Toll/IL-1 Signaling Is Critical for House Dust Mite–specific Th1 and Th2 Responses

Simon Phipps[1], Chuan En Lam[1], Gerard E. Kaiko[1], Shen Yun Foo[1], Adam Collison[1], Joerg Mattes[1], Jessica Barry[1], Sophia Davidson[1], Kevin Oreo[1], Lauren Smith[1], Ashley Mansell[2], Klaus I. Matthaei[3,4], and Paul S. Foster[1]

[1]Centre for Asthma and Respiratory Diseases, School of Biomedical Sciences, University of Newcastle, Newcastle; [2]Monash Institute of Medical Research, Monash University, Clayton; [3]Gene Targeting Group, John Curtin School of Medical Research, Australian National University, Canberra, Australia; and [4]Stem Cell Unit, Department of Anatomy, College of Medicine, and King Khalid University Hospital, King Saud University, Riyadh, Saudi Arabia

***Rationale*: One of the immunopathological features of allergic inflammation is the infiltration of helper T type 2 (Th2) cells to the site of disease. Activation of innate pattern recognition receptors such as Toll-like receptors (TLRs) plays a critical role in helper T type 1 cell differentiation, yet their contribution to the generation of Th2 responses to clinically relevant aeroallergens remains poorly defined.**
***Objectives*: To determine the requirement for TLR2, TLR4, and the Toll/IL-1 receptor domain adaptor protein MyD88 in a murine model of allergic asthma.**
***Methods*: Wild-type and factor-deficient ($^{-/-}$) mice were sensitized intranasally to the common allergen house dust mite (HDM) and challenged 2 weeks later on four consecutive days. Measurements of allergic airway inflammation, T-cell cytokine production, and airway hyperreactivity were performed 24 hours later.**
***Measurements and Main Results*: Mice deficient in MyD88 were protected from the cardinal features of allergic asthma, including granulocytic inflammation, Th2 cytokine production and airway hyperreactivity. Although HDM activated NF-κB in TLR2- or TLR4-expressing HEK cells, only in TLR4$^{-/-}$ mice was the magnitude of allergic airway inflammation and hyperreactivity attenuated. The diminished Th2 response present in MyD88$^{-/-}$ and TLR4$^{-/-}$ mice was associated with fewer OX40 ligand–expressing myeloid dendritic cells in the draining lymph nodes during allergic sensitization. Finally, HDM-specific IL-17 production and airway neutrophilia were attenuated in MyD88$^{-/-}$ but not TLR4$^{-/-}$ mice.**
***Conclusions*: Together, these data suggest that Th2- and Th17-mediated inflammation generated on inhalational HDM exposure is differentially regulated by the presence of microbial products and the activation of distinct MyD88-dependent pattern recognition receptors.**

Keywords: asthma; innate immunity; eosinophil; neutrophil

One of the immunopathological features of allergic inflammation is the infiltration of IL-13–producing T helper 2 (Th2) cells to the site of disease. Clinical investigations of allergic asthmatics show elevated IL-13 expression in the airway mucosa and bronchioalveolar lavage in response to allergen challenge (1, 2), whereas its overexpression in the mouse recapitulates many of the hallmark pathologic features of asthma (3). Moreover, a clinical trial using a recombinant human IL-4 variant to inhibit the binding of IL-4 and IL-13 to IL-4 receptor complexes found this therapy to effectively reduce the magnitude of the late-phase reaction to allergen challenge (4). However, it remains to be explained why the adaptive immune response generates IL-13–producing T cells to harmless proteins of plant and animal origin such as pollens, cats, and house dust mites.

Th1 responses that promote immunity from intracellular bacteria are primed in response to the Th1-inducing cytokine IL-12, which in turn is released by antigen-presenting cells (APCs) after recognition of conserved microbial motifs that activate pattern recognition receptors (PRRs), such as the Toll-like receptor (TLR) family (5). Similarly, emerging evidence has linked the activation of nucleotide-binding and oligomerization domain-2 to the production of IL-17 by memory T cells (6). However, although some novel Th2-inducing factors have been proposed (Jagged-2, IL-33, tumor necrosis factor–related apoptosis-inducing ligand, and thymic stromal lymphopoietin) (7–10) the innate recognition processes that induce their expression remain obscure. A landmark study by Eisenbarth and coauthors demonstrated that the development of experimental allergic asthma by direct inhalation of ovalbumin was dependent on the activation of TLR4 during sensitization (11). Moreover, whereas high concentrations of lipopolysaccharide promoted Th1 immunity characterized by IFN-γ production and neutrophilia, low concentrations of lipopolysaccharide promoted the production of Th2 cytokines and associated eosinophilia. Thus, distinct thresholds of PRR activation were associated with the generation of either Th1 or Th2 immunity. Studies have since demonstrated that other TLR ligands, in particular bacterial and synthetic products that activate TLR2, can promote or attenuate allergic airway inflammation (AAI) depending on the model systems and adjuvants employed (12–14). However, because these murine studies have almost exclusively used ovalbumin, the archetypal Th2-inducing antigen, it remains unknown whether the development of allergic inflammation to a clinically relevant allergen requires the activation of particular TLRs and/or the Toll/IL-1 receptor adaptor molecule, myeloid differentiation primary response gene 88 (MyD88).

