The TLR5 ligand flagellin promotes asthma by priming allergic responses to indoor allergens

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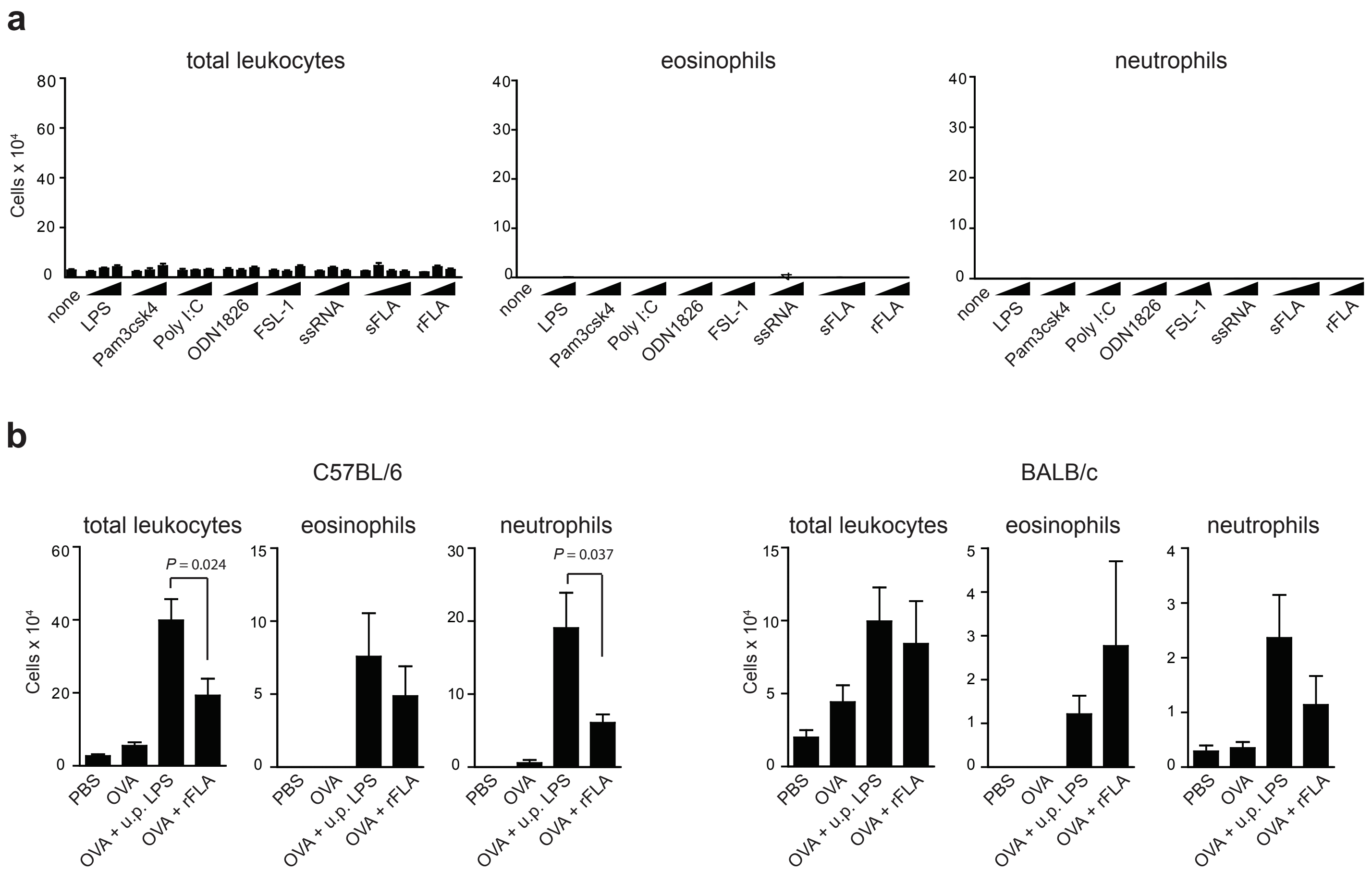
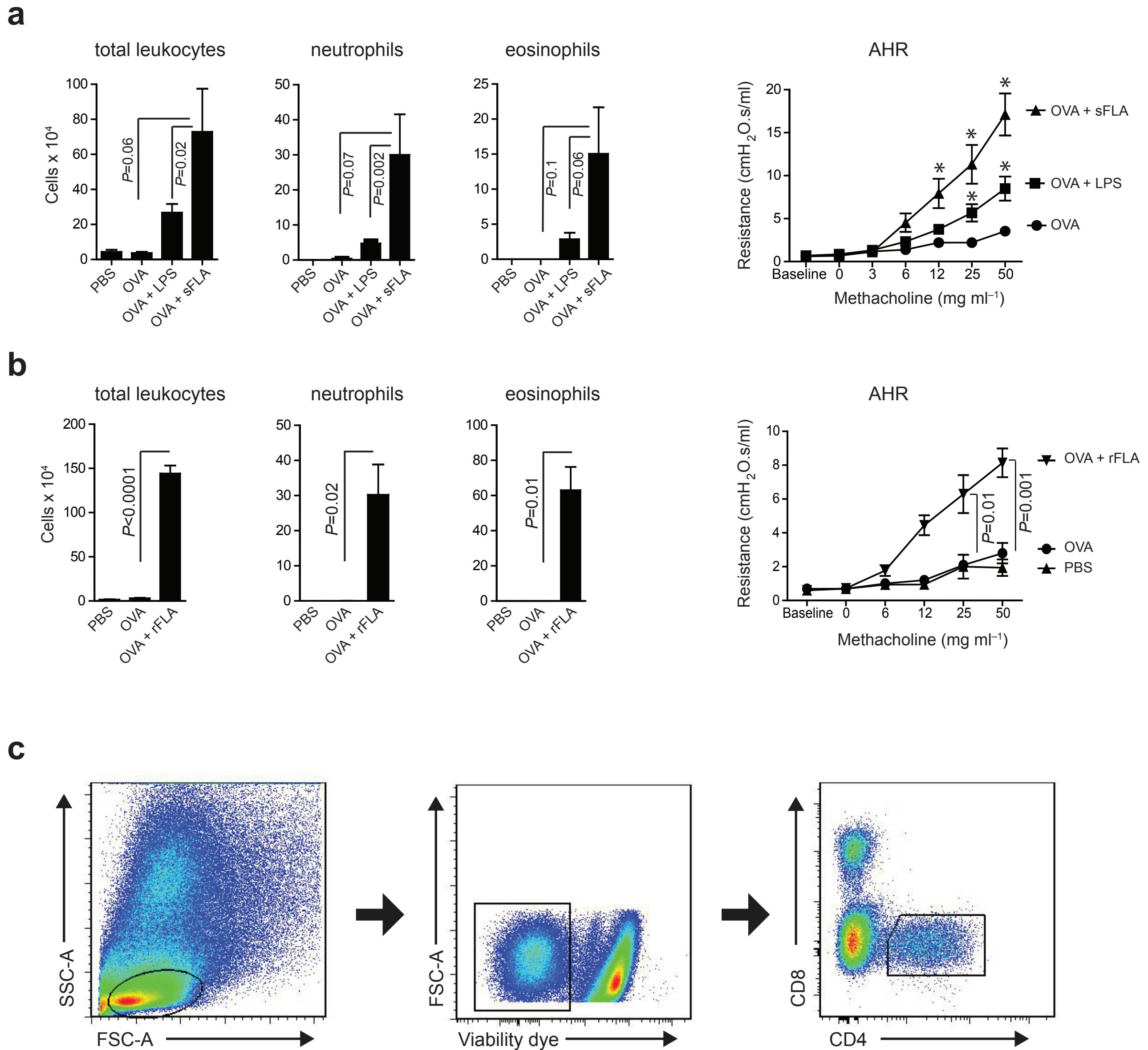
Supplementary Table 1. Allergen and endotoxin content of HDEs.

