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

Jaclyn W. McAlees[1], **Gregory S. Whitehead**[4], **Isaac T.W. Harley**[1], **Monica Cappelletti**[1], **Cheryl L. Rewerts**[1], **A. Maria Holdcroft**[1], **Senad Divanovic**[1], **Marsha Wills-Karp**[1,*], **Fred D. Finkelman**[1,2,3], **Christopher L. Karp**[1,*], and **Donald N. Cook**[4]

[1]Division of Immunobiology, Cincinnati Children's Hospital Research Foundation, Cincinnati, OH

[2]Department of Medicine, Cincinnati Veterans Affairs Medical Center, Cincinnati, OH

[3]Division of Allergy, Immunology and Rheumatology, Department of Internal Medicine, University of Cincinnati School of Medicine, Cincinnati, OH

[4]Laboratory of Respiratory Biology, National Institutes of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services, Research Triangle Park, NC

Abstract

Allergic asthma is a chronic, inflammatory lung disease. Some forms of allergic asthma are characterized by Th2-driven eosinophilia while 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 *Tlr4*^fl/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.

Corresponding Author: D.N.C., Laboratory of Respiratory Biology, National Institutes of Environmental Health Sciences National Institutes of Health, Department of Health and Human Services, 111 T.W. Alexander Dr. Building 101, E244, Research Triangle Park, NC 27709; Tel: (919) 541-4035. cookd@niehs.nih.gov.
*Current affiliations: Bill & Melinda Gates Foundation (C.L.K.), Johns Hopkins Bloomberg School of Public Health (M.W.-K.)

Disclosure: None to report

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 (Th)2 responses that result in eosinophilic inflammation, but, in many individuals, neutrophils are the predominant leukocytes in the airway (1). Several studies have suggested that neutrophilic forms of asthma arise from allergen-specific Th17 responses (2, 3), and are characterized by airway hyper-responsiveness and resistance to glucocorticoid therapy (4–6). These findings have been replicated in mouse models of experimental allergic asthma (4, 7). 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 TLRs (8). The best-characterized TLR in this regard is TLR4, which signals in response to LPS, a membrane component of Gram negative bacteria (9). However, the relationship between environmental LPS and asthma is complex. Whereas some epidemiologic studies have linked LPS exposure to an increased prevalence of asthma (10, 11), other studies have suggested that exposure to LPS decreases the risk of developing allergic asthma (12). 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 OVA, and lower doses promoting Th2 and Th17 responses (13, 14). 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 (15). 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 mice 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 (DCs) after irradiation (19). Furthermore, although AECs are widely regarded as radio-resistant, radiation induces profound transcriptional changes in these cells that have unknown effects on their function (20). Thus, while 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.

Materials and 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/6 J, *Tlr4*^{−/−} (B6.B10ScN-*Tlr4*[lps-del]/JthJ), B6(Cg)-*Tyr*[c-2J]/J, B6.Cg-Tg(ACTFLPe)9205Dym/J mice were purchased from Jackson Laboratories, Bar Harbor, ME. *Cre*-expressing mice were bred to conditionally mutant, *Tlr4*^{fl/fl} mice generated in collaboration with the Gene Targeted Mouse Service Core at the University of Cincinnati. The *Tlr4* targeting vector included two *Lox*P sites flanking exon 3, an FRT-flanked neomycin resistance gene (*Neo*) for positive selection, a thymidine kinase gene for negative selection, and 1.9 kb and 3.4 kb arms of genome-derived DNA homologous with the *Tlr4* locus. The targeting vector was introduced by electroporation into C57BL/6 mouse embryonic stem (ES) cells, and drug-resistant clones were screened by a PCR assay specific for the targeted *Tlr4* gene. Two cell lines whose correctly targeted *Tlr4*[fl] locus had been confirmed by Southern blot analysis were used to generate chimeras by injection into blastocysts from albino C57BL/6 (B6(Cg)-*Tyr*[c-2J]/J) mice. Two such chimeras were chosen to breed with B6(Cg)-*Tyr*[c-2J]/J mice, and ES 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(ACTFLPe)9205Dym/J mice to delete *neo* from the *Tlr4* locus. Offspring with the target *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 *Tlr4*^{fl/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 to 8 weeks of age with 900 rads over 12 minutes and injected 1 day later with 10^7 donor bone marrow cells. Mice were given acidified water, supplemented with 500 μg/ml neomycin for 2 weeks post-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 (21). To measure innate immune responses, mice were administered a single dose of 100 μg of *Dermatophagoides pteronyssinus* (Der p) 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 bronchoalveolar lavage (BAL) was performed at 2, 4, 6, 8 or 24 hours after challenge. For the model of HDM-mediated asthma, mice were given 100 μg Der p extracts on days 0, 7, 14, and BAL performed on day 17. For the OVA/LPS model of asthma, mice were given 100 μg LPS-depleted OVA (BioVendor Inc. Candler, NC)

together with 100 ng LPS from *E. 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 (21). Lungs were cultured for 24 hours in cRPMI (10% FBS, 0.1% 2-mercaptoethanol, 1000U/L Penicillin/Streptomycin, 10–30 μg/mL HDM or 10 μg/mL OVA), and cytokines in lung culture supernatants and BAL fluid (BALF) were quantified using Luminex or ELISA.

In vivo cytokine capture assay

Systemic cytokines were quantified using the In Vivo Cytokine Capture Assay (IVCCA) as previously described (22). All antibodies and standards were from eBioscience, San Diego, CA.

Airway epithelial cell cultures

Airway epithelial cells (AECs) were isolated and cultured as previously described (23). Briefly, 5 week-old tracheas were excised and disaggregated by 0.1% pronase (Roche) and DNase I (Sigma) digestion, followed by fibroblast removal by plastic adherence. MTECs were submersion cultured on type I collagen-coated, 0.4 μm pore transwell inserts (BD Pharmingen) until formation of tight junctions (R 1,000Ω; 7–14 days). An air-liquid interphase was established and maintained with daily basolateral media changes for 7 days before the AECs were stimulated apically with 10–100 ng/mL TLR4-specific ultrapure *E. coli* K12 LPS, 100 ng/mL Pam3CSK4 (Invivogen), 30 mg/mL HDM (Greer Laboratories) or vehicle control. Basolateral chemokine 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 (24). Cells were stimulated with LPS or Pam3Cys at the indicated concentrations for 24hrs and cytokine levels in supernatants were quantified by commercially available ELISA.

Cytokines

Cytokine concentrations in sample supernatants were quantified by enzyme-linked immunosorbent assay (ELISA) using commercially available ELISA kits from R&D Systems and BD Biosciences, 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(ng)/ml.

Gene expression analysis by qPCR

RNA was extracted using TRIzol Reagent (Invitrogen) according to manufacturer instructions. cDNA was generated as previously described (25) and subjected to qPCR analysis using Light Cycler 480 II (Roche). Sybr Green I Master mix (Roche) and the following primers pairs were used: TLR4: 5′-CATCCAGGAAGGCTTCCACA-3′ and 5′-

GGCGATACAATTCCACCTGC-3′; β-actin: 5′-GCCCCAGAGCAAGAGAGGTA-3′ and 5′-GGTTGGCCTTAGGTTTCAGG-3′.

