

The Toll-like receptor 5 ligand flagellin promotes asthma by priming allergic responses to indoor allergens

Rhonda H Wilson^{1,4}, Shuichiro Maruoka^{1,2,4}, Gregory S Whitehead¹, Julie F Foley¹, Gordon P Flake¹, Michelle L Sever¹, Darryl C Zeldin¹, Monica Kraft³, Stavros Garantziotis¹, Hideki Nakano¹ & Donald N Cook¹

Allergic asthma is a complex disease characterized by eosinophilic pulmonary inflammation, mucus production and reversible airway obstruction¹. Exposure to indoor allergens is a risk factor for asthma, but this disease is also associated with high household levels of total and particularly Gram-negative bacteria². The ability of bacterial products to act as adjuvants³ suggests they might promote asthma by priming allergic sensitization to inhaled allergens. In support of this idea, house dust extracts (HDEs) can activate antigen-presenting dendritic cells (DCs) *in vitro* and promote allergic sensitization to inhaled innocuous proteins *in vivo*⁴. It is unknown which microbial products provide most of the adjuvant activity in HDEs. A screen for adjuvant activity of microbial products revealed that the bacterial protein flagellin (FLA) stimulated strong allergic airway responses to an innocuous inhaled protein, ovalbumin (OVA). Moreover, Toll-like receptor 5 (TLR5), the mammalian receptor for FLA^{5,6}, was required for priming strong allergic responses to natural indoor allergens present in HDEs. In addition, individuals with asthma have higher serum levels of FLA-specific antibodies as compared to nonasthmatic individuals. Together, these findings suggest that household FLA promotes the development of allergic asthma by TLR5-dependent priming of allergic responses to indoor allergens.

To investigate the ability of microbial products to promote asthma-like responses in mice, we separately instilled individual products into the airway together with the innocuous protein, chicken ovalbumin (OVA). Control mice receiving either OVA alone or microbial products alone did not become sensitized and failed to develop airway inflammation after subsequent challenge with OVA (Fig. 1a–c and Supplementary Fig. 1a). However, instillation of some microbial products together with OVA caused the mice to become sensitized to this protein because after a subsequent OVA challenge, inflammatory leukocytes accumulated in the airway (Fig. 1c). Some mice displayed an allergic form of inflammation, as evidenced by large numbers of eosinophils in the airway (Fig. 1c). We observed strong allergic responses in mice receiving OVA together with standard FLA (sFLA) from *Salmonella typhimurium*, a partially purified product that contains lipopolysaccharide (LPS), or a highly purified recombinant

FLA (rFLA) produced in insect cells and lacking detectable LPS (see Online Methods for details). Ultrapure LPS, which signals through TLR4, was similar to rFLA in its ability to promote allergic responses (Supplementary Fig. 1b), suggesting that impurities in crude preparations of LPS can reduce its potency as an adjuvant.

The clinical features of asthma stem largely from the actions of CD4⁺ T helper type 2 (T_H2) cells that produce the cytokines interleukin-4 (IL-4), IL-5 and IL-13 and promote IgE synthesis, eosinophilic inflammation and mucus and airway hyperresponsiveness (AHR), respectively. IL-17 production by T_H17 cells also contributes to asthma severity by promoting neutrophil accumulation and AHR^{7–9}. We found that pulmonary IL-4, IL-5, IL-13 and IL-17 (Fig. 1d) and serum IgE (Fig. 1e) were all elevated in OVA-challenged mice previously sensitized with sFLA plus OVA or rFLA plus OVA. Mucus was present in the lungs of most mice that showed eosinophilic responses, but it was generally restricted to the large airways. However, mice sensitized with sFLA plus OVA also had mucus in preterminal and terminal bronchioles (Fig. 2a,b). Invasive measurements of airway resistance revealed that mice sensitized with either sFLA plus OVA (Fig. 2c) or rFLA plus OVA (Fig. 2d) developed strong AHR after a single OVA challenge, whereas mice sensitized to LPS plus OVA developed only modest AHR (Fig. 2c). Mice sensitized with OVA together with either sFLA or rFLA also displayed inflammation and AHR after OVA challenge on seven consecutive days (Supplementary Fig. 2a,b). T cells from lung-draining lymph nodes of these mice produced the Th2 cytokines IL-4, IL-5 and IL-13, as well as the Th17 cytokine IL-17, after restimulation with OVA *ex vivo* (Fig. 2e). Also, intracellular staining of CD4⁺ T cells from the lung revealed that they contain IL-13 and IL-17 (Fig. 2f and Supplementary Fig. 2c), confirming that inhaled FLA promotes T_H2 and T_H17 cell differentiation in adult mice.

Unlike highly purified rFLA, sFLA contains only 10% FLA and is contaminated with LPS (see Online Methods for details). However, *Tlr4*^{−/−} mice were readily sensitized to OVA when sFLA was used as an adjuvant, as evidenced by airway eosinophil accumulation in the airway following subsequent challenge with OVA (Fig. 3a). By contrast, *Tlr5*^{−/−} mice, which cannot respond to extracellular FLA⁶, had significantly lower levels of airway eosinophils and IL-5 than wild-type (WT) mice and showed a trend toward lower airway

¹Laboratory of Respiratory Biology, National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health, Research Triangle Park, North Carolina, USA. ²Division of Respiratory Medicine, Nihon University School of Medicine, Tokyo, Japan. ³Duke Asthma, Allergy and Airway Center, Duke University Medical Center, Durham, North Carolina, USA. ⁴These authors contributed equally to this work. Correspondence should be addressed to D.N.C. (cookd@niehs.nih.gov).