Sample #	Derp1 (ng/g)	Derf1 (ng/g)	Derp/Derf2 (ng/g)	Feld1 (ng/g)	Canf1 (ng/g)	Ratn1 (ng/g)	Blag2 (ng/g)	Alta1 (ng/g)	LPS (EU/g)
1	74.6	34	94	n.d.	n.d.	n.d.	n.d.	n.d.	16,557
2	21.5	n.d.	52.5	n.d.	n.d.	28.8	n.d.	n.d.	18,778
3	89.2	n.d.	24.2	n.d.	n.d.	4.6	n.d.	n.d.	165
4	98	99.2	37.5	2,375.3	n.d.	4.6	n.d.	14.3	8,744
5	4,141.1	n.d.	3,842.1	30.9	44.1	6.2	403.8	n.d.	38,077
6	30.4	38.5	82.9	n.d.	27.1	n.d.	228.3	n.d.	11,766
7	1,776.2	49.5	1872	n.d.	130.45	n.d.	361.4	8.6	29,508

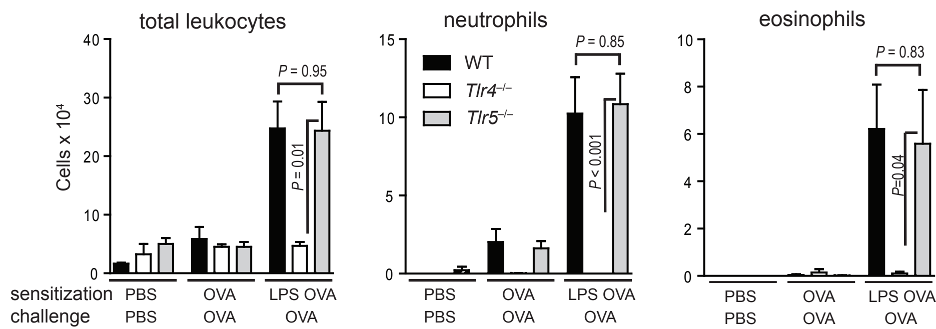
Supplementary Table 1. Allergen and endotoxin content of HDEs. Levels of the indicated allergens were measured by a multiplex assay. Shown are values present in dry house dust, as determined from dilutions of the extracts (see online methods). Der p, *Dermatophagoides pteronissinus* with protease activity; Feld, *Felis domesticus, Canf, Canis familiarus;* Blag, *Blattella germanica*; Ratn, *Rattus norvegicus*; Alta, *Alternaria alternata*; n.d., not detected.



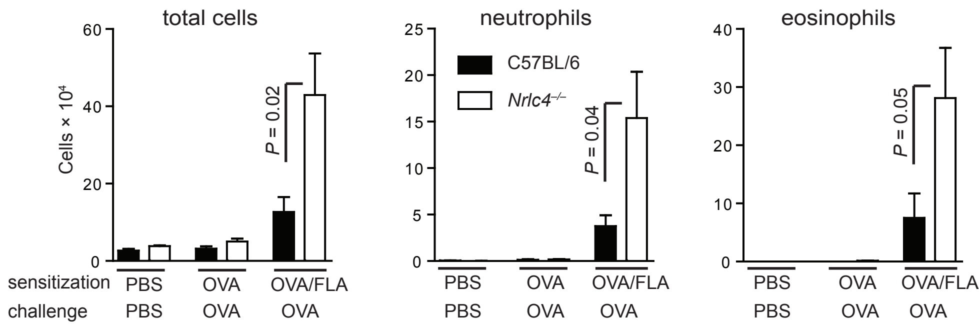
Supplementary Figure 1: Instillation of microbial products alone does not lead to allergic inflammation. (**a**) Airway inflammation in mice receiving airway instillations of 25 ng, 125 ng or 625 ng microbial product (◢).1250 ng of sFLA was also tested. The scale on the Y axis is the same as that for **Fig. 1** to allow ready comparison between the two sets of data. Mean levels of the indicated leukocyte subsets ± s.e.m. in BALF following a single OVA challenge are shown. Results shown are data from a single experiment. n = 5 mice per group. (**b**) Induction of allergic responses to OVA using ultra-pure (u.p.) LPS or rFLA as adjuvants in C57BL/6 and BALB/c mice.