Genomic DNA analysis

DNA was isolated from cells and tissues using the QIAamp DNA Mini Kit (Qiagen) according to manufacturer instructions and analyzed by PCR using the following primers: TLR4: 5′-CATCCAGGAAGGCTTCCACA-3′ and 5′-GGCGATACAATTCCACCTGC-3′; β-actin: 5′-CGCTCAGGAGGAGCAATGAT-3′ and 5′-TCCTTAGCTTGGTGAGGGTG-3′. Products were run on a 2% agarose gel and imaged.

Statistics

Data were analyzed by One-Way ANOVA, followed by Tukey's correction for multiple comparisons, or by unpaired Student's t-test, as appropriate.

Results

Generation of mice lacking *Tlr4* expression by airway epithelial or hematopoietic cells

We generated conditionally mutant *Tlr4* mice by flanking the third exon of *Tlr4* with *Lox*P sites (*Tlr4$^{fl/fl}$*) (Fig. 1a, b). This strategy was chosen because germline mutant mice lacking the third exon are unresponsive to LPS (9). To inactivate *Tlr4* in the airway epithelium, *Tlr4*$^{fl/fl}$ mice were bred to animals in which expression of *Cre recombinase* is controlled by the *Shh* locus (Shh-Cre mice) (26). *Shh* expression by the lung epithelium arises at E9.5 with full expression throughout the lung epithelium by E12.5 (27, 28). Thus, *Cre*-mediated genetic excisions are maintained in the progeny of these cells, which include all pulmonary epithelial cell lineages (29). Further, to inactivate *TLR4* in the hematopoietic compartment, *Tlr4*$^{fl/fl}$ mice were bred to transgenic animals expressing *Cre* under control of the HS21/45-vav1 oncogene promoter (Vav1-Cre mice) (30).

Selective *Tlr4* deletion in AECs of *Tlr4$^{fl/fl}$*Shh-Cre mice, and in hematopoietic cells of *Tlr4$^{fl/fl}$*Vav1-Cre mice, was confirmed by PCR analysis of genomic DNA (Fig. 1c) and by analysis of *Tlr4*-specific RNA prepared from AECs (Fig. 1d) and total splenocytes (Fig 1e). Inactivation of *Tlr4* was also confirmed by *in vitro* and *in vivo* functional studies. Bone marrow derived DCs (BMDCs) from *Tlr4$^{fl/fl}$*Shh-Cre mice and their Cre-negative *Tlr4$^{fl/fl}$* littermates produced comparable amounts of TNF-α after treatment with LPS, whereas BMDCs from *Tlr4$^{fl/fl}$*Vav1-Cre mice failed to produce this cytokine following LPS stimulation, although they responded strongly to the TLR2 ligand, Pam3Cys (Fig. 2a). Conversely, AECs from *Tlr4$^{fl/fl}$*Vav1-Cre and *Tlr4$^{fl/fl}$* mice produced comparable levels of the epithelium-derived chemokine, CCL20, in response LPS or HDM challenge, whereas AECs from *Tlr4$^{fl/fl}$*Shh-Cre mice had dramatically reduced responses to these stimuli (Fig. 2b). Lastly, *Tlr4$^{fl/fl}$*Shh-Cre mice, but not *Tlr4$^{fl/fl}$*Vav1-Cre, exhibited high levels of serum TNF-α after intraperitoneal injection of LPS mice (Fig. 2c). Taken together, these data indicate efficient functional deletion of TLR4 in AECs of *Tlr4$^{fl/fl}$*Shh-Cre mice and in hematopoietic cells of *Tlr4$^{fl/fl}$*Vav1-Cre mice.

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 ultra-pure LPS to *Tlr4$^{fl/fl}$*Shh-Cre mice and *Tlr4$^{fl/fl}$*Vav1-Cre mice by intratracheal aspiration. Two peaks of neutrophil accumulation were seen in the airways of *Tlr4$^{fl/fl}$* and *Tlr4$^{fl/fl}$*Shh-Cre mice; at 4 and 24 hours after LPS treatment (Fig. 3a). However, at both of these time points, *Tlr4$^{fl/fl}$*Vav1-Cre mice had significantly reduced numbers of these neutrophils, regardless of the amount of LPS used (Fig. 3b). Thus, in non-irradiated 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. *Tlr4$^{fl/fl}$*Shh- and Vav1-cre mice also had a trend towards reduced neutrophil recruitment to the lung following PBS treatment when compared to PBS-treated littermate controls, although these differences did not reach statistical significance. This trend might have been due to a role of *Tlr4* in neutrophilic responses to minor injury caused by repeated fluid installation into the lungs (31).

The markedly reduced neutrophil accumulation seen in *Tlr4$^{fl/fl}$*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 BAL fluid for CXCL1, CXCL2, and CXCL5. Each of these chemokines were significantly reduced in the airways of *Tlr4$^{fl/fl}$*Vav1-cre mice compared with *Tlr4$^{fl/fl}$* littermates, whereas concentrations of these chemokines in *Tlr4$^{fl/fl}$*Shh-cre mice were generally similar to their non-transgenic littermates (Fig. 3c). An exception was the reduction in CXCL1 seen in *Tlr4$^{fl/fl}$*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, *Tlr4$^{fl/fl}$*Vav1-cre mice also had significantly reduced amounts of IFN-γ, TNF-α and IL-6 in BAL fluid 24 hours after challenge (Fig. 3d), cytokines whose levels were unaffected in *Tlr4$^{fl/fl}$*Shh-cre mice. Collectively, these data demonstrate that hematopoietic cell expression of *Tlr4* is critical for LPS-driven recruitment of neutrophil the airway.

Tlr4 expression by hematopoietic cells regulates neutrophilic responses to HDM

HDM 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 cells in acute immune responses to HDM, we challenged the airways of mice with HDM and evaluated airway inflammation 24 hours later. As observed after LPS treatment, HDM-treated *Tlr4$^{fl/fl}$*Vav1-Cre mice had significantly fewer airway neutrophils than did their *Tlr4$^{fl/fl}$* littermate controls, whereas *Tlr4$^{fl/fl}$*Shh-Cre mice and control *Tlr4$^{fl/fl}$* littermates had similar numbers of neutrophils (Fig. 4a). To determine a possible cause for the decreased number of neutrophils recruited to the airways of *Tlr4$^{fl/fl}$*Vav1-cre mice, we quantified CXCL1 and CXCL2 in the BAL fluid. Both chemokines were reduced in *Tlr4$^{fl/fl}$*Vav1-cre mice when compared to non-transgenic littermates (Fig. 4b), whereas these chemokines were similar in *Tlr4$^{fl/fl}$*Shh-cre mice and non-transgenic *Tlr4$^{fl/fl}$* controls. Because IL-25 and IL-33 have been implicated in allergic sensitization through the airway

(32–37), we also quantified these cytokines in HDM-treated mice. *Tlr4$^{fl/fl}$*Shh-cre mice had reduced levels of IL-25 and IL-33 (Fig. 4c) in the BAL fluid, compared with *Tlr4$^{fl/fl}$* littermate controls, whereas *Tlr4$^{fl/fl}$*Vav1-cre mice and their *Tlr4$^{fl/fl}$* 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.