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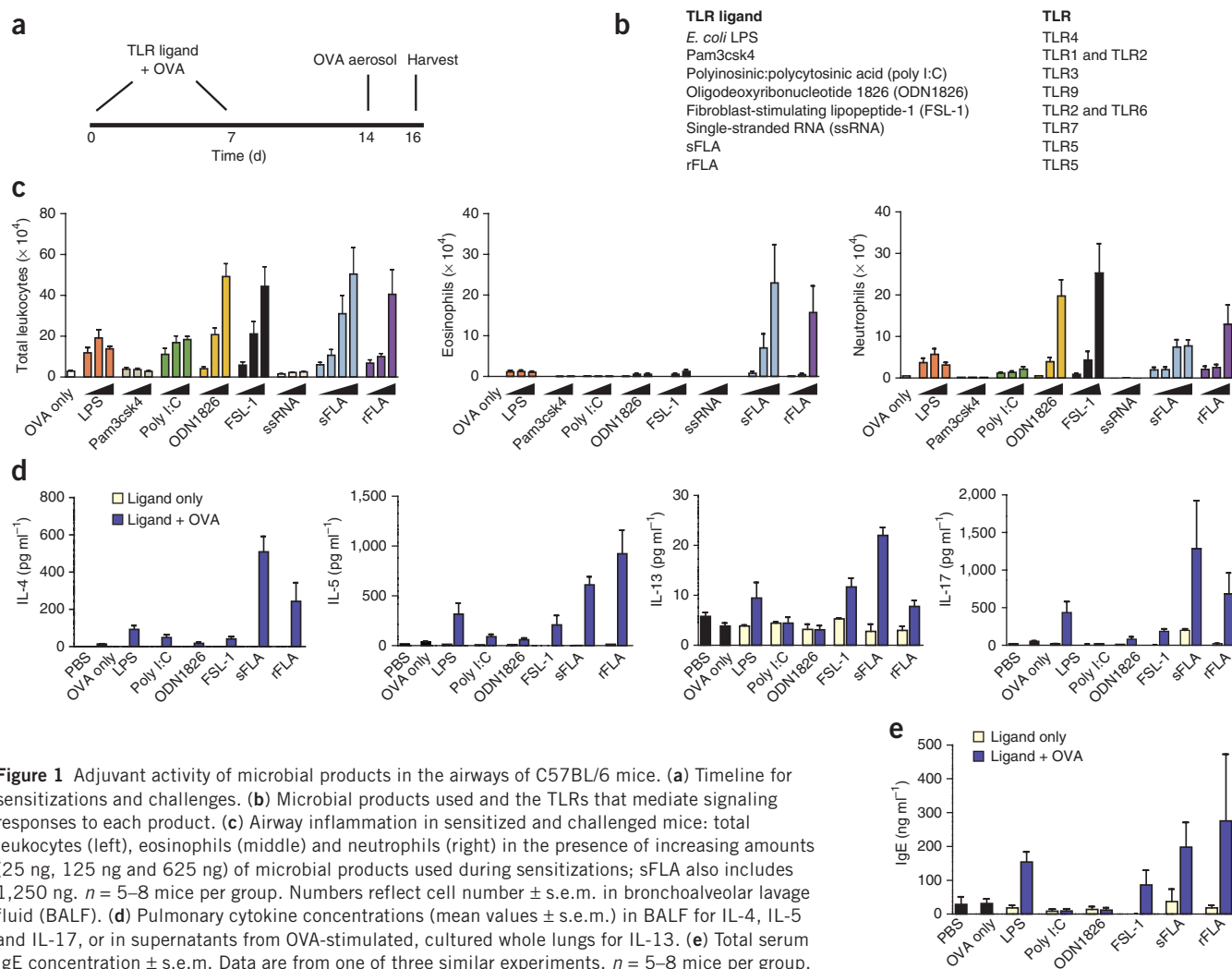