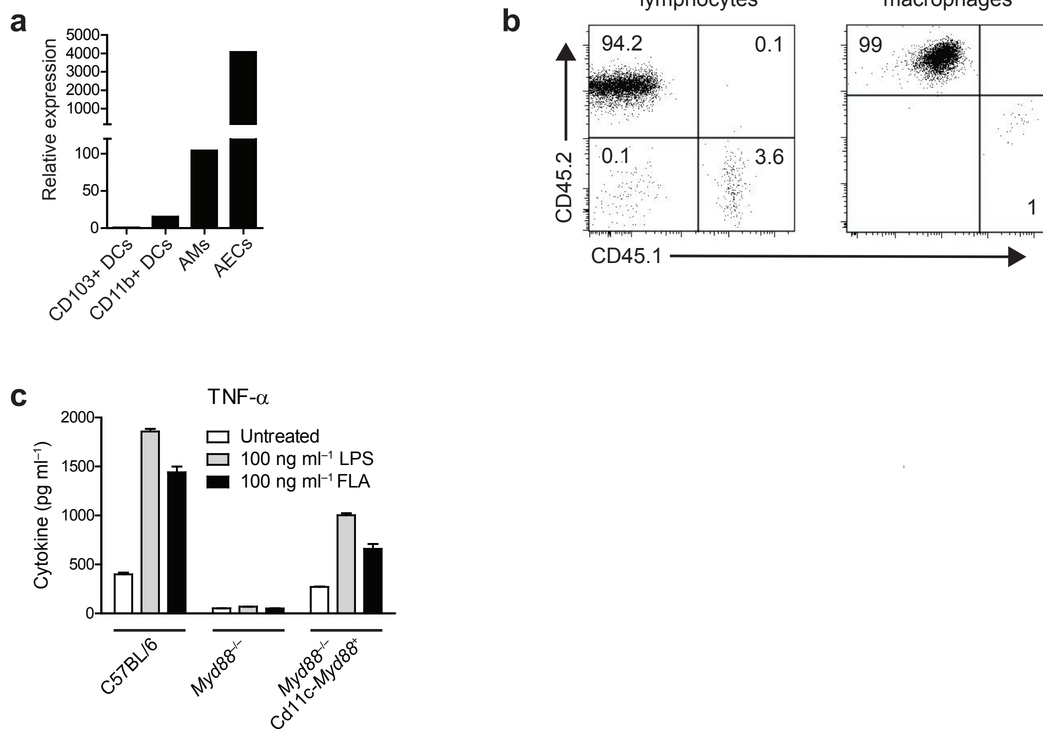
Supplementary Figure 2: FLA primes allergic airway disease. (**a**,**b**) Allergic responses in mice previously sensitized with (**a**) sFLA-OVA or (**b**) rFLA-OVA and challenged by OVA aerosol on seven consecutive days. Shown are mean values ± s.e.m. of leukocytes in the airway, and airway resistance for intubated mice inhaling air (baseline), or aerosols of PBS containing the indicated concentrations of methacholine. $n \geq 8$ mice/group. * $P < 0.01$ (*t* test) for differences between the indicated groups and mice sensitized and challenged with OVA only. (**c**) Gating strategy for live $CD4^+$ lymphocytes, using forward and side scatter (left), viability exclusion dye (middle), and staining for the CD4 and CD8 antigens.