TLR4-expressing airway epithelial and hematopoietic cells differentially regulate eosinophilic and neutrophilic allergic airway inflammation

To determine the role of TLR4 expression by airway epithelial and hematopoietic cells in a clinically relevant mouse model of asthma, we sensitized *Tlr4$^{fl/fl}$*Shh-cre mice and *Tlr4$^{fl/fl}$*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 approximately 100 ηg LPS per 100 μg of HDM, which is sufficient to promote adaptive immune responses to bystander allergens and drive *Tlr4*-dependent eosinophilia and neutrophilia in several mouse strains (13, 14, 18, 38) and (Fig. 5a). No differences between either of the present strains and their *Tlr4$^{fl/fl}$* littermates were seen when total leukocytes in the airway were quantified (Fig. 5b, c). However, cell differential analysis of the leukocytes in BAL revealed marked differences among these strains. *Tlr4$^{fl/fl}$*Vav1-cre mice had significantly fewer neutrophils in their airways than did their *Tlr4$^{fl/fl}$* littermates, whereas *Tlr4$^{fl/fl}$*Shh-cre mice had similar numbers of neutrophils compared with their non-transgenic littermates. Conversely, eosinophil numbers were significantly reduced in *Tlr4$^{fl/fl}$*Shh-cre mice compared with their *Tlr4$^{fl/fl}$* littermates, whereas the numbers of these cells were similar in *Tlr4$^{fl/fl}$*Vav1-cre and *Tlr4$^{fl/fl}$* mice. Thus, *Tlr4* expression in specific cell compartments impacts 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 we found to be significantly reduced in the lungs of *Tlr4$^{fl/fl}$*Shh-Cre mice compared to *Tlr4$^{fl/fl}$* littermate controls (Fig. 6a). Lungs from *Tlr4$^{fl/fl}$*Vav1-Cre mice also had reduced amounts of IL-33 (Fig. 6b), and produced significantly less of the neutrophilia promoting cytokines, IL-17A and IL-17F.

To compare our study of *Tlr4$^{fl/fl}$* mice to previous studies using bone marrow chimeric mice, we used 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 (Fig. 7a). However, WT→ *Tlr4*−/− mice displayed very little eosinophilia, in agreement with our finding of reduced eosinophilia in *Tlr4$^{fl/fl}$*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 *Tlr4^fl/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 *Tlr4^fl/fl* model, *Tlr4* expression in hematopoietic cells is critical for production of IL-17A and IL-17F (Fig. 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 ovalbumin (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 (Fig. 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 due to variable effects of radiation on structural and hematopoietic cell function (20). In the present study, we circumvented this complication by generating *Tlr4^fl/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, *Tlr4^fl/fl*Vav1-cre mice displayed significant reductions in airway neutrophilia (and the neutrophil chemokines CXCL1 and CXCL2), indicating that *Tlr4* expression in by hematopoietic cells is important for airway neutrophilic immune responses. However, neutrophilic inflammation was not completely absent in *Tlr4^fl/fl*Vav1-cre mice, and *Tlr4^fl/fl*Shh-cre mice lacking functional *Tlr4* in airway AECs had only 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. (16), but in opposition to the results of a bone marrow chimera study by Hammad et al. (17) 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 was more pronounced in *Tlr4*−/−→WT bone marrow chimeras than in *Tlr4^fl/fl*Vav1-cre mice, possibly because structural cells other

than AECs are damaged by the radiation and therefore less responsive to LPS in the chimeric mice. The decreased airway neutrophilia observed in mice lacking TLR4 in hematopoietic cells was associated with decreased concentrations of IL-17A and IL-17F, cytokines that are known to promote neutrophil accumulation in the lungs (14, 39, 40). This finding is consistent with a previous report showing that with high doses of LPS (10 – 15 μg), *Tlr4* expression by hematopoietic cells is primarily responsible for the production of IL-17 in an OVA/LPS model of asthma (18). A similar effect was not seen when the lower dose of 100 ng LPS was used, possibly because the timing of the OVA/LPS sensitizations used in that study leads to a weaker Th17-mediated neutrophilic response to OVA challenge than does the protocol employed in our study (Whitehead *et al*, unpublished observations). IL-17A and IL-17F induce neutrophil recruitment to the airways by inducing chemokine production, primarily by AECs (41). Taken 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 (42, 43), but there is only limited understanding of the specific pathways that might be involved. The conditionally targeted *Tlr4fl/fl* mice provide a novel resource to study how functional deletion of *Tlr4* in one cell type can alter the function of other cell types.

Studies of *Tlr4fl/fl* mice and bone marrow chimeric mice support the previous findings that *Tlr4* expression by AECs strongly contributes to the eosinophilic component of allergen-driven airway eosinophilia. This finding is consistent with previous work with bone marrow chimeric mice in the HDM (17) and OVA/LPS (18) model of allergic asthma. However, the reduction in eosinophils was less marked in the *Tlr4fl/fl*Shh-cre mice than in the WT→*Tlr4−/−* chimeric mice, suggesting that structural cells other than AECs also contribute to the HDM-mediated eosinophilic response. Our present study also confirms previous results suggesting that the absence of *Tlr4* expression in hematopoietic cells does not significantly affect HDM-driven airway eosinophilia (17).

A previous study attributed decreased eosinophilia in WT→*Tlr4−/−* bone marrow chimeric mice to reduced MHCII+CD11b+DC activation and reduced G-CSF, TSLP, 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 hours after a single HDM instillation. Thus, reductions in these cytokines might have contributed to the decreased eosinophilia seen after allergen challenge because they are reported to promote development of Th2-type immune responses that drive eosinophilia in the lung (44, 45). 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 non-transgenic *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→*Tlr4−/−* bone marrow chimeric mice compared to WT→WT control animals, while IL-4 and IL-13 were not significantly affected. This suggests that *Tlr4*-expressing radio-resistant cells in the lung - 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 individuals with distinct forms of asthma. Global blockade of TLR4 signaling in the airway has shown promise for diminishing allergic sensitization (17), but this approach might impair the function of cells that are important for host defense against pathogens. Selectively targeting TLR4 signaling in specific cell types that promote either neutrophilic or eosinophilic forms of asthma might reduce the severity of this disease while minimizing effects on host defense. Such a targeted approach might be particularly important for neutrophilic forms of asthma that 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 co-stimulatory 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 α/β T cells might also play a role. Defining roles for *Tlr4* expression in each of these cell types awaits the 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.

