid 24 h after challenge (Figure 3d), cytokines whose levels were unaffected in Tlr4fl/fl Shh-Cre mice. Collectively, these data demonstrate that hematopoietic cell expression of Tlr4 is critical for LPS-driven recruitment of neutrophils to the airway.

Tlr4 expression by hematopoietic cells regulates neutrophilic responses to HDM

HDMs represent a ubiquitous source of household allergens to which many patients with allergic asthma are sensitized. To determine the roles of Tlr4-expressing AECs and hematopoietic

Figure 3  Tlr4-expressing hematopoietic cells promote lipopolysaccharide (LPS)-mediated neutrophilic inflammation. (a) Time course of neutrophil recruitment following inhalation of 1 μg LPS. (b) LPS dose response for neutrophils in mice harvested 24 h after treatment. Chemokine and cytokine levels in the bronchoalveolar lavage fluid (BALF) at (c) 2 h and (d) 24 h after treatment with 1 μg LPS. IFN-γ, interferon-γ; IL-6, interleukin-6; ND, not detectable; PBS, phosphate-buffered saline; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-α. Data represent mean ± s.e.m. from one experiment representative of three experiments, with 4–12 mice per group. *P < 0.05; **P < 0.01; ***P < 0.001.
cells in acute immune responses to HDM, we challenged the airways of mice with HDM and evaluated airway inflammation 24 h later. As observed after LPS treatment, HDM-treated Tlr4fl/fl Vav1-Cre mice had significantly fewer airway neutrophils than did their Tlr4fl/fl littermate controls, whereas Tlr4fl/fl Shh-Cre mice and control Tlr4fl/fl littermates had similar numbers of neutrophils (Figure 4a). To determine a possible cause for the decreased number of neutrophils recruited to the airways of Tlr4fl/fl Vav1-Cre mice, we quantified CXCL1 and CXCL2 in the BAL fluid. Both chemokines were reduced in Tlr4fl/fl Vav1-Cre mice when compared with nontransgenic littermates (Figure 4b), whereas these chemokines were similar in Tlr4fl/fl Shh-Cre mice and nontransgenic Tlr4fl/fl controls. Because IL-25 and IL-33 have been implicated in allergic sensitization through the airway, we also quantified these cytokines in HDM-treated mice. Tlr4fl/fl Shh-Cre mice had reduced levels of IL-25 and IL-33 (Figure 4c) in the BAL fluid, compared with Tlr4fl/fl littermate controls, whereas Tlr4fl/fl Vav1-Cre mice and their Tlr4fl/fl littermates had similar amounts of these cytokines. Thus, Tlr4-expressing hematopoietic cells produce neutrophil-attracting chemokines in response to LPS, whereas Tlr4-expressing AECs produce Th2-promoting cytokines.

To determine the role of TLR4 expression by airway epithelial and hematopoietic cells in a clinically relevant mouse model of asthma, we sensitized Tlr4fl/fl Shh-Cre mice and Tlr4fl/fl Vav1-Cre mice with HDM on days 0 and 7, followed by challenge with this same allergen preparation on day 14. The commercial preparation of HDM we used contains ~100 ng LPS per 100 μg of HDM, sufficient to promote adaptive immune responses to bystander allergens and drive Tlr4-dependent eosinophilia and neutrophilia in several mouse strains (Figure 5a). No differences between either of the present strains and their Tlr4fl/fl littermates were seen when total leukocytes in the airway were quantified (Figure 5b,c). However, cell differential analysis of the leukocytes in BAL revealed marked differences among these strains. Tlr4fl/fl Vav1-Cre mice had significantly fewer neutrophils in their airways than did their Tlr4fl/fl littermates, whereas Tlr4fl/fl Shh-Cre mice had similar numbers of neutrophils compared with their nontransgenic littermates. Conversely, eosinophil numbers were significantly reduced in Tlr4fl/fl Shh-Cre mice compared with their Tlr4fl/fl littermates, TLR4-expressing airway epithelial and hematopoietic cells differentially regulate eosinophilic and neutrophilic allergic airway inflammation