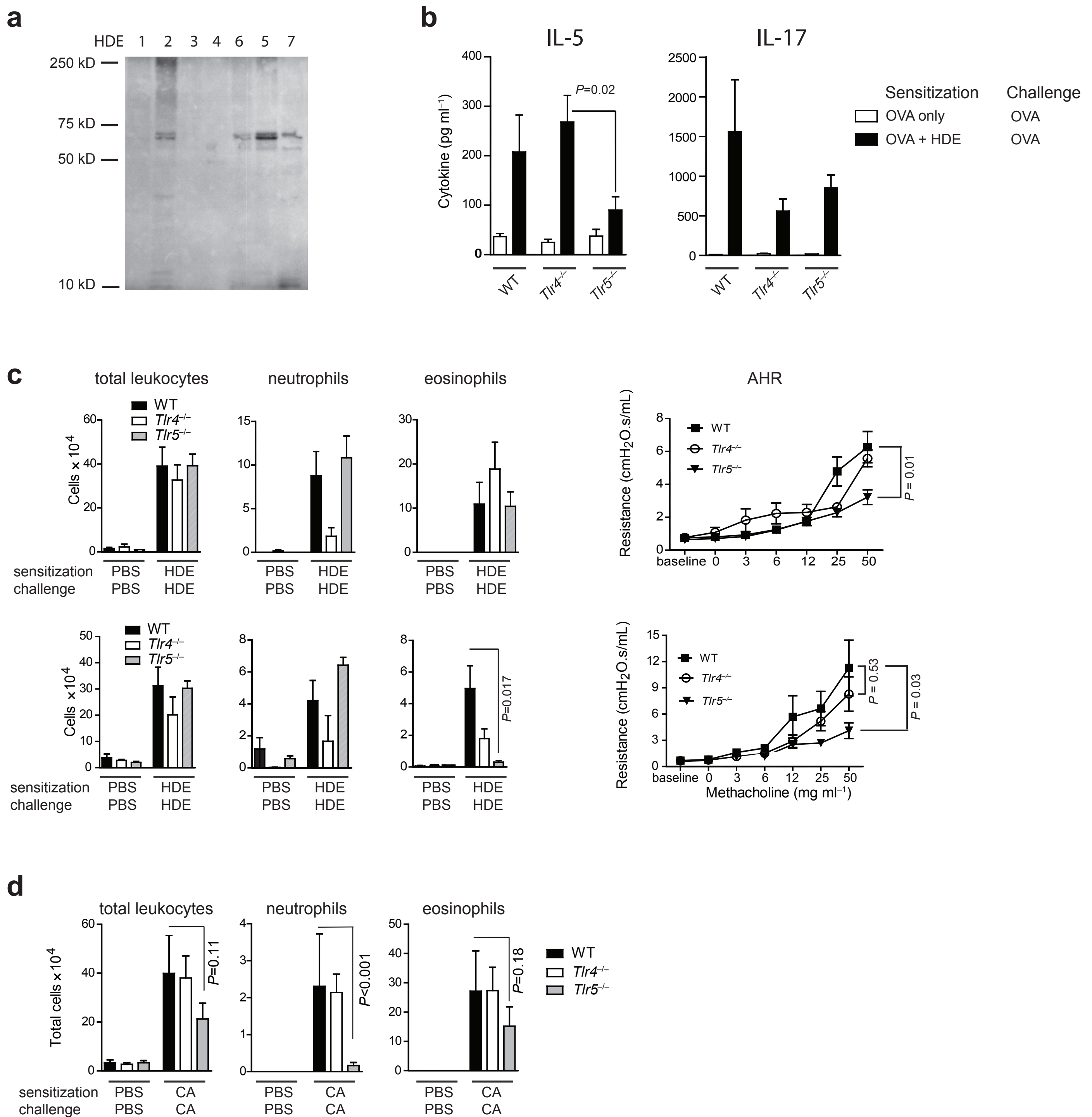
Supplementary Figure 3: *Tlr5*$^{-/-}$ mice develop allergic inflammation following sensitization with LPS-OVA. Mean numbers ± s.e.m. of inflammatory leukocytes in WT, *Tlr4*$^{-/-}$ and *Tlr5*$^{-/-}$ mice following allergic sensitization to OVA using as an adjuvant. *n* = 5 - 9 for LPS-OVA treated groups, *n* = 4 for negative control groups.