Acknowledgments

References

1. McGrath KW, Icitovic N, Boushey HA, Lazarus SC, Sutherland ER, Chinchilli VM, et al. A large subgroup of mild-to-moderate asthma is persistently noneosinophilic. Am J Respir Crit Care Med. 2012; 185(6):612–9. [PubMed: 22268133]

2. Barczyk A, Pierzchala W, Sozanska E. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. Respiratory medicine. 2003; 97(6):726–33. [PubMed: 12814161]

3. Bullens DM, Truyen E, Coteur L, Dilissen E, Hellings PW, Dupont LJ, et al. IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? Respiratory research. 2006; 7:135. [PubMed: 17083726]

4. McKinley L, Alcorn JF, Peterson A, Dupont RB, Kapadia S, Logar A, et al. TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. J Immunol. 2008; 181(6):4089–97. [PubMed: 18768865]

5. Green RH, Brightling CE, Woltmann G, Parker D, Wardlaw AJ, Pavord ID. Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. Thorax. 2002; 57(10):875–9. [PubMed: 12324674]

6. Haldar P, Pavord ID. Noneosinophilic asthma: a distinct clinical and pathologic phenotype. J Allergy Clin Immunol. 2007; 119(5):1043–52. quiz 53–4. [PubMed: 17472810]

7. Lajoie S, Lewkowich IP, Suzuki Y, Clark JR, Sproles AA, Dienger K, et al. Complement-mediated regulation of the IL-17A axis is a central genetic determinant of the severity of experimental allergic asthma. Nat Immunol. 2010; 11(10):928–35. [PubMed: 20802484]

8. Karp CL. Guilt by intimate association: what makes an allergen an allergen? J Allergy Clin Immunol. 2010; 125(5):955–60. quiz 61–2. [PubMed: 20381850]

9. Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, et al. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J Immunol. 1999; 162(7):3749–52. [PubMed: 10201887]

10. Ross MA, Curtis L, Scheff PA, Hryhorczuk DO, Ramakrishnan V, Wadden RA, et al. Association of asthma symptoms and severity with indoor bioaerosols. Allergy. 2000; 55(8):705–11. [PubMed: 10955695]

11. Thorne PS, Kulhankova K, Yin M, Cohn R, Arbes SJ Jr, Zeldin DC. Endotoxin exposure is a risk factor for asthma: the national survey of endotoxin in United States housing. Am J Respir Crit Care Med. 2005; 172(11):1371–7. [PubMed: 16141442]

12. Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. N Engl J Med. 2002; 347(12):869–77. [PubMed: 12239255]

13. Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. J Exp Med. 2002; 196(12):1645–51. [PubMed: 12486107]

14. Wilson RH, Whitehead GS, Nakano H, Free ME, Kolls JK, Cook DN. Allergic sensitization through the airway primes Th17-dependent neutrophilia and airway hyperresponsiveness. Am J Respir Crit Care Med. 2009; 180(8):720–30. [PubMed: 19661246]

15. Trompette A, Divanovic S, Visintin A, Blanchard C, Hegde RS, Madan R, et al. Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. Nature. 2009; 457(7229):585–8. [PubMed: 19060881]

16. Hollingsworth JW, Chen BJ, Brass DM, Berman K, Gunn MD, Cook DN, et al. The critical role of hematopoietic cells in lipopolysaccharide-induced airway inflammation. Am J Respir Crit Care Med. 2005; 171(8):806–13. [PubMed: 15618460]

17. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. Nat Med. 2009; 15(4):410–6. [PubMed: 19330007]

18. Tan AM, Chen HC, Pochard P, Eisenbarth SC, Herrick CA, Bottomly HK. TLR4 signaling in stromal cells is critical for the initiation of allergic Th2 responses to inhaled antigen. J Immunol. 2010; 184(7):3535–44. [PubMed: 20194715]

19. Hahn I, Klaus A, Maus R, Christman JW, Welte T, Maus UA. Dendritic cell depletion and repopulation in the lung after irradiation and bone marrow transplantation in mice. Am J Respir Cell Mol Biol. 2011; 45(3):534–41. [PubMed: 21177980]

20. Citrin DE, Shankavaram U, Horton JA, Shield W 3rd, Zhao S, Asano H, et al. Role of type II pneumocyte senescence in radiation-induced lung fibrosis. Journal of the National Cancer Institute. 2013; 105(19):1474–84. [PubMed: 24052614]

21. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, et al. Interleukin-13: central mediator of allergic asthma. Science. 1998; 282(5397):2258–61. [PubMed: 9856949]

22. Finkelman, F.; Morris, S.; Orekhova, T.; Sehy, D. The in vivo cytokine capture assay for measurement of cytokine production in the mouse. In: Coligan, John E., et al., editors. Current protocols in immunology. Vol. Chapter 6. 2003. p. 28

23. Lewkowich IP, Lajoie S, Stoffers SL, Suzuki Y, Richgels PK, Dienger K, et al. PD-L2 modulates asthma severity by directly decreasing dendritic cell IL-12 production. Mucosal immunology. 2013; 6(4):728–39. [PubMed: 23149662]

24. Divanovic S, Trompette A, Atabani SF, Madan R, Golenbock DT, Visintin A, et al. Negative regulation of Toll-like receptor 4 signaling by the Toll-like receptor homolog RP105. Nat Immunol. 2005; 6(6):571–8. [PubMed: 15852007]

25. Harley IT, Giles DA, Pfluger PT, Burgess SL, Walters S, Hembree J, et al. Differential colonization with segmented filamentous bacteria and Lactobacillus murinus do not drive

divergent development of diet-induced obesity in C57BL/6 mice. Molecular metabolism. 2013; 2(3):171–83. [PubMed: 24049732]

26. Harfe BD, Scherz PJ, Nissim S, Tian H, McMahon AP, Tabin CJ. Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. Cell. 2004; 118(4):517–28. [PubMed: 15315763]

27. Bitgood MJ, McMahon AP. Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. Developmental biology. 1995; 172(1):126–38. [PubMed: 7589793]

28. Harris KS, Zhang Z, McManus MT, Harfe BD, Sun X. Dicer function is essential for lung epithelium morphogenesis. Proc Natl Acad Sci U S A. 2006; 103(7):2208–13. [PubMed: 16452165]

29. Morrisey EE, Hogan BL. Preparing for the first breath: genetic and cellular mechanisms in lung development. Developmental cell. 2010; 18(1):8–23. [PubMed: 20152174]

30. de Boer J, Williams A, Skavdis G, Harker N, Coles M, Tolaini M, et al. Transgenic mice with hematopoietic and lymphoid specific expression of Cre. European journal of immunology. 2003; 33(2):314–25. [PubMed: 12548562]

31. Jiang D, Liang J, Fan J, Yu S, Chen S, Luo Y, et al. Regulation of lung injury and repair by Toll-like receptors and hyaluronan. Nat Med. 2005; 11(11):1173–9. [PubMed: 16244651]

32. Bartemes KR, Kita H. Dynamic role of epithelium-derived cytokines in asthma. Clinical immunology. 2012; 143(3):222–35. [PubMed: 22534317]

33. Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S, et al. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. Immunity. 2001; 15(6):985–95. [PubMed: 11754819]

34. Tamachi T, Maezawa Y, Ikeda K, Kagami S, Hatano M, Seto Y, et al. IL-25 enhances allergic airway inflammation by amplifying a TH2 cell-dependent pathway in mice. J Allergy Clin Immunol. 2006; 118(3):606–14. [PubMed: 16950278]

35. Ballantyne SJ, Barlow JL, Jolin HE, Nath P, Williams AS, Chung KF, et al. Blocking IL-25 prevents airway hyperresponsiveness in allergic asthma. J Allergy Clin Immunol. 2007; 120(6):1324–31. [PubMed: 17889290]