Figure 4  Role of Tlr4-expressing airway epithelial cells (AECs) and hematopoietic cells in innate immune responses to house dust mite (HDM). (a) Neutrophils in bronchoalveolar lavage (BAL) fluid of mice 24 h after a single instillation of HDM. (b) CXCL1 and CXCL2 and (c) interleukin (IL)-25 and IL-33 levels in the BAL fluid. PBS, phosphate-buffered saline; TLR4, Toll-like receptor 4. Data represent mean ± s.e.m. from one experiment representative of two experiments, with 6–8 mice per group. *P < 0.05; **P < 0.01.
whereas the numbers of these cells were similar in Tlr4fl/fl Vav1-Cre and Tlr4fl/fl mice. Thus, Tlr4 expression in specific cell compartments affects distinct arms of the airway immune response, with expression in hematopoietic cells promoting neutrophilic inflammation and expression in epithelial cells promoting eosinophilic inflammation.

To examine whether differences in cytokine and chemokine levels might have led to qualitative differences in airway inflammation, whole lungs from HDM-sensitized and -challenged mice were restimulated in vitro with HDM. Surprisingly, IL-33 was the only cytokine that we found significantly reduced in the lungs of Tlr4fl/fl Shh-Cre mice compared with Tlr4fl/fl littermate controls (Figure 6a). Lungs from Tlr4fl/fl Shh-Cre mice also had reduced amounts of IL-33 (Figure 6b), and produced significantly less of the neutrophilia-promoting cytokines, IL-17A and IL-17F.

To compare our study of Tlr4fl/fl mice with previous studies using bone marrow chimeric mice, we used wild-type (WT) and Tlr4−/− animals to generate reciprocal bone marrow chimeras and studied them in the HDM-driven model of allergic asthma. As expected, when WT marrow was transferred into irradiated WT recipients (WT→WT), HDM-treated mice developed both eosinophilic and neutrophilic airway inflammation (Figure 7a). However, WT→Tlr4−/− mice displayed very little eosinophilia, in agreement with our finding of reduced eosinophilia in Tlr4fl/fl Shh-Cre mice and confirming an important role of Tlr4 expression in airway structural cells for eosinophilic inflammation. Conversely, neutrophilic airway inflammation was not significantly affected in the WT→Tlr4−/− mice, but was completely abrogated in Tlr4−/−→WT mice, whereas eosinophils were unaffected. This finding was also consistent with our observation that neutrophilia is reduced in Tlr4fl/fl Vav1-Cre mice, confirming that Tlr4 expression in hematopoietic cells is critical for the neutrophilic component of airway inflammation. Analysis of cytokines in lungs prepared from bone marrow chimeric mice revealed that, as seen in the Tlr4fl/fl model, Tlr4 expression in hematopoietic cells is critical for production of IL-17A and IL-17F (Figure 7b).

To confirm that our results were not unique to the HDM model of allergic inflammation, we carried out experiments in a different established mouse model of asthma in which inhaled LPS promotes Th2 and Th17 responses to inhaled OVA. These experiments confirmed that, as we had seen with the HDM model, Tlr4 expression in hematopoietic cells is critical for production of IL-17A and IL-17F and associated neutrophilic
inflammation, whereas Tlr4 expression in hematopoietic cells is critical for production of IL-5 and eosinophilic inflammation (Figure 8).

DISCUSSION

Previous studies employing bone marrow chimeric mice have led to diverse conclusions regarding the contribution of Tlr4-expressing cell compartments to leukocyte recruitment in animal models of acute inflammation and allergic airway inflammation. Some of this diversity is likely because of variable effects of radiation on structural and hematopoietic cell function. In this study, we circumvented this complication by generating Tlr4fl/fl mice in which deletion of Tlr4 signaling can be achieved in specific cell types without irradiation. With regard to acute immune responses to inhaled LPS, Tlr4fl/flVav1-Cre mice displayed significant reductions in airway neutrophilia (and the neutrophil chemokines CXCL1, CXCL2, and CXCL5), indicating that Tlr4 expression by hematopoietic cells is important for airway neutrophilic immune responses. However, neutrophilic inflammation was not completely absent in Tlr4fl/flVav1-Cre mice, and Tlr4fl/flShh-Cre mice lacking functional Tlr4 in airway AECs had modest reductions in neutrophilia and CXCL1. Thus, in these experiments, Tlr4 expression by hematopoietic cells was primarily responsible for neutrophil recruitment to airways in both genetic and radiation chimera models of hematopoietic cell deletion of TLR4 expression. This conclusion is concordant with a previous study of bone marrow chimeric mice by Hollingsworth et al., but in opposition to the results of a bone marrow chimera study by Hammad et al. that reported airway structural cells were necessary and sufficient for airway neutrophilia. The reasons underlying the latter, discrepant, results remain unclear.