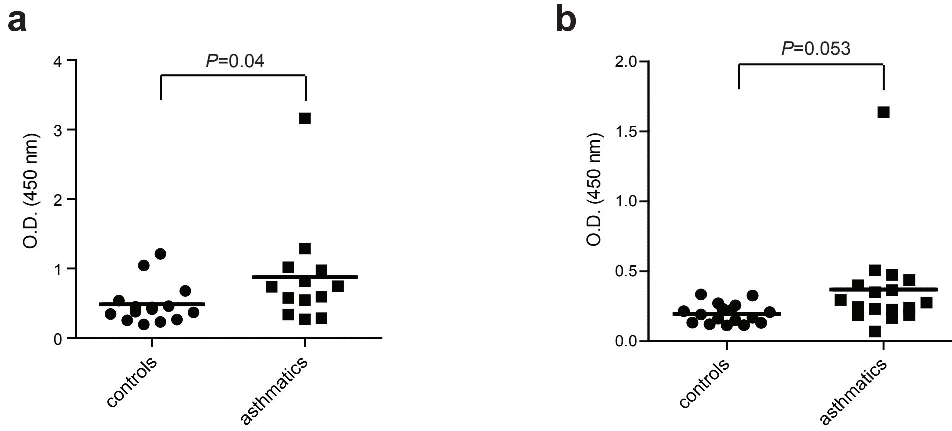
Supplementary Figure 4: Nlrc4 is dispensable for FLA-mediated allergic sensitization through the airway. Mean numbers ± s.e.m. of the indicated leukocyte subsets in BALF following a single OVA challenge. Data are from two experiments. *n* = 9 for sFLA-OVA treated mice, *n* = 4 for negative control groups.



Supplementary Figure 5: Requirement of *Tlr5* expression on cell compartments for FLA-mediated allergic sensitization through the airway. (**a**) *Tlr5* expression in select cell subsets prepared from the lung. Values are shown relative to $CD103^+$ dendritic cells (DC). AM, alveolar macrophages; AEC, airway epithelial cells (**b**) Reconstitution of irradiated recipient mice with donor bone marrow. Shown are representative flow plots for T cells and pulmonary macrophages from an irradiated WT CD45.1 mouse receiving WT CD45.2 marrow. Similar reconstitution levels were seen for other genotypes. (**c**) Function of *Cd11c-Myd88* transgene. $CD11c^+$ cells were prepared from lungs of the indicated mouse strains and treated *ex vivo* with LPS or FLA. TNF-α in cell supernatants were analyzed by ELISA.



Supplementary Figure 6: Role of FLA as an environmental adjuvant. (**a**) Western blot of multiple HDEs probed with a different anti-FLA antibody than that used in Fig.4b. (**b**) Cytokines in lungs of mice following HDE-OVA sensitization and OVA challenge. *n* = 7 - 10 mice/group for HDE-OVA sensitizations, 4 per group for OVA only sensitizations. (**c**) Mean levels ± s.e.m. of inflammatory leukocytes in BALF (left) and AHR (right) are shown for mice sensitized and challenged with HDE#4 (top) and HDE#5 (bottom). (*n* = 5 – 12 mice/group). (**d**) Mean levels ± s.e.m. of inflammatory leukocytes in BALF and of AHR are shown for cockroach antigen-sensitized and -challenged mice. *n* = 5 – 12 mice/group.



Supplementary Figure 7. Titers of antibodies to microbial products in asthmatic and non-asthmatic subjects. (**a**) Antibodies to FLA from *P. aeruginosa*. (**b**) Antibodies to LPS from *E. coli*.

ONLINE SUPPLEMENTARY METHODS

Flow cytometry. After blocking, surface antigens were stained using antibodies against mouse CD4 (clone RM4-5; BD Pharmingen) and CD8α (clone 5H10; Caltag) or the appropriate isotype control. Dead cells were stained with Fixable Viability Dye eFluor® 780 (eBioscience) and excluded from analysis. Intracellular staining was done using antibodies against IL-13 (clone eBio13A; eBioscience) and IL-17A (clone TC11-18H10; BD Pharmingen).