36. Kurowska-Stolarska M, Kewin P, Murphy G, Russo RC, Stolarski B, Garcia CC, et al. IL-33 induces antigen-specific IL-5+ T cells and promotes allergic-induced airway inflammation independent of IL-4. J Immunol. 2008; 181(7):4780–90. [PubMed: 18802081]

37. Ramaprakash H, Shibata T, Duffy KE, Ismailoglu UB, Bredernitz RM, Moreira AP, et al. Targeting ST2L potentiates CpG-mediated therapeutic effects in a chronic fungal asthma model. The American journal of pathology. 2011; 179(1):104–15. [PubMed: 21640974]

38. Whitehead GS, Thomas SY, Cook DN. Modulation of Distinct Asthmatic Phenotypes in Mice by Dose-Dependent Inhalation of Microbial Products. Environmental health perspectives. 2013

39. Fogli LK, Sundrud MS, Goel S, Bajwa S, Jensen K, Derudder E, et al. T cell-derived IL-17 mediates epithelial changes in the airway and drives pulmonary neutrophilia. J Immunol. 2013; 191(6):3100–11. [PubMed: 23966625]

40. Miyamoto M, Prause O, Sjostrand M, Laan M, Lotvall J, Linden A. Endogenous IL-17 as a mediator of neutrophil recruitment caused by endotoxin exposure in mouse airways. J Immunol. 2003; 170(9):4665–72. [PubMed: 12707345]

41. Liang SC, Long AJ, Bennett F, Whitters MJ, Karim R, Collins M, et al. An IL-17F/A heterodimer protein is produced by mouse Th17 cells and induces airway neutrophil recruitment. J Immunol. 2007; 179(11):7791–9. [PubMed: 18025225]

42. Upham JW, Stick SM. Interactions between airway epithelial cells and dendritic cells: implications for the regulation of airway inflammation. Current drug targets. 2006; 7(5):541–5. [PubMed: 16719765]

43. Lambrecht BN, Hammad H. The role of dendritic and epithelial cells as master regulators of allergic airway inflammation. Lancet. 2010; 376(9743):835–43. [PubMed: 20816550]

44. Guo L, Wei G, Zhu J, Liao W, Leonard WJ, Zhao K, et al. IL-1 family members and STAT activators induce cytokine production by Th2, Th17, and Th1 cells. Proc Natl Acad Sci U S A. 2009; 106(32):13463–8. [PubMed: 19666510]

45. Angkasekwinai P, Park H, Wang YH, Wang YH, Chang SH, Corry DB, et al. Interleukin 25 promotes the initiation of proallergic type 2 responses. J Exp Med. 2007; 204(7):1509–17. [PubMed: 17562814]
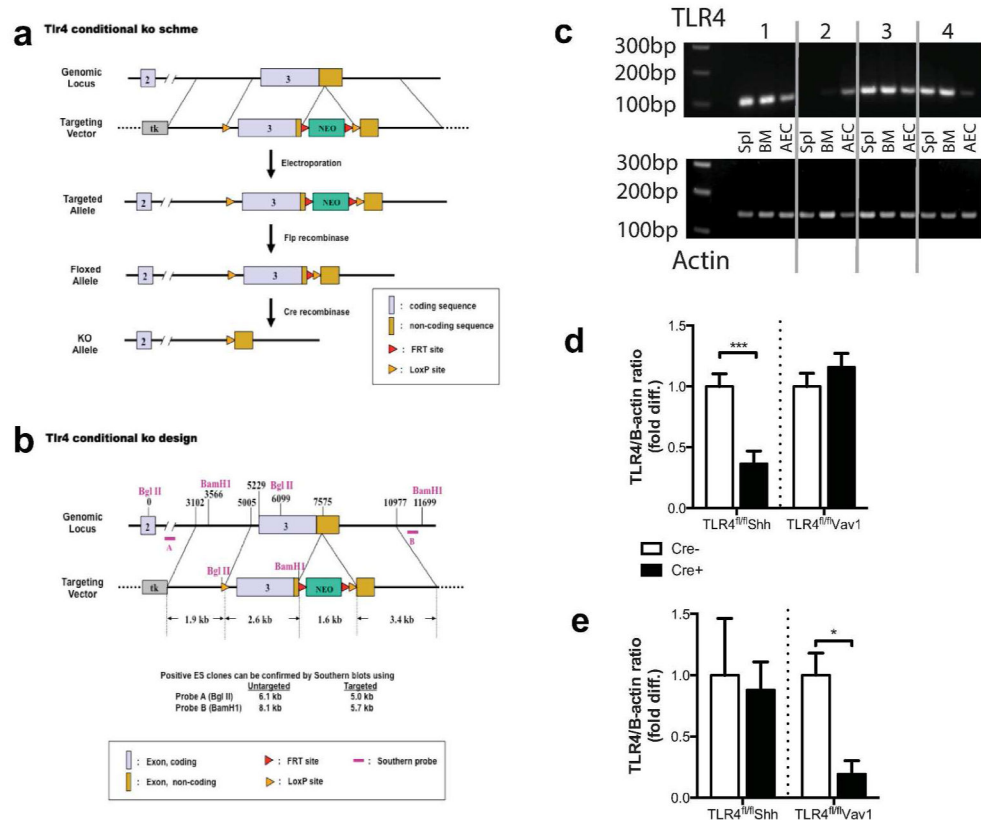
Figure 1. Generation of a *Tlr4*^{fl/fl} mouse