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The activation of pattern recognition receptors shapes the development of helper T-cell type 1 (Th1) and Th17 immunity. However, the role of these receptors in the development of Th2 responses to clinically relevant aeroallergens is less well defined.

What This Study Adds to the Field

Toll-like receptor-4 deficiency attenuates eosinophilia and T-cell–derived IL-5 production, but not neutrophilia and IL-17. Thus, altered innate signaling may contribute to the heterogeneous inflammation observed in asthma.

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Correspondence and requests for reprints should be addressed to P. Foster, Ph.D., and S. Phipps, Ph.D., Centre for Asthma and Respiratory Diseases, David Maddison Clinical Sciences Building, Watt Street, Newcastle, NSW, 2300 Australia. E-mail: paul.foster@newcastle.edu.au and simon.phipps@newcastle.edu.au

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Using an improved acute mouse model in which sensitization was induced directly via the airways, in the absence of exogenous adjuvants such as LPS or aluminum hydroxide, we demonstrated that induction of the cardinal features of allergic asthma, with the exception of goblet cell hyperplasia (GCH), were dependent on MyD88. Although HDM extract stimulated HEK cells expressing TLR2 and TLR4 to activate NF-κB, only the loss of TLR4 attenuated the magnitude of AAI and airway hyperreactivity (AHR). Notably, the diminished Th2 response was associated with fewer OX40 ligand $(OX40L)^+$ myeloid DCs in the draining lymph nodes. Furthermore, antigen-specific production of IL-17 and neutrophilia were attenuated in MyD88- but not TLR4-deficient mice, suggesting that in response to HDM inhalation, divergent pathways are activated to mediate the generation of Th2 and Th17 responses. Thus this study shows an important contribution of innate host defense pathways in the generation of aberrant Th2 responses.

METHODS

Animals

All mice were housed at the John Curtin School of Medical Research specific pathogen–free facility (Canberra, ACT, Australia). $MyD88^{-/-}$, $TLR2^{-/-}$, and $TLR4^{-/-}$ mice were a generous gift from S. Akira (Osaka University, Osaka, Japan). All strains were backcrossed to the BALB/c background for 10 generations. All experiments were approved by the University of Newcastle Animal Care and Ethics Committee and performed at the University of Newcastle (Newcastle, NSW, Australia).

Cell Culture

RAW-Elam cells or human embryonic kidney cells (HEK-293) stably transfected with TLR2, TLR3, TLR4, TLR5, or TLR9 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) and maintained in a 37°C humidified atmosphere of 5% CO_2. We determined NF-κB–dependent gene expression using the 5× κB-luciferase reporter construct (Stratagene, La Jolla, CA) as described previously (15). Cells were seeded at 2×10^4 cells per well and stimulated with various concentrations of HDM, saline, or relevant TLR ligand for 6 hours and assayed for luciferase activity with a FLUOstar Optima (BMG Technologies, Offenburg, Germany) after incubation in luciferase assay reagent (Promega, Madison, WI).

Induction of Allergic Airway Inflammation

House dust mite (HDM) (*Dermatophagoides pteronyssinus*) extract was obtained from Greer Labs (Lenoir, NC) as a lyophilized preparation of milled mites and four batches of HDM were used in total during the study. Six- to 8-week-old mice were sensitized through the nares with vehicle (endotoxin-free saline) or HDM extract on Days 0, 1, and 2 (25 μg of total protein in 50 μl; *Der p 1* constituted ~10% of protein weight). This dose of HDM contained about 44 ng of LPS as determined by *Limulus* amebocyte assay (AMS Laboratories Pty Ltd, Sydney, Australia). On Days 14, 15, 16, and 17 mice were intranasally challenged with either vehicle or HDM extract (5 μg in 50 μl) and AHR was assessed 24 hours later if required. All mice were killed by intraperitoneal injection of sodium pentobarbitone on Day 18.

Flow Cytometry

Lung cells were mashed through a cell strainer and red blood cells were lysed with NH_4Cl. Cells were seeded into U-bottom 96-well plates at 10^6 per well, and preincubated with Fcγ receptor block in phosphate-buffered saline (PBS)−2% fetal calf serum (FCS) medium before a 20-minute incubation with one or more of the following fluorochrome-labeled antibodies (BD Biosciences [San Jose, CA] unless otherwise stated): fluorescein isothiocyanate (FITC)–B220 (clone RA3-6B2), peridinin chlorophyll protein–conjugated Gr-1 (clone RB6-8C5), FITC-conjugated CD3 (clone 145-2C11), allophycocyanin-conjugated CD4 (clone RM4-5), phycoerythrin-conjugated CD24 (clone M1/69; eBioscience, San Diego, CA), FITC-conjugated CD11c (clone HL3), peridinin chlorophyll protein–conjugated CD11b (clone M1/70), allophycocyanin-conjugated pDC antigen (clone JF05-1C2.4.1; Miltenyi Biotec, Bergisch Gladbach, Germany). After three washes in PBS−2% FCS medium cells were resuspended in PBS–formalin (0.5%, vol/vol) and analyzed with a BD FACSCanto within 3 days.