A novel finding of our study is that in mouse models of allergic asthma, Tlr4 expression by hematopoietic cells and AECs drives distinct arms of the airway immune responses.
Specifically, whereas neutrophil recruitment following allergen sensitization and challenge was primarily dependent on hematopoietic cell expression of Tlr4, eosinophilic inflammation was chiefly dependent on Tlr4 expression by AECs. Moreover, this dichotomy was observed in two different models of allergic asthma, suggesting that TLR4 signaling in these two cell types leads to fundamentally distinct outcomes. The deficit in neutrophil recruitment after HDM sensitization and challenge than does the protocol employed in our study a weaker Th17-mediated neutrophilic response to OVA. IL-17F induce neutrophil recruitment to the airways by together with these previous observations, our current findings suggest that chemokine production by AECs is at least partially dependent on TLR4 responsiveness in hematopoietic cells. The concept of cross-talk between AECs and hematopoietic cells has been proposed previously, but there is only limited understanding of the specific pathways that might be involved. The conditionally targeted Tlr4 mouse model of asthma.18 A similar effect was not seen in previous work with bone marrow chimeric mice in the HDM-driven airway eosinophilia. This finding is consistent with previous results suggesting that the absence of Tlr4 with bone marrow chimeric mice to reduced MHCII expression by hematopoietic cells does not significantly affect HDMediated eosinophilic response. Our present study also confirms previous results suggesting that the absence of Tlr4 expression in hematopoietic cells does not significantly affect HDMedriven airway eosinophilia.17

A previous study attributed decreased eosinophilia in WT → Tlr4 bone marrow chimeric mice to reduced MHCII+ CD11b+ dendritic cell activation and reduced granulocyte colony-stimulating factor, thymic stromal lymphopoietin, IL-25, and IL-33 production after HDM sensitization. Here, we also found that selective deletion of functional Tlr4 from AECs led to reduced IL-25 and IL-33 in the BAL fluid 24 h after a single HDM instillation. Thus, reductions in these cytokines might have contributed to decreased eosinophilia seen after

Figure 8 Allergic responses to ovalbumin/lipopolysaccharide (OVA/LPS) sensitization and challenge in bone marrow chimeric mice. (a) Airway inflammation and (b, c) cytokine production in the OVA/LPS model of asthma. Cytokines are from cultures of whole lungs restimulated ex vivo for 5 days with OVA. IL, interleukin; TLR4, Toll-like receptor 4; WT, wild type. Data shown represent mean ± s.e.m., and are from one experiment representative of three experiments. N = 2–4 mice/per group. *P < 0.05; **P < 0.01; ***P < 0.001.
allergen challenge because they are reported to promote development of Th2-type immune responses that drive eosinophilia in the lung.39,40 However, the amounts of IL-4, IL-13, and IL-5 were comparable in lungs of HDM-challenged Tlr4fl/fl Shh-Cre mice and their nontransgenic Tlr4fl/fl littermates, suggesting that Th2 cell development might not be affected by the lack of Tlr4 expression in AECs. Unlike in Tlr4fl/fl/Shh-Cre mice, IL-5 was significantly reduced in lungs of HDM-sensitized and -challenged WT → Tlr4fl/fl (bone marrow chimeric mice compared with WT→WT control animals, whereas IL-4 and IL-13 were not significantly affected. This suggests that Tlr4-expressing radioresistant cells in the lungs—other than AECs—regulate IL-5 production. These data extend previous studies by showing that TLR4 expression in AECs is necessary for allergen-driven lung eosinophilia, although the molecular mechanisms that drive this response in the present model remain unclear.