(**a**) Structure of the targeting vector and (**b**) strategy used to create *Tlr4*^{fl/fl} mice. (**c**) PCR-based assay confirming selective deletion of *Tlr4* from genomic DNA from the indicated cell types; total splenocytes, Spl; bone marrow, BM; and airway epithelial cells, AECs. Section 1, *Tlr4*^{fl/fl}; Section 2, *Tlr4*^{fl/fl}Vav1-Cre; Section 3, *Tlr4*^{fl/fl}; Section 4, *Tlr4*^{fl/fl}Shh-Cre. *Tlr4* mRNA levels in (**d**) AECs and (**e**) total splenocytes. Data shown represent mean values ±SEM, and are from one experiment, representative of 2–3. $N = 4$ to 6 mice per group. ***$P<0.001$.
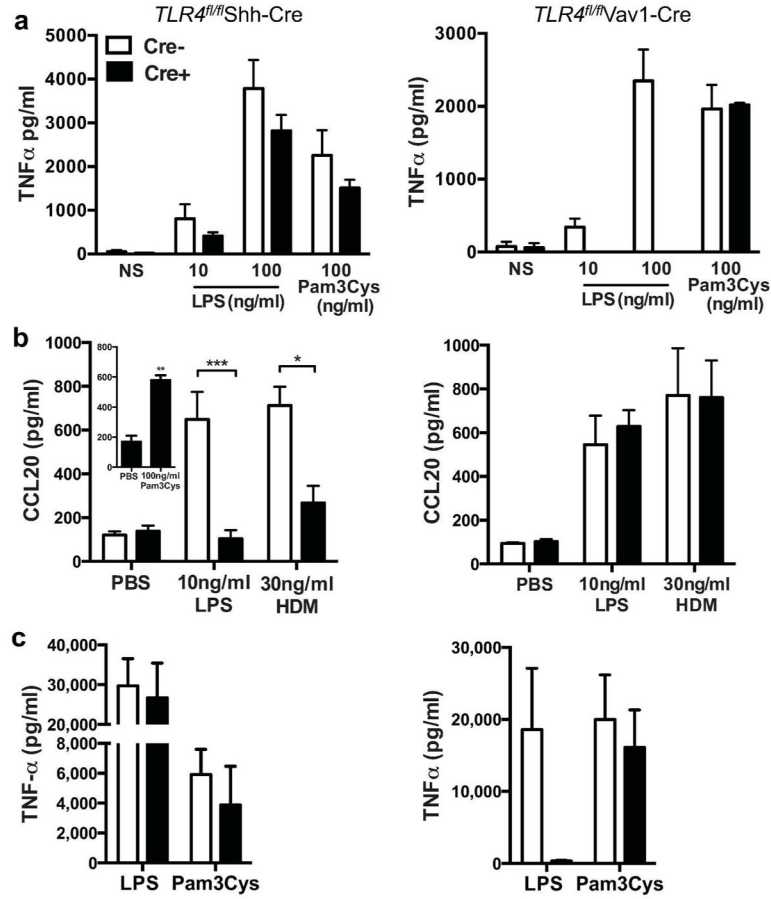
Figure 2. LPS-responsiveness of cells from *TLR4^{fl/fl}*Shh-Cre and *TLR4^{fl/fl}*Vav1-Cre mice
(**a**) TNFα production by LPS- and Pam3Cys-treated BMDCs (*n*=4–6 mice). (**b**) CCL20 production by LPS-, HDM- and Pam3Cys-treated AECs (*n*=6–10 mice/group). (**c**) Serum TNFα in mice 24 h after i.p. injection of LPS (*n*=6–8 mice/group). Data represent mean values ±SEM from one of 2–3 experiments yielding similar results. *P<0.05; **P<0.01; ***P<0.001.
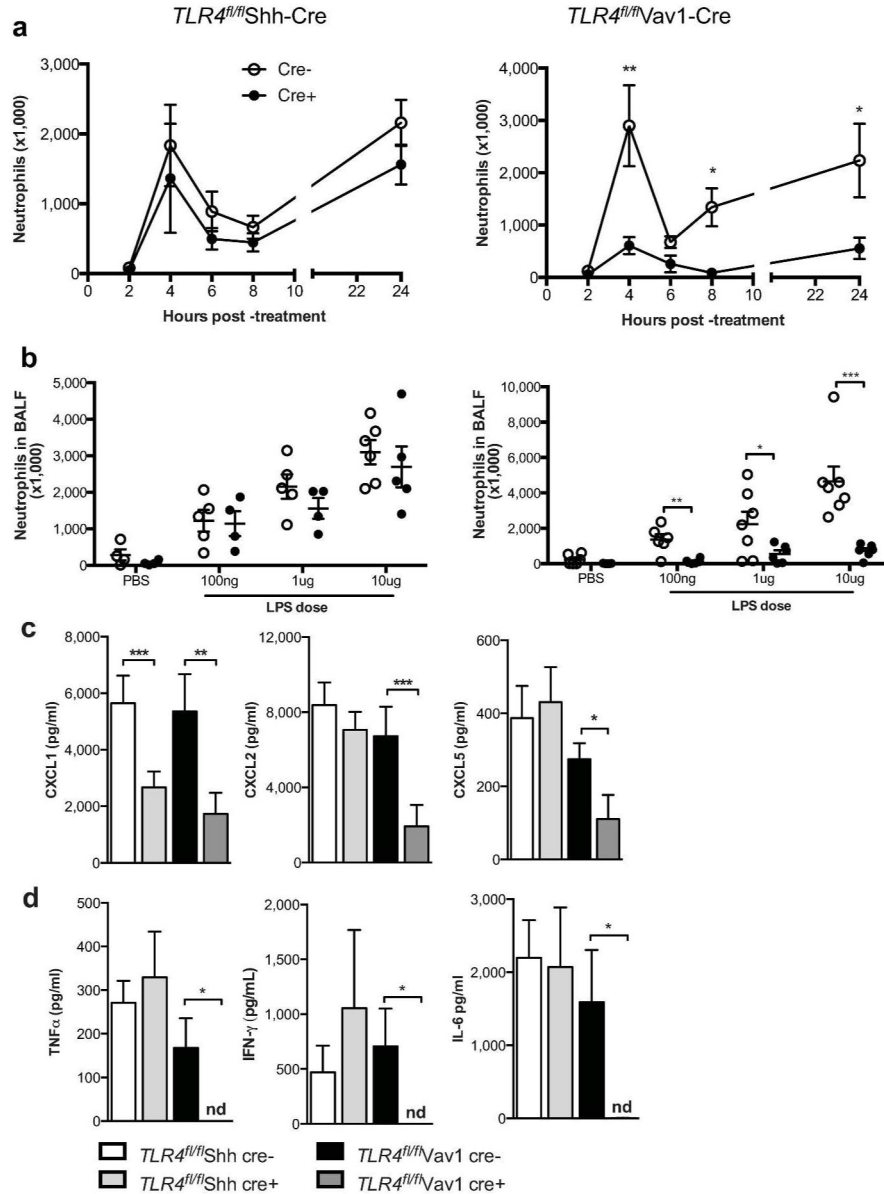
Figure 3. *Tlr4*-expressing hematopoietic cells promote LPS-mediated neutrophilic inflammation
(**a**) Time course of neutrophil recruitment following inhalation of 1 µg LPS. (**b**) LPS dose-response for neutrophils in mice harvested 24 h after treatment. Chemokine and cytokine levels in the BAL fluid at (**c**) 2 h and (**d**) 24 h after treatment with 1 µg LPS (n.d., not detectable). Data represent mean values ±SEM from one experiment, representative of three, with 4–12 mice per group. *$P<0.05$; **$P<0.01$; ***$P<0.001$.
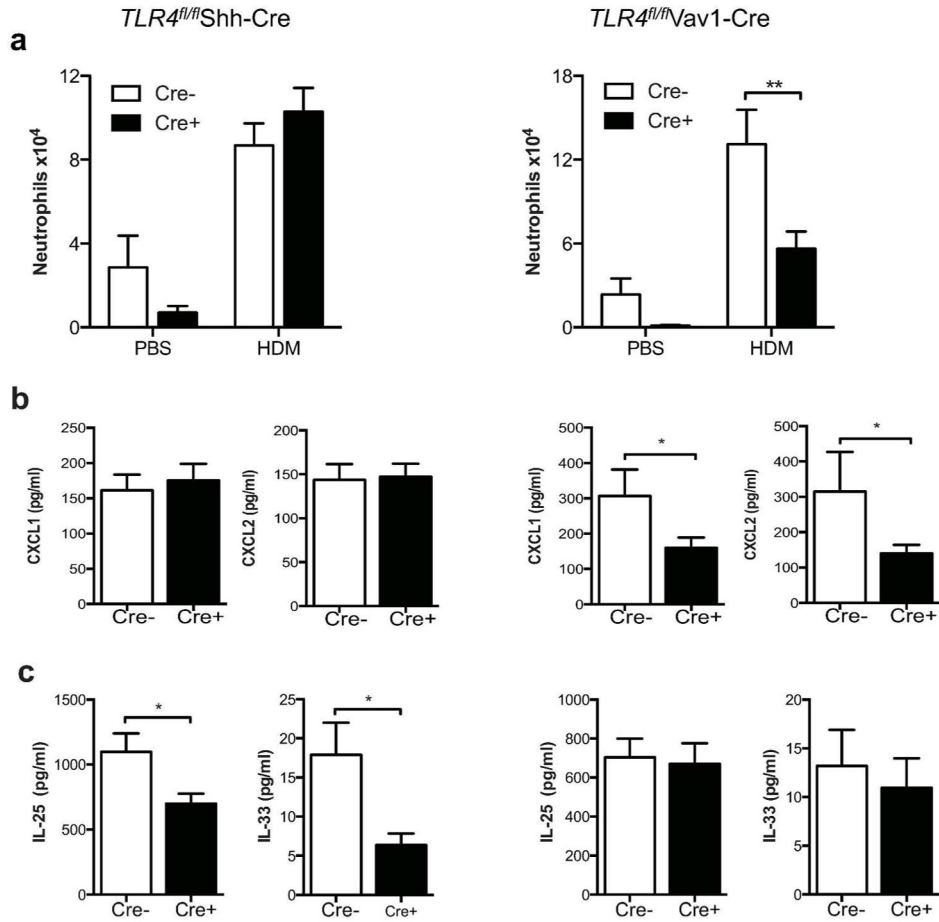
Figure 4. Role of *Tlr4*-expressing AECs and hematopoietic cells in innate immune responses to HDM