Draining Lymph Node Cell Culture and Measurement of T-cell Cytokine Production

Draining lymph nodes were removed and cells were dissociated by applying gentle pressure with a syringe plunger over a cell strainer. Red blood cells were depleted by NH_4Cl lysis. Cells were resuspended in complete Hanks' balanced salt solution (*N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid, sodium pyruvate, L-glutamine, 10% [vol/vol] FCS, penicillin–streptomycin–neomycin, 2-mercaptoethanol) seeded into a flat-bottomed 96-well plate at 0.5×10^6 per well and treated with HDM extract (50 μg/ml) or diluent (complete Hanks' balanced salt solution). After a 4-day incubation cell-free supernatants were collected and stored at −80°C before cytokine detection by ELISA as described previously (10).

Enumeration of Mucous Cell Hyperplasia

Lung tissue samples representing the central and peripheral airways were fixed in 10% phosphate-buffered formalin, sectioned, and stained with periodic acid–Schiff or chromotrope 2R to enumerate mucin-producing goblet cells or eosinophils, respectively. Positive cells were expressed as cells per 100 μm of epithelial basement membrane length.

Measurement of HDM-specific IgG1

Ninety-six–well plates were coated overnight with either HDM extract (50 μg/ml) or unlabeled anti-IgG of corresponding isotype for standard wells. Nonspecific binding sites were blocked with PBS–bovine serum albumin (2%, wt/vol) before addition of serum or standards. After detection with biotinylated anti-IgG1 wells were incubated with streptavidin–horseradish peroxidase for 30 minutes and then developed with tetramethylbenzidine substrate. Optical density was determined at 450 nm with a microplate reader (Bio-Rad, Hercules, CA).

Measurement of AHR

Mice were mechanically ventilated and AHR to inhaled β-methacholine (determined by alterations in resistance and compliance) was measured as described previously (10). Responses to methacholine are expressed as the percentage change over saline control (baseline).

Statistical Analysis

Data are presented as means ± SEM. Data sets were analyzed by Student *t* test, except for those obtained by measuring AHR, which were analyzed by analysis of variance. The software package GraphPad Prism 3.01 (GraphPad Software, San Diego, CA) was used for all data analysis and preparation of graphs.

RESULTS

Acute Intranasal Administration of HDM Extract Induces Allergic Inflammation in the Absence of a Classical Adjuvant

Repeated daily inhalational exposure to HDM extract over a period of 5 weeks induces many of the hallmark pathologic features of AAI and airway remodeling (16). To address the role of innate PRRs in the development of AAI, we first sought to develop an acute disease model. Mice were sensitized with either HDM extract or vehicle (saline) on Days 0–2, and then challenged to HDM or vehicle on Days 14–17 and killed on Day 18 after measurement of AHR if required (Figure 1A). We observed a significant increase in the numbers of eosinophils located in the lamina propria in the antigen-sensitized/antigen-challenged mice as compared with the vehicle-sensitized/vehicle-challenged (vehicle control) and vehicle-sensitized/antigen-challenged (challenged control) groups (Figure 1B). Consistent with the findings of Cates and colleagues (17), we observed a significant increase in the