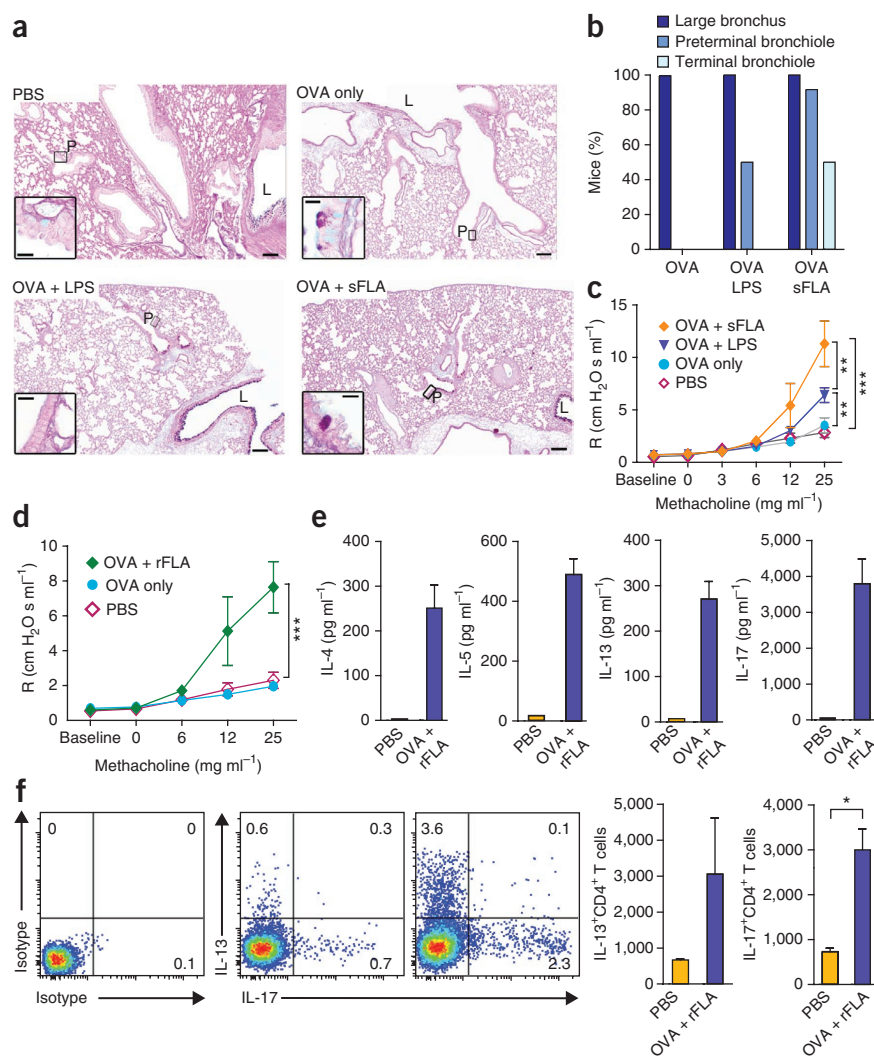
Figure 1 Adjuvant activity of microbial products in the airways of C57BL/6 mice. **(a)** Timeline for sensitizations and challenges. **(b)** Microbial products used and the TLRs that mediate signaling responses to each product. **(c)** Airway inflammation in sensitized and challenged mice: total leukocytes (left), eosinophils (middle) and neutrophils (right) in the presence of increasing amounts (25 ng, 125 ng and 625 ng) of microbial products used during sensitizations; sFLA also includes 1,250 ng. $n = 5-8$ mice per group. Numbers reflect cell number $\pm$ s.e.m. in bronchoalveolar lavage fluid (BALF). **(d)** Pulmonary cytokine concentrations (mean values $\pm$ s.e.m.) in BALF for IL-4, IL-5 and IL-17, or in supernatants from OVA-stimulated, cultured whole lungs for IL-13. **(e)** Total serum IgE concentration $\pm$ s.e.m. Data are from one of three similar experiments. $n = 5-8$ mice per group.

IL-17 and serum IgE (**Fig. 3a,b**). By contrast, numbers of airway neutrophils were lower in *Tlr4*^{-/-} mice than in WT mice, suggesting that LPS contributed to the TH17 response. The reduced allergic response of *Tlr5*^{-/-} mice sensitized with OVA plus sFLA was not due to a general immune deficit in these animals because, unlike *Tlr4*^{-/-} mice, the *Tlr5*^{-/-} mice were readily sensitized to OVA when we used LPS as an adjuvant (**Supplementary Fig. 3**). The inflammasome-associated molecule NLR family CARD domain-containing protein 4 (*Nlr4*) responds to intracellular FLA^{10,11}, but we found that *Nlr4*^{-/-} mice were efficiently sensitized by OVA-FLA and developed strong allergic responses to subsequent OVA challenge (**Supplementary Fig. 4**). By contrast, similarly treated mice lacking myeloid differentiation factor 88 (*Myd88*), an adapter protein used by all TLRs except TLR3, had virtually no eosinophils, neutrophils or inflammatory cytokines in the lung after sensitization with OVA plus rFLA and challenge with OVA (**Fig. 3a,b**). Together, these data suggest that although multiple microbial products contribute to the adjuvant activity of sFLA, the primary adjuvant component in this preparation is FLA. This is consistent with our finding that rFLA, which lacks detectable LPS, can prime allergic responses. In addition to monomeric FLA, airway instillations of heat-killed *Pseudomonas aeruginosa* (PA01), a ubiquitous flagellated strain of bacteria, also primed allergic responses to OVA, whereas a similar strain lacking

FLA (PA01ΔfliC) was significantly less effective at stimulating airway eosinophilia and IgE (**Fig. 3c**). Thus, multiple forms of FLA can prime allergic responses to inhaled OVA.