(**a**) Neutrophils in BAL fluid of mice 24 h after a single instillation of HDM. (**b**) CXCL1 and CXCL2 and (**c**) IL-25 and IL-33 levels in the BAL fluid. Data represent means ±SEM from one experiment, representative of two, with 6–8 mice per group. *$P<0.05$; **$P<0.01$.
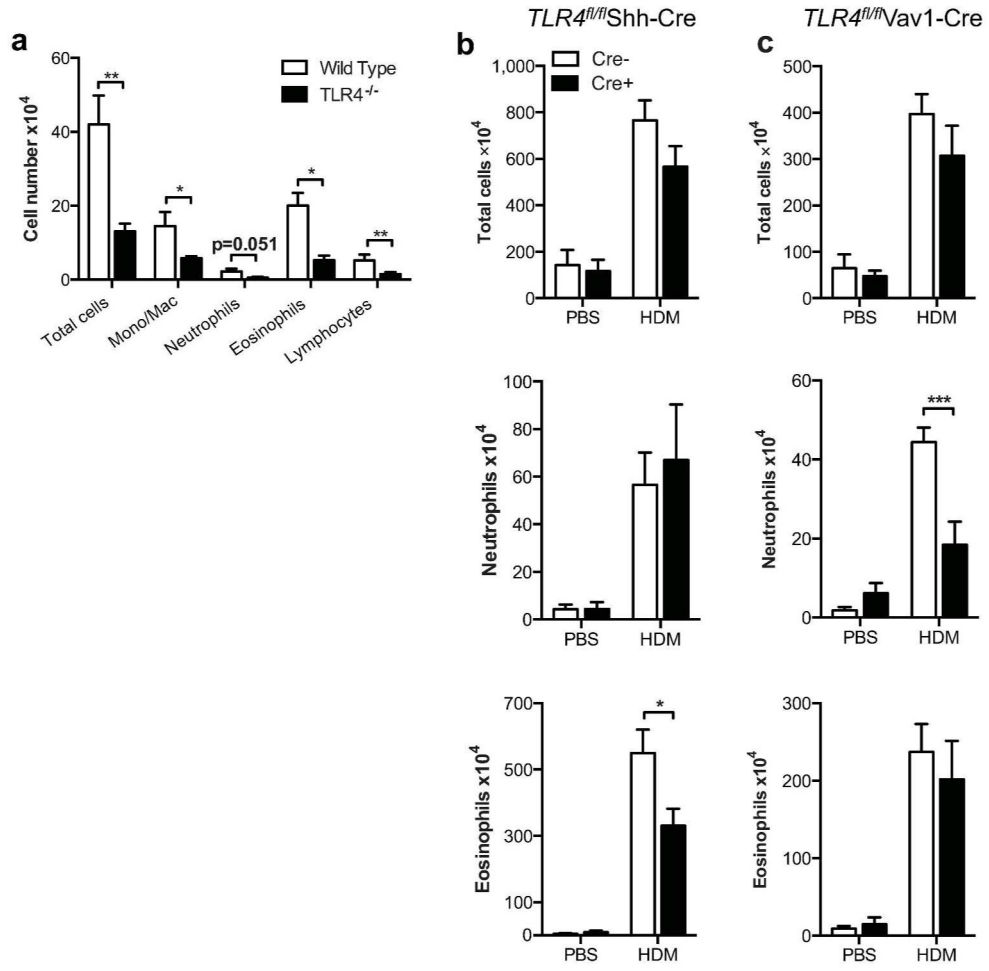
Figure 5. *Tlr4*-expressing AECs and hematopoietic cells control distinct aspects of HDM-driven allergic airway inflammation