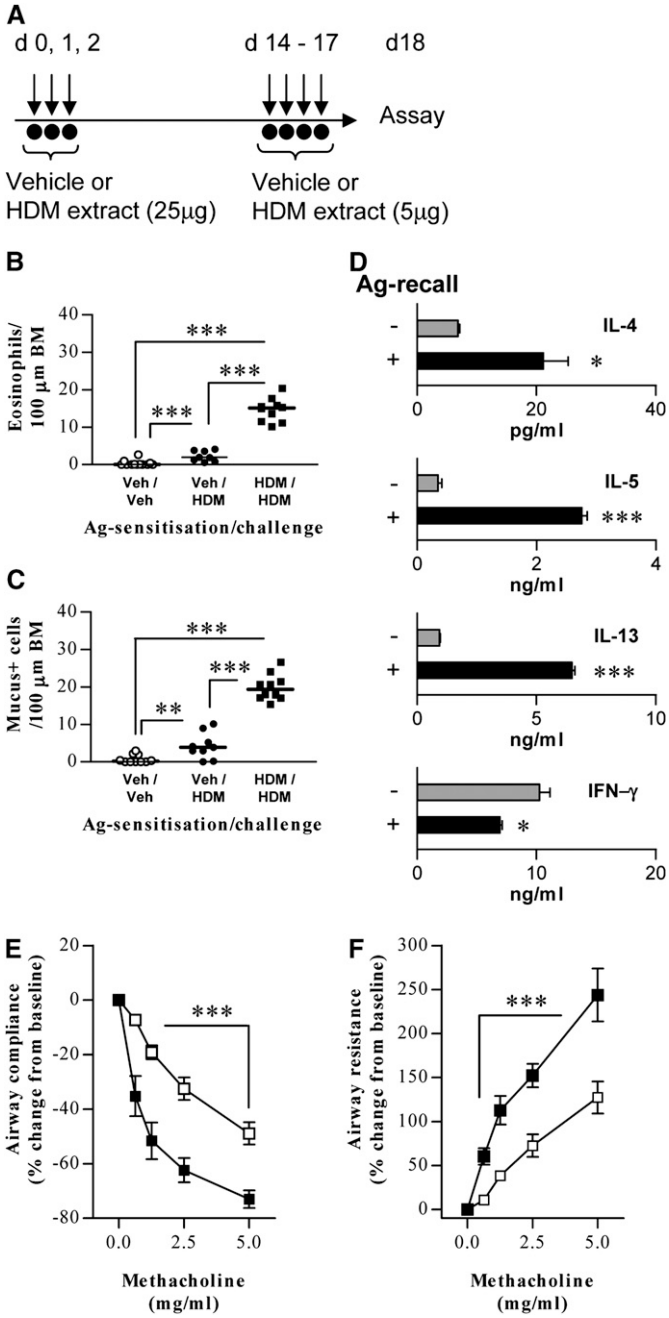

Figure 1. Acute house dust mite (HDM) immunization and challenge induce allergic airway inflammation and airway hyperreactivity. (*A*) Study design. (*B* and *C*) Lung sections from vehicle control (Veh/Veh), antigen-challenged control (Veh/HDM), and antigen-sensitized/antigen-challenged (HDM/HDM) mice were stained with chromotrope 2R (*B*) or periodic acid–Schiff (*C*) and eosinophilia in the airway mucosa and mucus-positive cells in the airway epithelia were assessed histologically. (*D*) Cytokine production in draining lymph node cells restimulated *ex vivo* for 4 days with (*solid bars*) or without (*shaded bars*) HDM. (*E* and *F*) Antigen-sensitized/antigen-challenged (*solid squares*) and "vehicle control" (*open squares*) mice were mechanically ventilated and airway compliance (*E*) and resistance (*F*) were determined by exposure to various doses of methacholine. Alterations in lung function are expressed as a percentage change from the baseline measurement (water) in response to inhaled methacholine. Data represent means ± SEM, 4–12 mice in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Ag = antigen; BM = basement membrane; veh = vehicle.

numbers of eosinophils in the challenged-only as compared with vehicle control mice, indicating that low-dose administration of HDM (over the course of four consecutive days) was able to elicit a weak innate inflammatory response. Similarly, assessment of airway mucins by periodic acid–Schiff staining revealed significantly elevated numbers of goblet cells in the antigen-sensitized/antigen-challenged mice as compared with antigen-challenged only controls (Figure 1C). Again, HDM-challenged only mice also demonstrated a significant, albeit mild, increase in mucus-secreting cells as compared with vehicle controls. *Ex vivo* HDM stimulation of draining lymph node (DLN) cells induced the production of Th2-associated cytokines IL-4, IL-5, and IL-13 but not the Th1 cytokine IFN-γ in antigen-sensitized/antigen-challenged mice (Figure 1D). Lung function measured in response to aerosolized methacholine showed increased resistance and decreased compliance in antigen-sensitized/antigen-challenged mice as compared with vehicle-exposed mice (Figures 1E and 1F), consistent with the development of allergic inflammation. Thus, acute sensitization via inhaled HDM in the absence of a classical adjuvant induced many of the cardinal features of allergic asthma.

HDM-induced Allergic Inflammation Is Dependent on the Adaptor Molecule MyD88

To investigate whether the development of this allergic response was dependent on the TLR signaling adaptor protein MyD88, we employed MyD88-deficient mice and followed the same study design. Although antigen-sensitized/antigen-challenged MyD88$^{-/-}$ mice exhibited similar numbers of mucus-secreting cells as antigen-sensitized/antigen-challenged MyD88$^{+/+}$ (wild-type) mice (Figure 2A, *left*), the numbers of eosinophils infiltrating the lamina propria was significantly attenuated (Figure 2B, *right*) between these groups. To quantify total numbers of eosinophils and neutrophils in the lung we used flow cytometry; gating granulocytes by forward and side scatter as well as anti–Gr-1 and anti-CD24 staining (Figure 2C, *top*). Using this approach we found that both eosinophils (Gr-1^{med}CD24^{hi}) (18, 19) and neutrophils (Gr-1^{hi}CD24^{med}) were significantly increased in response to antigen immunization/antigen challenge in wild-type mice as compared with control treatments, and that this infiltration was significantly abrogated in the absence of MyD88 for both granulocyte populations (Figure 2C, *bottom*).

Th2 and Th17 Responses Are Attenuated in MyD88-deficient Mice

Th2 responses have been linked to and dissociated from MyD88 signaling (20–23), although Th2 cytokine–induced AAI induced by inhalation of ovalbumin (OVA) was found to be dependent on MyD88 (24). However, a caveat of this study was the necessity for spiking of the antigen with low-dose LPS. To determine whether MyD88 was required for HDM-mediated Th2 responses, the production of T-cell cytokines from antigen-stimulated lung DLN cells was assessed. Antigen-sensitized/challenged MyD88$^{-/-}$ mice presented decreased LN cellularity (Figure 3A) and produced significantly less IL-4, IL-5, and IL-13 whereas the secretion of IFN-γ was significantly increased (Figures 3B–3E). We obtained similar results (decreased levels) when cytokine production was analyzed by intracellular cytokine staining, confirming that MyD88 contributed to HDM-induced generation of the Th2 cytokines IL-4, IL-5, and IL-13, whereas IFN-γ was not affected (data not shown). Th2 cytokines in antigen-challenged only mice were also diminished in the absence of MyD88, whereas vehicle-challenged mice failed to generate IL-4, IL-5, or IL-13 as expected (data not shown). We also measured IL-17, which is increased in the sputum and