The two major DC populations in the lung (CD103⁺ and CD11b^{hi})¹² expressed low but detectable levels of *Tlr5* RNA (**Supplementary Fig. 5a**). Alveolar macrophages expressed intermediate levels, and airway epithelial cells expressed the highest levels of *Tlr5* mRNA. To investigate the requirement of different FLA-responsive cell compartments during allergic sensitization, we used WT and *Tlr5*^{-/-} mice to generate reciprocal bone marrow-chimeric mice (**Supplementary Fig. 5b**). WT mice receiving WT marrow showed the strongest eosinophilic responses to rFLA-OVA sensitization and OVA challenge. Compared to these animals, WT mice receiving *Tlr5*^{-/-} marrow, as well as *Tlr5*^{-/-} mice receiving WT marrow, had lower numbers of eosinophils, although this difference did not reach statistical significance (**Fig. 3d**). *Tlr5*^{-/-} mice receiving marrow from *Tlr5*^{-/-} mice had almost no eosinophilic inflammation (**Fig. 3d**). This suggests that *Tlr5* expression in radiosensitive hematopoietic cells and radio-resistant structural cells contributes to FLA-mediated allergic sensitization, in agreement with a previous report¹³. Mice in which *Myd88* expression is restricted to *Cd11c*-expressing cells¹⁴ did not become sensitized to OVA when we used rFLA as an adjuvant (**Fig. 3e**), although CD11c⁺ cells prepared from lungs of these mice were able to respond to FLA, as evidenced by production of tumor

Figure 2 FLA promotes asthma-like responses to OVA. **(a)** Periodic acid-Schiff and Alcian blue staining of mucus-producing cells in the airway. Representative low-magnification images are shown (scale bars, 50 μm). Insets show expanded images of the indicated regions (scale bars, 10 μm). L, large airway; P, preterminal bronchioles. **(b)** Percentage of mice with mucus staining in the indicated regions of the lung. $n = 8$ –10 mice per group. **(c,d)** Mean values $\pm$ s.e.m. of airway resistance (R) for intubated mice inhaling air (baseline) or aerosols of PBS containing the indicated concentrations of methacholine. $n \geq 8$ mice per group. **(e)** Cytokine concentrations (mean values $\pm$ s.e.m.) in cultures of total cell suspensions from lymph nodes excised from mice sensitized with PBS ($n = 1$) or OVA + rFLA ($n = 5$). Cells were stimulated *ex vivo* with OVA. **(f)** Intracellular staining for cytokines in pulmonary T cells. Shown are representative flow plots with percentages of positive cells indicated in each quadrant and bar histograms of mean $\pm$ s.e.m. numbers of CD4⁺ cells staining for IL-13 (left) and IL-17 (right). $n = 2$ for PBS group; $n = 3$ for OVA + rFLA-treated group. Data are from one of three similar experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.



necrosis factor- α (Supplementary Fig. 5c). Thus, FLA responsiveness in DCs and alveolar macrophages is not sufficient for FLA-mediated allergic sensitization through the airway. Previous reports have indicated that the airway epithelium contributes to innate immune responses to FLA^{15,16} and to LPS-mediated T_H2 responses by rapidly releasing proinflammatory cytokines¹⁷. We found that the amounts of IL-25 and IL-33 in the airway were strongly increased within 2 h of rFLA instillation, whereas amounts of thymic stromal lymphopoietin (TSLP) increased within 4 h of treatment compared to those in PBS-treated animals (Fig. 3f). We also observed increased amounts of TSLP in supernatants of rFLA-treated primary airway epithelial cells from WT mice, but not in their *Tlr5*^{-/-} counterparts (Fig. 3g). No increases were seen for IL-25 or IL-33 in these primary cell cultures (data not shown). Together, these results suggest that FLA might promote allergic sensitization to inhaled allergens by inducing secretion of proinflammatory cytokines by epithelial cells.

Having found that multiple forms of FLA can function as strong adjuvants in the airway, we next tested whether FLA is a naturally occurring, environmental adjuvant. Common house dust represents a readily available source of airborne material that is inhaled daily. Several different HDEs promoted allergic responses to inhaled OVA, although they differed in potency (Fig. 4a). Western blot analysis (Fig. 4b and Supplementary Fig. 6a) revealed immunoreactive proteins within the expected molecular weight range of FLA, from 30 kDa to 65 kDa¹⁸. The two major bands observed might represent FLA from different species of bacteria or different forms of FLA from a single species. To determine the individual contributions of LPS and FLA to the adjuvant activity of HDEs, we examined HDE-mediated allergic responses to OVA in *Tlr4*^{-/-} and *Tlr5*^{-/-} mice. After sensitization with HDE plus OVA, followed by OVA challenge, *Tlr4*^{-/-} mice had fewer airway neutrophils and showed a trend toward lower

concentrations of IL-17 in bronchoalveolar lavage fluid (BALF) compared with WT mice (Fig. 4c and Supplementary Fig. 6b). However, numbers of airway eosinophils and the concentration of IL-5 in BALF were not lower in *Tlr4*^{-/-} mice compared with WT mice (Fig. 4c and Supplementary Fig. 6b), indicating that, for this extract, LPS is dispensable for HDE-mediated eosinophilic airway inflammation but is required for neutrophilic airway inflammation and might contribute to AHR. By contrast, *Tlr5*^{-/-} mice had fewer airway eosinophils (Fig. 4c) and had lower concentrations of IL-5 than did WT or *Tlr4*^{-/-} mice (Supplementary Fig. 6b). WT and *Tlr4*^{-/-} mice sensitized with HDE plus OVA developed AHR after OVA challenge, whereas this response was markedly reduced in *Tlr5*^{-/-} mice (Fig. 4d). These findings suggest that FLA is a major component of HDE for priming asthma-like responses to inhaled antigens.