Heat-killed *Pseudomonas areruginosa*-mediated sensitization to OVA. We used PA01, a widely-used strain of *P. aeruginosa*, and PA01Δ*fliC*, a mutant strain that lacks the flagellin-encoding *fliC* gene. The bacteria were grown overnight in Luria Bertoni broth, diluted 100-fold, transferred to fresh medium and grown for another 3 h. Bacterial counts were inferred from OD_{600nm}, based on previous empirical data, and confirmed by plating of an aliquot of the culture for colony counting on the following day. Bacteria were centrifuged at 7000 rpm for 3 m, suspended in PBS, and incubated for 1 h at 56º C to kill the organisms, which were then centrifuged once more and resuspended in PBS. For allergic sensitizations, mice received various numbers of heat-killed bacteria together with 100 μg OVA on days 0 and 7. For challenge, the mice were exposed to an aerosol of 1% OVA for 1 h on day 14.

Analysis of airway inflammation, cytokines, and IgE. Measurements of IL-4, IL-5, IL-17 in the BALF were assessed at 4 h post-challenge, using a commercial multiplexed fluorescent bead-based immunoassay (Bio-Rad Laboratories, Hercules, CA), according to the manufacturers' instructions. IL-13 was measured in supernatants of whole lung and lymph node cultures by an enzyme-linked immunosorbent assay (ELISA) using an anti-IL-13 antibody (eBioscience). TNF-

α in CD11c^{+} cell supernatants was measured by ELISA (Biolegend), and serum IgE by ELISA (BD Bioscience).

Isolation and culture of alveolar type II cells. Lungs were perfused and instilled with 1.5 ml of dispase II (1.5 U/ml) (Roche) via the trachea, which was immediately closed with a ligature. Intact lungs were removed and incubated in culture tubes with an additional 2 ml of dispase II for 45 minutes at 37º C for digestion. After incubation, lungs were minced in a solution of Dulbecco's modified Eagle's medium (DMEM; Invitrogen), 25 mM Hepes (Sigma), 1% penicillin/streptomycin and 100U/ml DNase I (Sigma), then filtered through a 70 µM cell strainer and incubated in Mouse IgG (Jackson, ImmunoResearch Laboratories, Inc.) coated plates for two hours at 37º C. Non-adherent cells were removed, washed and re-suspended in DMEM with 10% fetal bovine serum (FBS; certified, Invitrogen), 25 mM Hepes, 1% penicillin/streptomycin and 10 ng/ml keratinocyte growth factor (Sigma). Cells were cultured at an approximate density of 2.5×10^{5} cells/cm^{2} in plates pre-coated with 30 µg/ml bovine collagen I (Invitrogen) and 10 µg/ml fibronectin (EMD4biosciences) at 37º C in an atmosphere of 5% CO_2. Media was changed after the first day of culture and every two days thereafter until cells reached confluence.

Isolation/culture of pulmonary dendritic cells and macrophages. CD11c^{+} DCs were fractionated into their CD11b^{+} and CD103^{+} subsets by flow cytometry-based cell sorting as previously described[1] and analyzed for *tlr5* expression by qPCR. For TNF-α analysis, CD11c^{+} cells were isolated by a magnetic bead-based cell sorting system (Miltenyi) using a biotin-conjugated antibody against mouse CD11c (clone HL3; BD Pharmingen). Cells were cultured in

200 µl of RPMI with 10% FBS (certified, Invitrogen), 50 µM β-mercaptoethanol and penicillin streptomycin at a density of 1×10^5 cells/well in a 96-well U-bottom plate (BD Biosciences).

Human studies. Non-smoking volunteers underwent a comprehensive screening protocol to define asthma and atopy phenotypes. Atopy was defined as having seasonal symptoms requiring medications and a positive skin-prick to house dust mite and at least 3 other aeroallergens. Asthmatic subjects had mild-moderate disease as defined by NHLBI EPR3 guidelines (http://www.nhlbi.nih.gov/guidelines/asthma/asthgdln.pdf).

LITERATURE CITED

1 Nakano, H. *et al.* Pulmonary CD103(+) dendritic cells prime Th2 responses to inhaled allergens. *Mucosal Immunol*, doi:10.1038/mi.2011.47 (2011).