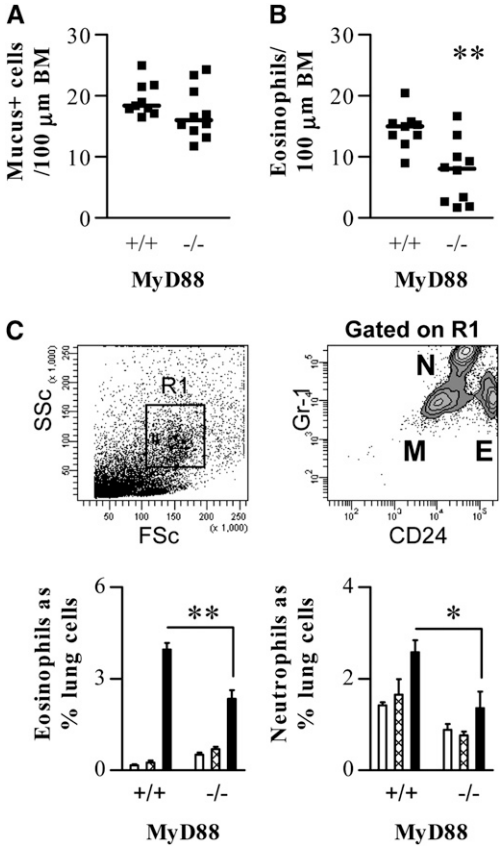

Figure 2. House dust mite (HDM)-induced allergic airway inflammation is dependent on MyD88. (*A* and *B*) Lung sections from antigen-sensitized/antigen-challenged MyD88-sufficient ($^{+/+}$) and MyD88-deficient ($^{-/-}$) mice were stained with periodic acid–Schiff (*A*) or chromotrope 2R (*B*), and mucus-positive cells in the airway epithelia and eosinophilia in the airway mucosa were assessed histologically. (*C*) Lungs of vehicle control (*open bars*), challenged control (*cross-hatched bars*), and antigen-sensitized/antigen-challenged (*solid bars*) mice were mechanically dispersed through a cell strainer and granulocyte populations were measured by flow cytometry based on scatter properties and expression of CD24 and Gr-1. Data represent means ± SEM (4–12 mice in each group) and the significance of differences was calculated between MyD88$^{+/+}$ and MyD88$^{-/-}$ strains of HDM-sensitized/challenged mice: *$P < 0.05$, **$P < 0.01$. BM = basement membrane; E = eosinophils; FSc = forward scatter; M = macrophage/monocytes; N = neutrophils; SSc = side scatter.

bronchoalveolar lavage fluid of patients with asthma as compared with healthy control subjects (25). IL-17 was produced in an antigen-specific manner, and was also dependent on the expression of MyD88 (Figure 3F). In combination with the diminished DLN hyperplasia in MyD88-deficient mice, the weak proliferation of T cells after antigen stimulation (data not shown) suggested that the absolute numbers of HDM-specific cells was greatly reduced in the absence of this molecule. Notably, the numbers of infiltrating T cells in the lung were also significantly reduced in the absence of MyD88 (Figure 3G). Thus, sensitization to a common aeroallergen induced via the target organ induced the generation of a Th2 response, and this was almost completely dependent on the adaptor protein MyD88. Th2 responses and production of IL-13 are closely associated with the development of AHR (26) and therefore we next investigated whether lung compliance and resistance were MyD88 dependent. No significant difference in baseline resistance and compliance was observed between vehicle- and HDM-exposed mice, or between different mouse strains. However, antigen-sensitized/antigen-challenged MyD88-deficient mice were significantly protected from HDM-induced AHR as compared with MyD88-sufficient controls (Figures 3H and 3I).

The Generation of Antigen-specific IgG1 Is MyD88 Dependent

To examine whether TLR activation affected the humoral response we assessed the infiltration of B cells/plasma ($CD19^+B220^+$) cells in the lungs and the pattern of antigen-specific antibody isotypes. The number of B cells was significantly elevated in antigen-sensitized/antigen-challenged mice as compared with vehicle and antigen-challenged control groups, and this response was significantly abrogated in the absence of MyD88 (Figure 3J). Notably, histopathology revealed that a number of the plasma cells were localized within the airway mucosa, suggesting that antibody production was active at the site of inflammation (data not shown). The concentration of HDM-specific IgG1, a Th2-associated immunoglobulin, was significantly elevated in antigen-sensitized/antigen-challenged MyD88-suficient but not MyD88-deficient mice (Figure 3K). In agreement with the findings of others (21), MyD88-deficient mice exhibited extremely high preimmune serum concentrations of total IgE (data not shown). The concentration of HDM-specific IgE and IgG2a (Th1 associated) was below the detection limit of the ELISA.

Myeloid and Plasmacytoid DC Recruitment Is Attenuated in the Absence of MyD88

To determine whether the dampened HDM-specific T- and B-cell responses were related to APCs in the lung, we stained the lungs for myeloid dendritic cells (mDCs; $FSc^{high}CD11c^{high}$, $autofluorescence^{low}$) and plasmacytoid dendritic cells (pDCs; $CD11c^{low}B220^+pDCA^+$). The lungs of antigen-sensitized/antigen-challenged wild-type mice had markedly elevated numbers of mDCs in comparison with either vehicle of challenge controls. Notably, mDC numbers were significantly reduced in MyD88-deficient mice as compared with MyD88-suficient mice (Figure 3L), consonant with the important role of this DC subtype in the pathogenesis of AAI (27, 28). In addition, we observed a significant increase in the numbers of lung pDCs after HDM immunization/challenge, an effect that was completely ablated in MyD88-deficient mice (Figure 3M).