Our novel finding that Tlr4 expression in hematopoietic cells and AECs direct distinct arms of the airway immune response has important implications for the therapeutic treatment of asthma. Global blockade of TLR4 has been reported to promote Th17 cells, in turn promoting neutrophilic cytokines such as IL-1 and IL-6 that promote the development of Th17 cells, which are resistant to glucocorticoid therapy.4–6 We have not yet identified the specific hematopoietic cell type(s) expressing Tlr4 that are necessary for the induction of neutrophilic inflammation. Dendritic cells represent a compelling hypothesis in this regard—responding to LPS by increasing costimulatory molecule expression and the production of cytokines such as IL-1 and IL-6 that promote the development of allergen-specific Th17 cells, in turn promoting neutrophilic asthma. However, macrophages, innate lymphoid, and/or γδ T cells might also play a role. Defining roles for Tlr4 expression in each of these cell types awaits further generation of appropriate cell-specific Cre TLR4-floxed crosses. Identification of the specific hematopoietic cell type(s) requiring Tlr4 expression for the induction of neutrophilic asthma will be critical for a deeper understanding of asthma and for designing novel strategies for therapeutic intervention.

METHODS
Mice. Animals were housed under specific pathogen-free conditions at the National Institute of Environmental Health Science or Cincinnati Children’s Research Foundation and used between 5 and 12 weeks of age. All animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee of the respective institutions.

Mice expressing Cre recombinase in the Shh locus (B6.Cg- Shh-tm1(EGFP/cre)Cjt/J) or from the Vav1 promoter B6.Cg-Tg(Vav1-Cre) A2Kio/J, as well as C57BL/6J, Tlr4−/− (B6.B10ScN-Tlr4−/−/C0), B6(Cg-Tyrγr2−/−/J), and B6.Cg-Tg(ActFltlp)9205Dym/J mice, were purchased from Jackson Laboratories (Bar Harbor, ME). The Cre-expressing mice were bred to conditionally mutant, Tlr4fl/fl mice generated in collaboration with the Gene Targeted Mouse Service Core at the University of Cincinnati and are available at Jackson Laboratories (strain #024872). The Tlr4 targeting vector included two LoxP sites flanking exon 3, an FRT-flanked neomycin resistance gene (Neo) for positive selection, a thymidine kinase gene for negative selection, and 1.9 and 3.4 kb arms of genome-derived DNA homologous with the Tlr4 locus. The targeting vector was integrated into the C57BL/6 mouse embryonic stem cells, and drug-resistant clones were screened by a PCR assay specific for the targeted Tlr4 gene. Two cell lines whose correctly targeted Tlr4 locus had been confirmed by Southern blot analysis were used to generate chimeras by injection into blastocysts from albino C57BL/6 (B6(Cg)- Tyrγr2−/J) mice. Two such chimeras were chosen to breed with B6(Cg)- Tyrγr2−/J mice, and embryonic stem cell-derived offspring were identified by their black coat color. Black mice bearing the targeted Tlr4 gene were then bred to FlpE recombinase-encoding B6.Cg-Tg(ActFltlp)9205Dym/J mice to delete neo from the Tlr4 locus. Offspring with the targeted Tlr4 locus, but lacking neo, were crossed to B6.Cg-Tg(Vav1-cre)A2Kio/J and B6.Cg-Shh-tm1(EGFP/cre)Cjt/J mice and the offspring interbred to obtain homozygous Tlr4fl/fl mice with the desired Cre-expressing transgene.

Reciprocal bone marrow chimeric animals were generated using WT C57BL/6J and Tlr4−/− donors and recipient mice. Recipient animals were irradiated at 6–8 weeks of age with 900 rad over 12 min and injected 1 day later with 107 donor bone marrow cells. Mice were given acidified water, supplemented with 500 μg/ml−1 neomycin for 2 weeks after irradiation. The animals were used in experiments after allowing for a minimum of 10 weeks for hematopoietic reconstitution, as confirmed by flow cytometry (data not shown).