Airway inflammation in the HDM model of asthma. (**a**) Mean cell numbers ±SEM are shown for the indicated cell types in BAL fluid from WT and *Tlr4*^{−/−} mice. Data are from one experiment, representative of two. (**b**) *Tlr4*^{fl/fl}Shh-cre and (**c**) *Tlr4*^{fl/fl}Vav1-cre mice sensitized and challenged with HDM extracts. Values for the indicated cell types represent mean cell numbers ±SEM from one experiment, representative of three. $N = 6$ –12 mice/ group. *$P<0.05$; **$P<0.01$; ***$P<0.001$.
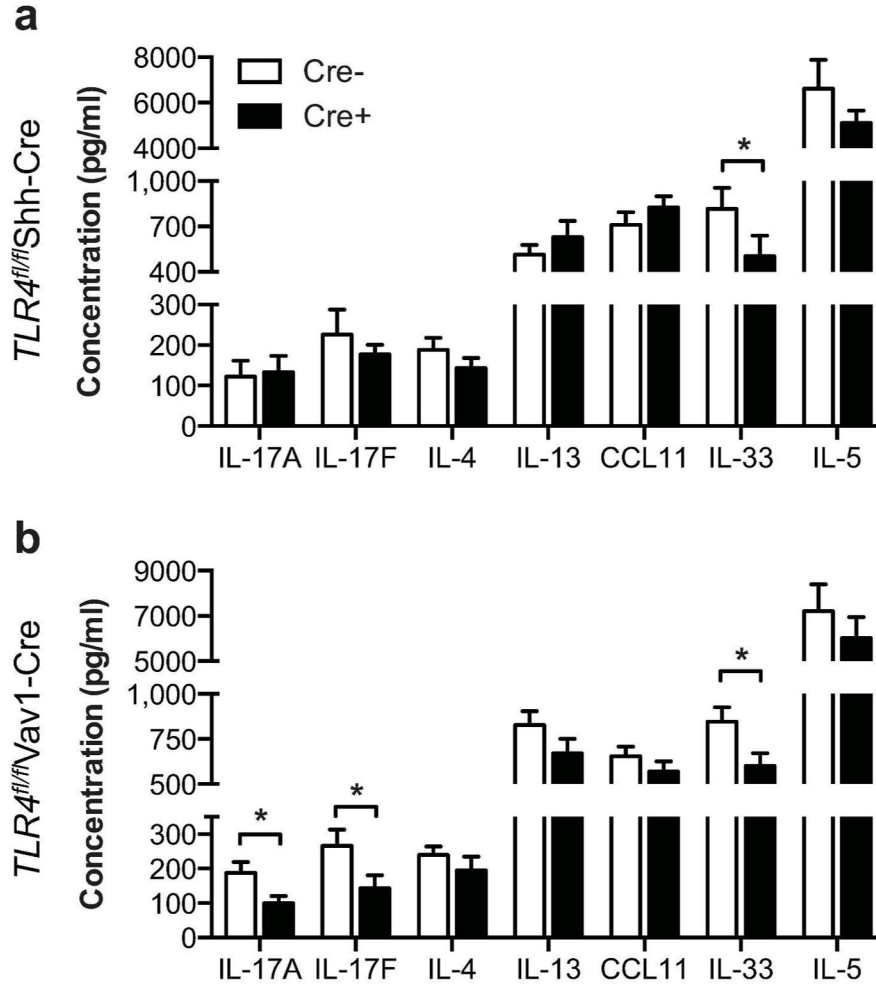
Figure 6. Effect of *Tlr4*-expressing cell types on cytokine and chemokine production in the lung
Cytokines and chemokines in the supernatants of whole lungs restimulated ex vivo for 5 days with HDM from (**a**) *Tlr4*$^{fl/fl}$Shh-cre and (**b**) *Tlr4*$^{fl/fl}$Vav1-cre mice. Data represent mean concentrations ±SEM from one experiment, representative of two. $N = 6$ to 12 mice/ group. $*P<0.05$.

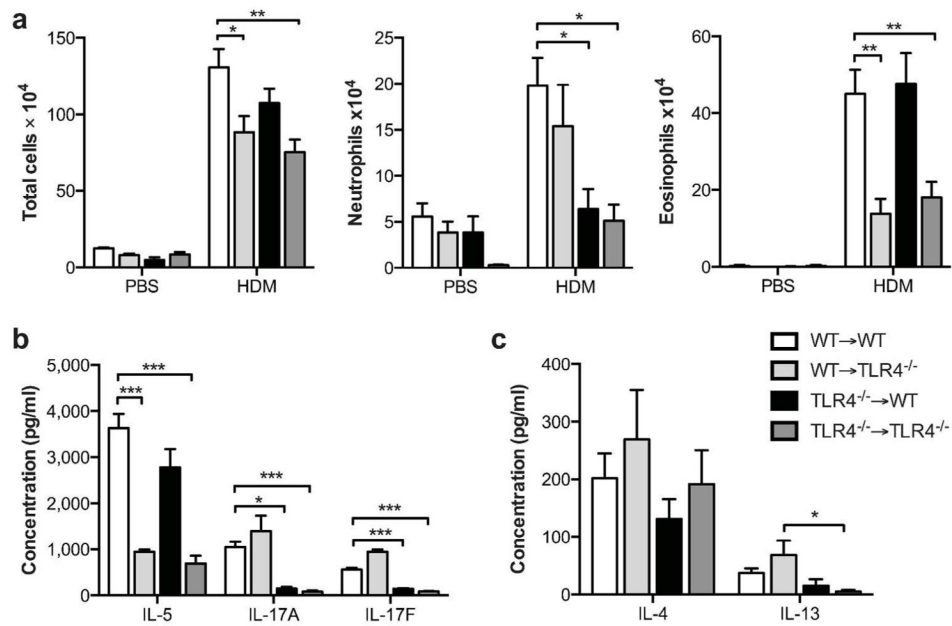**Figure 7. Allergic responses to HDM sensitization and challenge in bone marrow chimeric mice**
(**a**) Airway inflammation and (**b, c**) cytokine production in the HDM model of asthma. Cytokines are from cultures of whole lungs restimulated *ex vivo* for 5 days with HDM. Data represent mean values ±SEM from one experiment, representative of three. *P<0.05; **P<0.01; ***P<0.001.
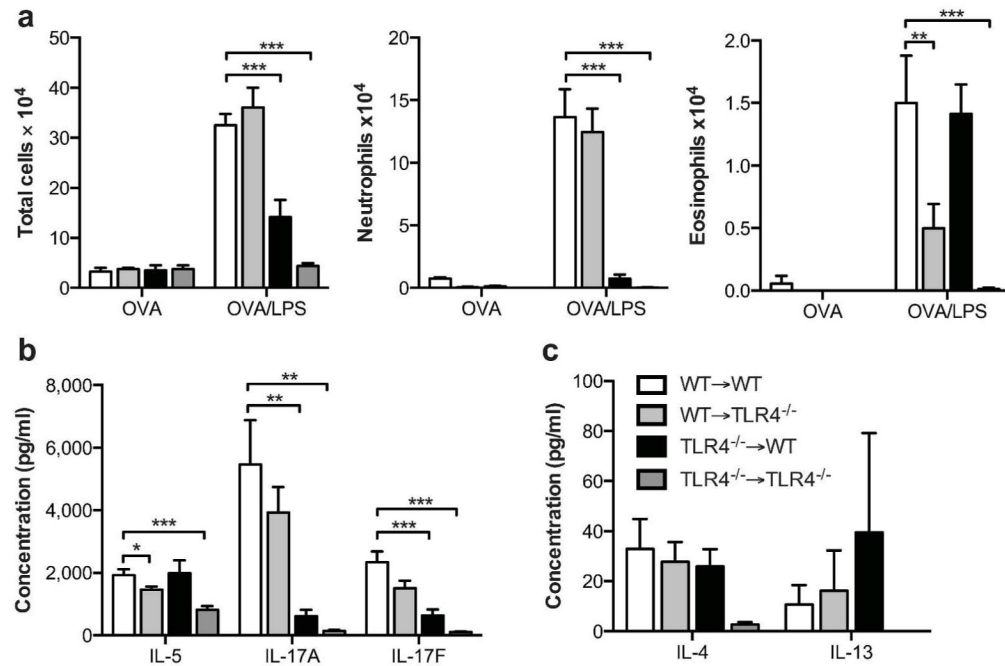
Figure 8. Allergic responses to OVA/LPS sensitization and challenge in bone marrow chimeric mice

(**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. Data shown represent mean values ±SEM, and are from one experiment, representative of three. $N = 2$ to 4 mice/per group. *$P<0.05$; **$P<0.01$; ***$P<0.001$