HDM Extract Activates TLR2 and TLR4 on HEK-293 Cells

Because MyD88 is a common intracellular signaling molecule to the structurally related receptors for the TLR and IL-1 cytokine family (e.g., IL-1, IL-18, and IL-33) (5), we next examined whether HDM extract could directly induce the activation of individual TLRs using HEK-293 cells transfected with an NF-κB–reporting luciferase construct. The degree of immunogenicity between batches of HDM was assessed by induction of NF-κB activation in RAW-elam cells and found to be comparable (Figure 4A). HEK-293 cells expressing TLR2 or TLR4 but not TLR3, TLR5, or TLR9 were activated in a dose-dependent manner by HDM (Figures 4B–4F). All batches of HDM used during the study demonstrated the same pattern of TLR activation and the positive controls for the individual reporter cell lines induced the activation of luciferase as expected. Neutrophil elastase has been shown to indirectly activate TLR2 through the generation of an endogenous fragment of the IL-8 receptor CXCR1 (29). To exclude the possibility that HDM activated TLR2 or TLR4 via the generation of an endogenous danger signal, the HDM extract was pretreated with the cysteine protease–specific inhibitor E-64. Even in the

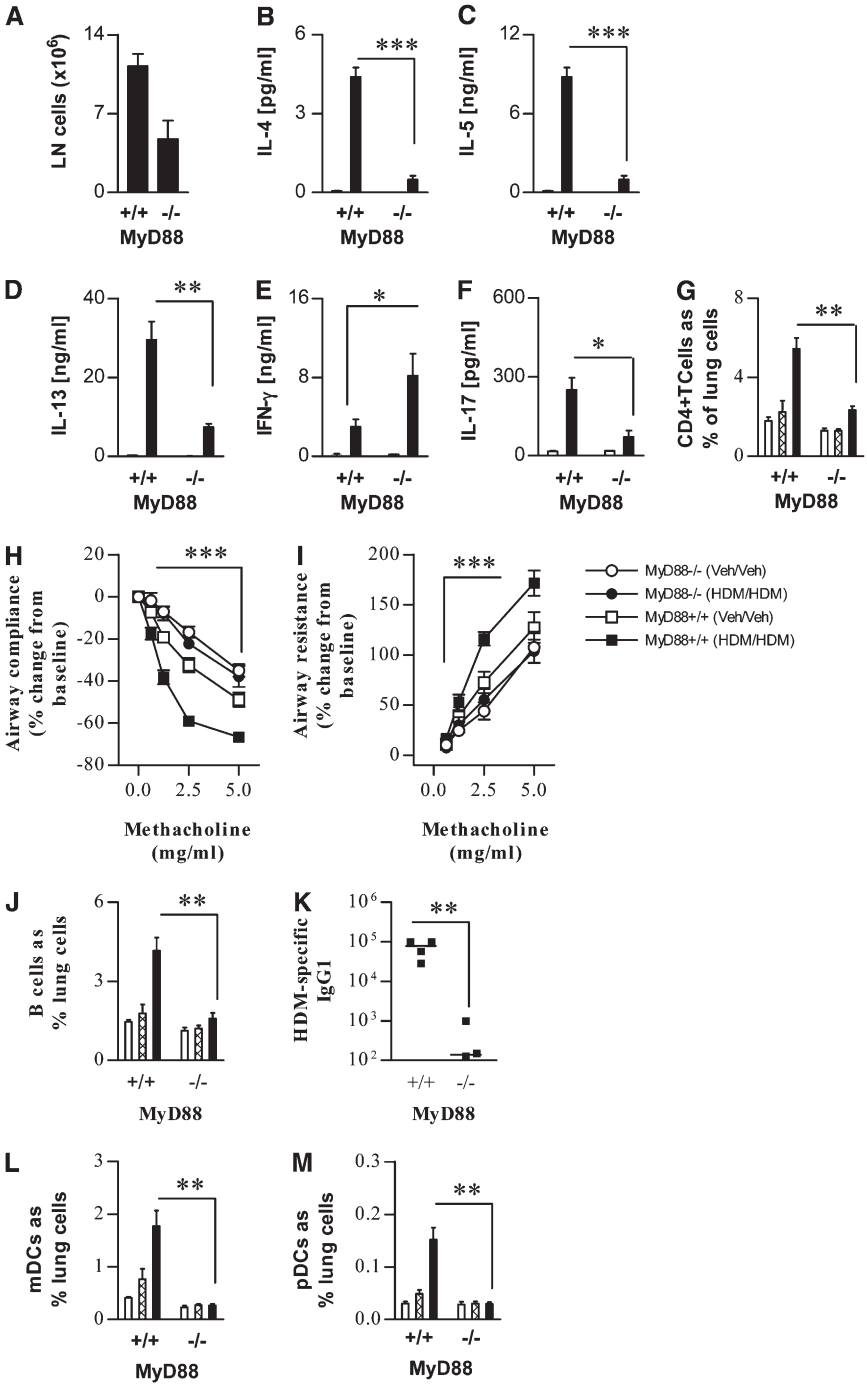

Figure 3. House dust mite (HDM)-induced helper T-cell type 2 (Th2) responses and airway hyperreactivity are MyD88 dependent. (*A–F*) Draining lymph nodes were collected from the trachea of antigen-sensitized/antigen-challenged MyD88-sufficient ($^{+/+}$) or MyD88-deficient ($^{-/-}$) mice, counted (*A*), and cultured with HDM for 4 days. Supernatants were analyzed for (*B*) IL-4, (*C*) IL-5, (*D*) IL-13, (E) IFN-γ, and (*F*) IL-17 by ELISA. *Shaded bars* represent non–antigen-stimulated cells, *solid bars* represent HDM-stimulated cells. (*G*) Lung cells from vehicle control (*open bars*), challenged control (*cross-hatched bars*), and antigen-sensitized/antigen-challenged (*solid bars*) mice were stained for T-cell markers CD3 and CD4. (*H* and *I*) Antigen-sensitized/antigen-challenged and "vehicle control" MyD88-sufficient ($^{+/+}$, *squares*) or MyD88-deficient ($^{-/-}$, *circles*) mice were mechanically ventilated and airway compliance and resistance were determined (*see* Figure 1 caption). Statistical comparisons were made between antigen-sensitized/antigen-challenged MyD88$^{+/+}$ and MyD88$^{-/-}$ mice. (*J–M*) Lung cells from mice treated as described in (*B*) were stained for (*J*) B cells (CD19^{+}CD3^{-}) and for (*L*) mDC (FSc^{hi}CD11c^{hi}) and (*M*) pDC (FSC^{med}pDCAg^{hi}B220^{+}) populations. (*K*) HDM-specific IgG1 responses were measured in sera by ELISA. Data represent means ± SEM (4–10 mice in each group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. LN, lymph node; mDC, myeloid dendritic cells; pDC, plasmacytoid dendritic cells; Veh = vehicle.