Models of airway disease. Mice were anesthetized with inhaled isoflurane and all reagents were administered via intratracheal aspiration.41 To measure innate immune responses, mice were administered a single dose of 100 μg of Dermatophagoides pteronyssinus extracts (Greer Laboratories, Lenoir, NC) or 1 μg of TLR4-specific ultrapure LPS (Invivogen, San Diego, CA) in a total volume of 40 μl, and BAL was performed at 2, 4, 6, 8, or 24 h after challenge. For the model of HDM-mediated asthma, mice were given 100 μg Derma- topagoides pteronyssinus extracts on days 0, 7, and 14, and BAL performed on day 17. For the OVA/LPS model of asthma, mice were given 100 μg LPS-depleted OVA (BioVendor, Candler, NC) together with 100 ng LPS from Escherichia coli (Sigma, St Louis, MO) on days 0, 7, and 14 and BAL was performed on day 16. Cell differential analysis was carried out as described previously.41 Lungs were cultured for 24 h in RPMI (10% fetal bovine serum, 0.1% 2-mercaptoethanol, 1,000 U ml−1 penicillin/streptomycin, 10–30 μg/ml−1 HDF, or 10 μg/ml−1 OVA) and cytokines in lung culture supernatants and BAL fluid (BALF) were quantified using Luminex or enzyme-linked immunosorbent assay (ELISA).

In vivo cytokine capture assay. Systemic cytokines were quantified using the in vivo cytokine capture assay as previously described.42 All antibodies and standards were from eBioscience (San Diego, CA).

AEC cultures. AECs were isolated and cultured as previously described.43 Briefly, 5-week-old tracheas were excised and disaggregated by 0.1% pronase (Roche, Indianapolis, IN) and DNase I digestion, followed by fibroblast removal by plastic adherence. Mouse tracheal epithelial cells (MTECs) were submergence cultured on type I collagen-coated, 0.4 μm pore transwell inserts (Corning, Tewksbury, MA) until formation of tight junctions (R ≥ 1,000 Ω; 7–14 days). An air–liquid interface was established and maintained with daily basolateral media changes for 7 days before the AECs were stimulated apically with 10–100 ng ml−1 TLR4-specific ultrapure E. coli K12 LPS, 100 ng ml−1 Pam3CSK4 (Invivogen), 30 mg ml−1 HDL (Greer Laboratories), or vehicle control. Basolateral chemokine

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and cytokine production was quantified by commercially available Luminex or ELISA assays.

**Bone marrow-derived dendritic cell culture.** Dendritic cells were generated from bone marrow using standard protocols as previously described.44 Cells were stimulated with LPS or Pam3Cys at the indicated concentrations for 24 h and cytokine levels in supernatants were quantified by commercially available ELISA.

**Cytokines.** Cytokine concentrations in samples were quantified by ELISA using commercially available ELISA kits from R&D Systems (Minneapolis, MN) and BD Biosciences (San Jose, CA) or using Milliplex Multiplex kits (Millipore, Billerica, MA) according to the manufacturer’s protocol and read using luminex technology on the Bio-Plex (Bio-Rad, Hercules, CA). Concentrations were calculated from standard curves using recombinant proteins and expressed in pg/ml⁻¹.

**Gene expression analysis by quantitative PCR.** RNA was extracted using TRizol Reagent (Invitrogen, Waltham, MA) according to the manufacturer’s instructions. Complementary DNA was generated as previously described and subjected to quantitative PCR analysis using Light Cycler 480 II (Roche). Sybr Green I Master mix (Roche) and the following primers pairs were used: TLR4: 5'-CATTACAGG AAGGCTTCCA-A-3' and 5'-GGGATACACCTCCACCTG-3'; β-actin: 5'-GCACCAGCAAGAGGTA-3' and 5'-GGTGGG CTTAGGGTTCCAGG-3'.

**Genomic DNA analysis.** DNA was isolated from cells and tissues using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions and analyzed by PCR using the following primers: TLR4: 5'-CATTACAGGAAGGCTTCCA-A-3' and 5'-GG CGATACATCTCCACCTG-3'; β-actin: 5'-GGTCAGCAGGAGGCG AATGAT-3' and 5'-TCCCTTACGTTGAGGGTG-3'. Products were run on a 2% agarose gel and imaged.

**Statistics.** Data were analyzed by one-way analysis of variance, followed by Tukey’s correction for multiple comparisons, or by unpaired Student’s t-test, as appropriate.

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**DISCLOSURE**
The authors declared no conflict of interest.

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