OVA is widely used in animal models of allergic pulmonary inflammation, but it is not a clinically relevant allergen for asthma. Household dust typically contains many allergens, including those derived from dust mites, cockroaches and animal dander. Multiple instillations of HDE alone (without OVA) is sufficient to trigger allergic responses in mice¹⁹. Each of the seven HDEs we tested contained several allergens, as well as various levels of LPS (Supplementary Table 1). To determine if LPS and FLA promote sensitization to these natural indoor allergens, we tested responses in *Tlr4*^{-/-} and *Tlr5*^{-/-} mice to

an HDE (#7) that contained multiple indoor allergens. In WT mice, sensitization and challenge with HDE alone elicited eosinophilic and neutrophilic airway inflammation (Fig. 4e). However, some of this neutrophilic inflammation was due to innate immune responses to LPS, because WT mice, but not *Tlr4*^{-/-} mice, displayed airway neutrophil infiltration after only a single HDE instillation (Fig. 4e, middle). By contrast, eosinophil accumulation in the airways of WT mice was dependent on adaptive immune responses because these

cells were present only in HDE-challenged mice that had been previously sensitized with HDE (Fig. 4e, right). Like WT mice, *Tlr4*^{-/-} mice had large numbers of eosinophils in the airway after HDE challenge, suggesting that LPS in this extract was dispensable for eosinophilic responses. However, *Tlr5*^{-/-} mice had significantly fewer eosinophils in the airway than did WT mice (Fig. 4e, right). These data suggest that interactions between FLA and TLR5 promote the eosinophilic component of allergic responses to indoor allergens.