presence of this inhibitor, HDM extract was able to induce the activation of both TLR2 and TLR4 (data not shown).

HDM-induced Eosinophilia but Not Goblet Cell Hyperplasia Is TLR4 Dependent

To determine whether the TLR2 and/or TLR4 activity present in the HDM extract was a prerequisite for the generation of AAI and Th2 immunity we next subjected mice deficient in TLR2 or TLR4 to the same acute inhalational model of HDM-induced allergic asthma. In agreement with the redundant role of MyD88 in the development of GCH, neither TLR2 nor TLR4 deficiency was protective from the development of this tissue tropism (Figure 5A). These data were confirmed by measuring MUC5a gene expression (data not shown). In contrast, peribronchial eosinophilia was significantly diminished in the absence of TLR4 as compared with wild-type or TLR2-deficient mice (Figure 5B). However, neutrophil infiltration was not significantly altered in the absence of TLR4 as determined by flow cytometry (Figure 5C).

Th2 Responses Are Reduced in TLR4- but Not TLR2-deficient Mice

After acute inhalation of HDM, the magnitude of lymph node hyperplasia did not vary between wild-type, TLR2-deficient, or TLR4-deficient mice (Figure 6A). In addition, the proliferative response to recall antigen did not differ among strains either (data not shown), indicating that the absence of an individual TLR failed to affect the immunogenicity of the HDM extract. To determine whether the absence of individual TLRs altered the differentiation of helper T cells into effector cells, we next

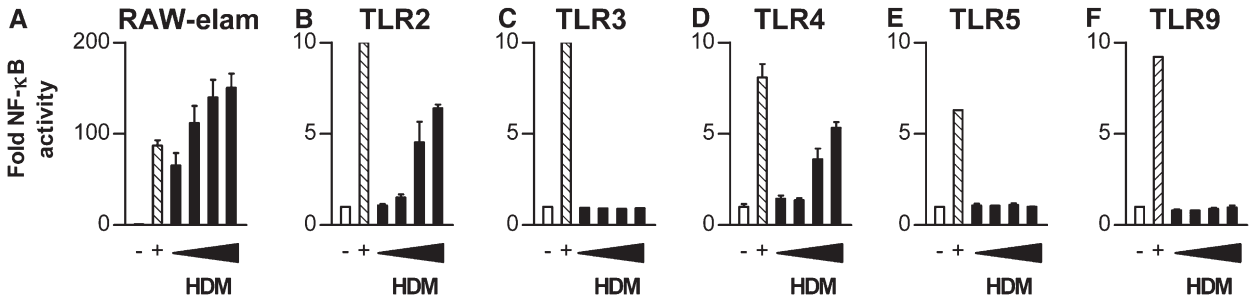

Figure 4. House dust mite (HDM) extract induces the activation of Toll-like receptor-2 (TLR2) and TLR4 but not TLR3, -5, or -9. RAW-Elam cells (*A*) or HEK-293 cells stably transfected with (*B*) TLR2, (*C*) TLR3, (*D*) TLR4, (*E*) TLR5, or (*F*) TLR9 were cultured in Dulbecco's modified Eagle's medium at 2×10^4 cells per well and stimulated with various concentrations of HDM (*solid bars, from left to right*: 0.1, 0.5, 5, and 20 μg/ml), saline (–), or relevant TLR ligand (positive control, +) for 6 hours at 37°C. Samples were tested in triplicate for each dose of ligand, and the mean ± SEM of one representative experiment shown. All four batches of HDM used in the study had comparable activity.