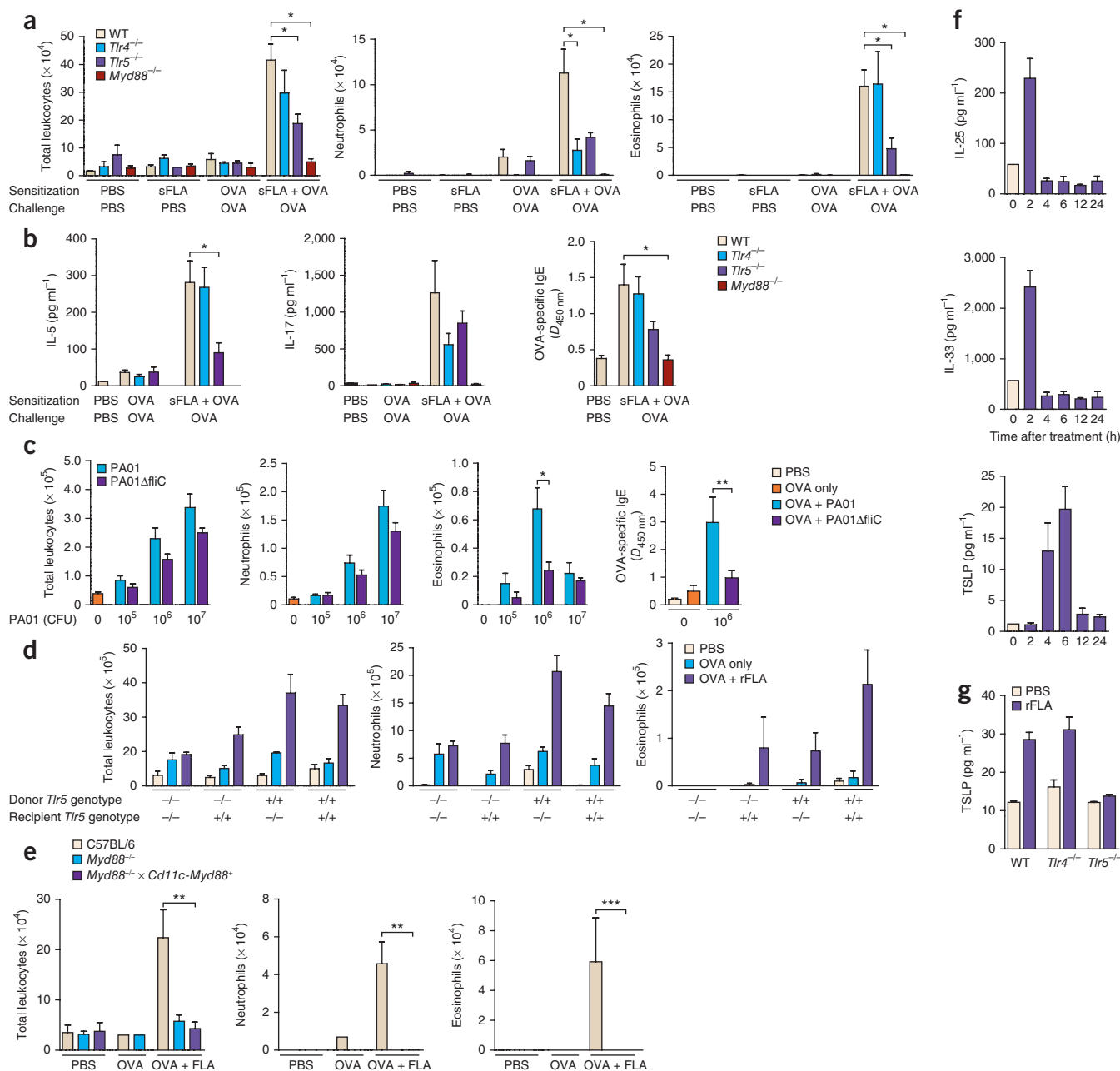
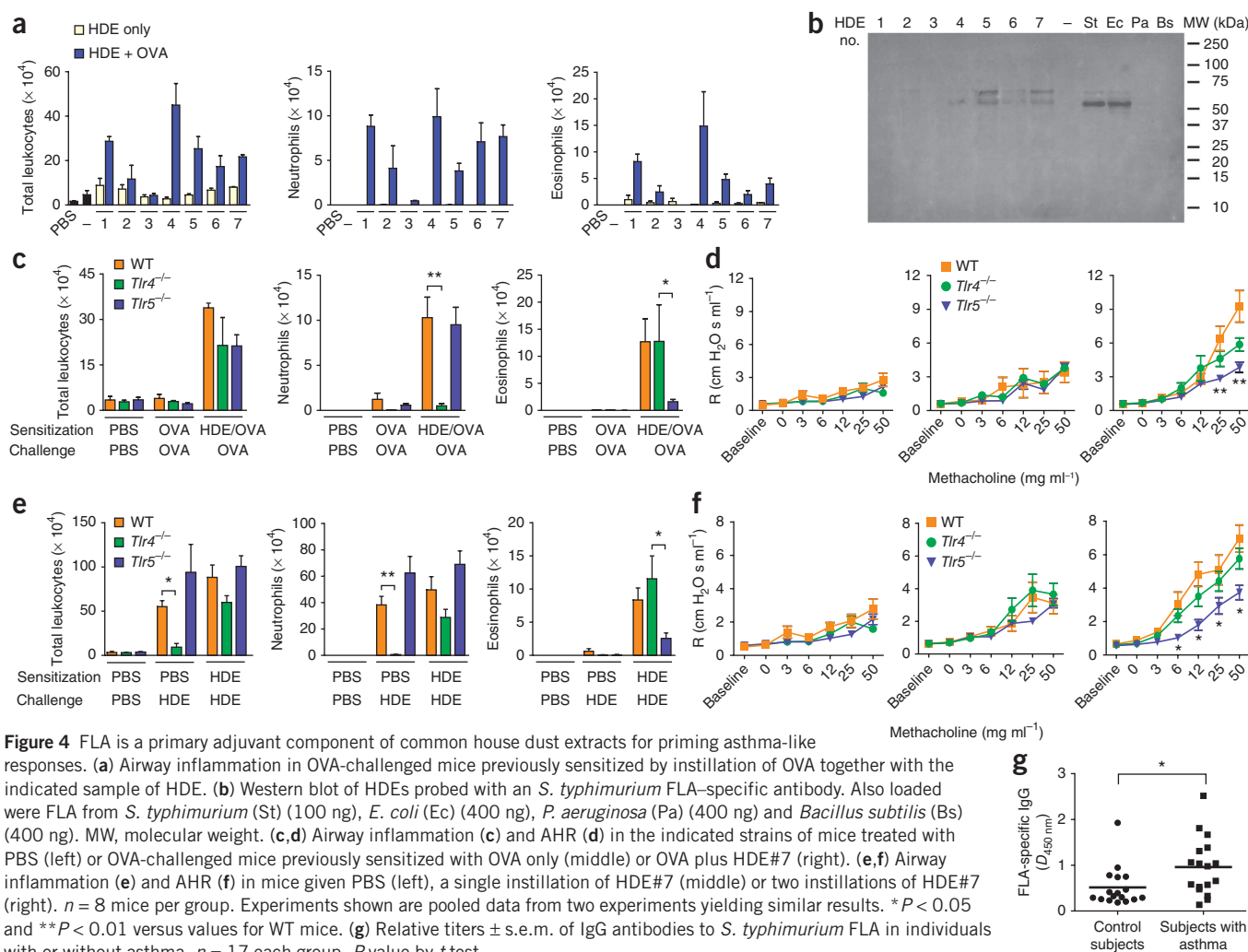


Figure 3 FLA possesses potent, TLR5-dependent adjuvant activity in the airway. (a) Mean numbers $\pm$ s.e.m. of total leukocytes (left), neutrophils (middle) and eosinophils (right) in mice sensitized and challenged as indicated. $n = 6$ mice per group. (b) Concentrations of IL-5 (left) and IL-17 (middle) in BALF and relative amounts of serum OVA-specific IgE (right) in mice of the indicated genotypes. (c) Left to right: total leukocytes, neutrophils, eosinophils and OVA-specific IgE in mice sensitized to OVA using either WT (PAO1) or FLA-deficient (PAO1Δflc) heat-killed *P. aeruginosa* as an adjuvant. (d) Total leukocytes (left), neutrophils (middle) and eosinophils (right) in bone marrow-chimeric mice generated with WT and *Tlr5*^{-/-} mice, sensitized as indicated. (e) Total leukocytes, neutrophils and eosinophils in mice expressing a *Myd88* transgene in CD11c⁺ cells only. Experiments were done at least twice with similar results. $n \geq 6$ mice per group. * $P < 0.05$. Values $\pm$ s.e.m. of cytokines in the BALF at the indicated time points after rFLA instillation. (g) TSLP concentration in supernatants of FLA-treated primary airway epithelial cells from the indicated mice.



When sensitized and challenged with HDE, WT mice developed strong AHR (Fig. 4f), whereas mice exposed to HDE on a single occasion did not develop AHR (Fig. 4f). These results indicate that adaptive immune responses to HDE are required for AHR. *Tlr4*^{-/-} mice also developed AHR after repeated instillation of HDE, although their responses were slightly decreased compared with WT mice. Therefore, LPS in this HDE probably contributes to, but is not essential for, HDE-induced AHR. By contrast, AHR was markedly lower in *Tlr5*^{-/-} mice compared to WT mice, again indicating that FLA present in this HDE promotes asthma-like responses to natural indoor allergens. We confirmed these findings using two additional extracts (Supplementary Fig. 6c). Although the absolute levels of inflammation and AHR varied according to the individual HDE used, *Tlr5*^{-/-} mice had less AHR or eosinophilia than WT mice for each of the three extracts tested. *Tlr5*^{-/-} mice also showed less airway inflammation than WT mice following sensitization and challenge with a preparation of cockroach antigens (Supplementary Fig. 6d).

We reasoned that if FLA-TLR5 interactions contribute to human asthma, individuals exposed to high amounts of FLA might be at increased risk for developing this disease. We therefore assessed titers of antibodies specific to FLA in sera from humans with mild to moderate asthma as an indirect measure of their exposure to FLA. Compared with age- and gender-matched subjects with healthy airways, subjects with asthma had significantly higher titers of

antibodies to FLA (Fig. 4g and Supplementary Fig. 7a), suggesting that exposure to FLA might be a risk factor for asthma. An alternative explanation is that antibodies to FLA reflect exposure to bacterial products in general. Individuals with asthma also had slightly higher levels of antibodies to LPS than did control subjects, but this difference did not reach statistical significance (Supplementary Fig. 7b). When taken together with the reduced eosinophilic and AHR responses of *Tlr5*^{-/-} mice to HDE-mediated allergic sensitization, our data suggest that environmental FLA participates directly in priming allergic responses to indoor allergens.

The relationship between allergic asthma and TLR signaling is complex. Whereas some studies have found that exposure to LPS protects against the development of asthma^{20–22}, others have found that LPS levels positively correlate with asthma^{23,24}. These contradictory conclusions might be due to differences in timing or amounts of LPS, which can affect the nature of immune responses²⁵. Recent evidence suggests that gene-environment interactions can also have an impact on the effect of TLR ligand exposure on allergic asthma^{26–28}. Although several polymorphisms have been identified in the human *TLR5* gene²⁹, it remains to be determined whether gene-gene or gene-environment interactions also affect the relationship between FLA exposure and allergic asthma.

Previous reports have shown that, depending on the experimental setting, FLA can prime T_H1 responses^{30,31}, T_H2 responses³², or both T_H1 and T_H17 responses³³. Direct comparison of these

studies is confounded by differences in FLA preparation and routes of administration. In addition, the context of FLA presentation is probably important. Thus, soluble monomeric FLA can prime T_H2 responses, whereas FLA associated with *S. typhimurium* is reported to prime T_H1 responses³⁴. Similarly, a fusion protein of rFLA and OVA suppressed T_H2 responses in a mouse model of intestinal allergy, whereas concurrent administration of OVA and rFLA as separate molecules can enhance responses³⁵. Although the evolutionary benefit of FLA-mediated eosinophilic responses in the airway is unclear, eosinophils are reported to promote clearance of some bacteria³⁶.

There is no known single structural feature that is common to all allergens. Some possess protease activity, whereas others possess carbohydrate moieties, bind lipids or interact with a TLR signaling complex^{37,38}. It is currently unknown whether FLA can promote sensitization to all allergens residing in house dust or only to a subset of them. Future studies that address the relationships between FLA, allergens and other TLR ligands should improve understanding of how various environmental exposures impact affect initiation of allergic responses that ultimately lead to allergic asthma.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Note: Supplementary information is available in the [online version of the paper](#).

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AUTHOR CONTRIBUTIONS

R.H.W. and S.M. conducted the majority of the experiments, designed experiments and helped prepare the manuscript. G.S.W. performed some assays and mouse experiments, including most airway physiology experiments. D.C.Z. and M.L.S. provided dust samples from US households, and M.L.S. performed the Multiplex Array for Indoor Allergens. J.F.F. and G.P.F. analyzed and graded lung sections for mucus production and took photographs. H.N. helped with flow cytometry and experimental design. M.K. and S.G. provided sera from patients with asthma and control subjects. D.N.C. conceived of the project and helped with experimental design and writing of the manuscript. All authors discussed the results and commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals and allergen models. Five- to eight-week old C57BL/6 and *Tlr5*^{-/-} mice were purchased from Jackson Laboratories. Male mice between 6 and 12 weeks of age were used in all experiments and were treated in accordance with the NIEHS Institutional Animal Care and Use Committee. We sensitized mice by oropharyngeal instillation⁹ of 100 µg low-endotoxin OVA (<1 EU per mg OVA) (Hyglos GmbH) in a 50-µl volume on days 0 and 7. Microbial products tested include *E. coli* LPS (Sigma) and ultrapure LPS (List Biological Laboratories). Also tested were triacylated peptide, Pam3csk4; poly(I:C); sFLA from *S. typhimurium*; endotoxin-free rFLA from *S. typhimurium*; endotoxin-free FLA from *B. subtilis*; FSL-1 (Pam2CGDPKHKFS), the N-terminal portion of the 44-kDa lipoprotein LP44 of *Mycoplasma salivarium*; ssRNA40; and ODN1826, all from InvivoGen. The sFLAS contained (<http://www.invivogen.com/fla-st>), whereas endotoxin was undetectable in rFLA (<http://www.invivogen.com/recfla-st>). Except where otherwise indicated, we challenged mice with a 1% OVA (Sigma) aerosol for 1 h on day 14 and analyzed them on day 16. For multiple exposures, mice were challenged for 30 min daily and analyzed 1 d after the last exposure. Other mice were sensitized on days 0 and 7 with 10 µg cockroach antigen (Greer Inc.) treated with ProteoSpin endotoxin removal kit (Norgen Biotek), challenged on days 14–17 with 10 µg of the antigen, and BALF collected 3 d later.

Histology. Lungs were fixed in 10% formalin, processed and embedded in paraffin. Left-lobe longitudinal sections 5–7 µm thick were stained with Alcian blue and periodic acid-Schiff. We scored the airways in a blinded fashion, and considered a single positive mucus-staining cell in an airway diagnostic of a positive result.

Airway physiology. We assessed responses to aerosolized methacholine using the Flexivent mechanical ventilator system (Scireq) as previously described³⁹.

Cytokine analysis. We analyzed cytokines in BALF and whole-lung cultures by ELISA or multiplexed bead-based assays as described previously⁹. For analysis of lymph node cytokines, we injected mice intravenously with 10⁶ naive, OVA-specific CD4⁺ T cells from OT-II mice 1 d before allergic sensitization. Mediastinal lymph nodes were collected 3 d later and cultured for 3 d in complete RPMI medium containing 1 mg ml⁻¹ OVA. IL-25, IL-33 and TSLP in BALF were measured by ELISA (R&D Systems).

Flow cytometry. We prepared lymphocytes from the lung, blocked the cells and stained them for surface antigens and intracellular cytokines as previously described⁹. Stained cells were collected using a BD LSR II cytometer (BD Biosciences), and data were analyzed using FlowJo 7.2.2 software (Tree Star, Inc.).

House dust extract preparation and analyses. HDEs were prepared as described previously^{40,41}. Endotoxin activity was measured by a *Limulus* amoebocyte

lysate QCL-1000 assay (Lonza). Allergen concentrations were measured by Multiplex Array for Indoor Allergens (Indoor Biotechnologies).

Bone marrow chimeric mice. We irradiated mice with 900 rad, injected them 1 d later with 10⁷ bone marrow cells, and gave them acidified water supplemented with 500 µg ml⁻¹ neomycin. Reconstitution of lung leukocytes was verified to be greater than 94% by flow cytometry, on the basis of staining with antibodies against CD45.2 (clone 104; eBioscience) and CD45.1 (clone A20; eBioscience).

Immunoblotting. HDEs (20 µg per sample) were loaded on a 4–12% Bis-Tris polyacrylamide gel (Bio-Rad Laboratories) and resolved by electrophoresis. Proteins were transferred to a PVDF membrane, blocked for 1 h, probed overnight with 0.1 µg ml⁻¹ anti-flagellin FliC (clone X5A12; InvivoGen or clone FLIC-1; Biolegend), followed by Mouse Trueblot Ultra anti-mouse immunoglobulin conjugated to horseradish peroxidase (1:1,000 dilution; eBioscience). Chemiluminescence by Lumigen PS-3 (GE Healthcare) was used to detect immunoreactive proteins.

ELISA for flagellin-specific IgG. Microplates were coated with 100 ng per well rFLA (InvivoGen), FLA from *P. aeruginosa* or *E. coli* LPS and probed with human sera (1:500 dilution), followed by horseradish peroxidase-conjugated anti-human IgG (BD Pharmingen).

Human studies. The protocol was approved by the Institutional Review Board at NIEHS and Duke University Medical Center. Informed consent was obtained from human subjects. Individuals with asthma had physician-diagnosed asthma and AHR (defined as a 20% decrease in forced expiratory volume at 1 s (FEV1) after inhalation of <8 mg/ml of methacholine). Normal subjects had no asthma or allergic rhinitis and tested negative for methacholine challenge and allergen skin tests. Nonfasting blood samples were drawn from the antecubital vein into a clot-activating container. Serum was separated and stored at -80 °C until assayed.

Statistics. Data are expressed as mean ± s.e.m. Statistical differences between groups were generally calculated using the unpaired, two-tailed Student's *t* test. Where multiple groups were compared, we used analysis of variance. *P* values of less than 0.05 were considered statistically significant.

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

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