measured T-cell cytokine production. HDM-stimulated DLN cells from antigen-sensitized/antigen-challenged TLR2-deficient mice produced the same amount of Th2-associated cytokines as wild-type mice (Figures 6B–6D) but a reduction in the production of IFN-γ (Figure 6E). In contrast, TLR4-deficient mice generated significantly lower concentrations of IL-4, IL-5, and IL-13 as compared with wild-type mice, whereas the production of IFN-γ was unaffected (Figures 6B–6E), consistent with the reduced eosinophilia observed in the airways (Figure 5B). Because TLR4-deficient mice exhibited diminished Th2 responses in the presence of lymphoid hyperplasia and unaltered antigen-specific proliferation when stimulated *ex vivo*, we questioned whether these cells were producing IL-17. Intriguingly, HDM-specific IL-17 production was unaffected by the absence of TLR4 (Figure 6F), indicating that the generation of Th2, but not Th17, responses was dependent on TLR4. To determine whether this was functionally relevant we measured airway responsiveness to methacholine. Both HDM-induced airway compliance and airway resistance were significantly attenuated in the absence of TLR4 (Figures 6G and 6H) as

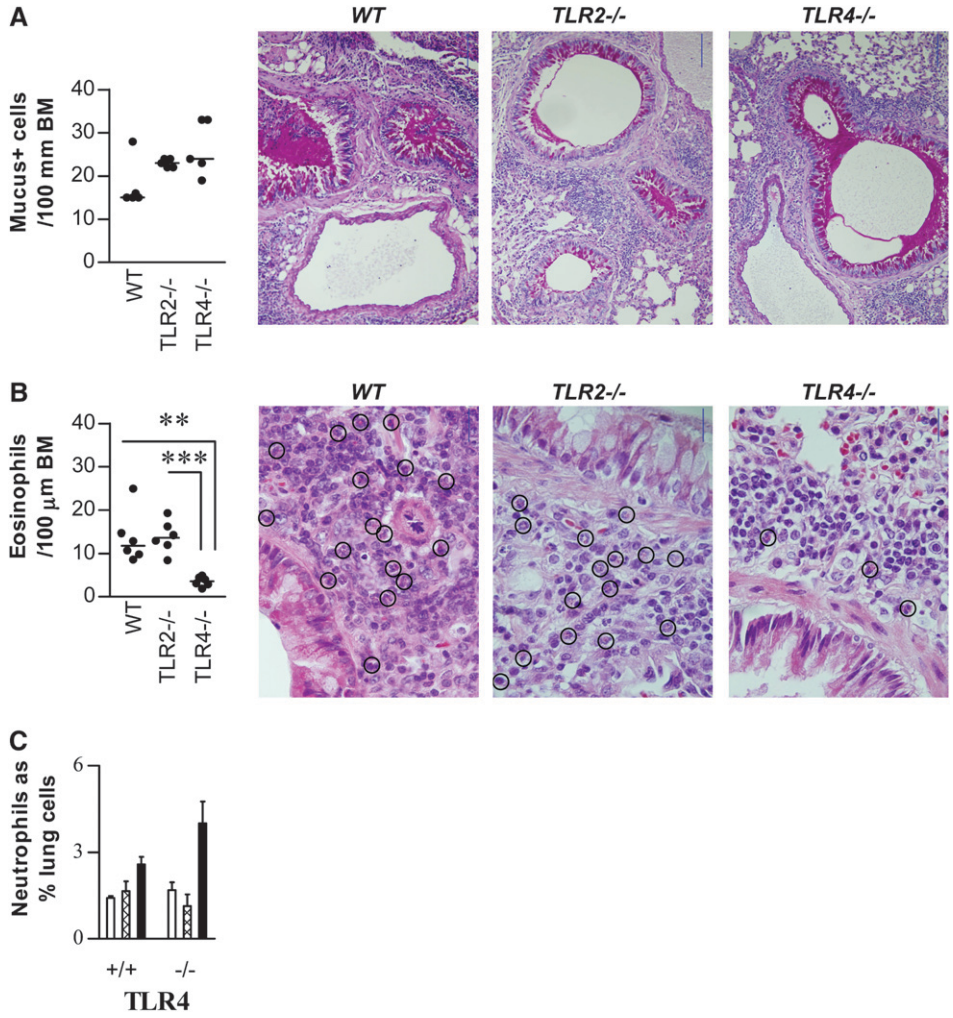

Figure 5. Toll-like receptor-4 (TLR4) but not TLR2 contributes to house dust mite (HDM)-induced allergic airway inflammation (AAI). (*A* and *B*) Lung sections were stained with (*A*) periodic acid–Schiff (PAS) or (*B*) chromotrope 2R and mucus-positive cells in the airway epithelia and numbers of mucosal eosinophils were assessed histologically. Only antigen-sensitized/challenged groups are shown. Representative photographs of mucus-positive cells and eosinophilia (*circles*) from wild-type, TLR2-deficient, and TLR4-deficient mice are shown [(*A*) *scale bar*, 200 μm; original magnification, ×100; (*B*) *scale bar*, 20 μm; original magnification, ×1,000]. (*C*) Lungs of vehicle control (*open bars*), challenged control (*cross-hatched bars*), and antigen-sensitized/antigen-challenged (*solid bars,*) TLR4-sufficient ($^{+/+}$) and TLR4-deficient ($^{-/-}$) mice were digested and neutrophil populations were measured by flow cytometry as described in the caption to Figure 2. Data represent means ± SEM (four to nine mice in each group) and statistical differences were calculated between wild-type and gene-deficient strains of HDM-sensitized/challenged mice: $^{**}P < 0.01$, $^{***}P < 0.001$. BM, basement membrane; WT, wild type.

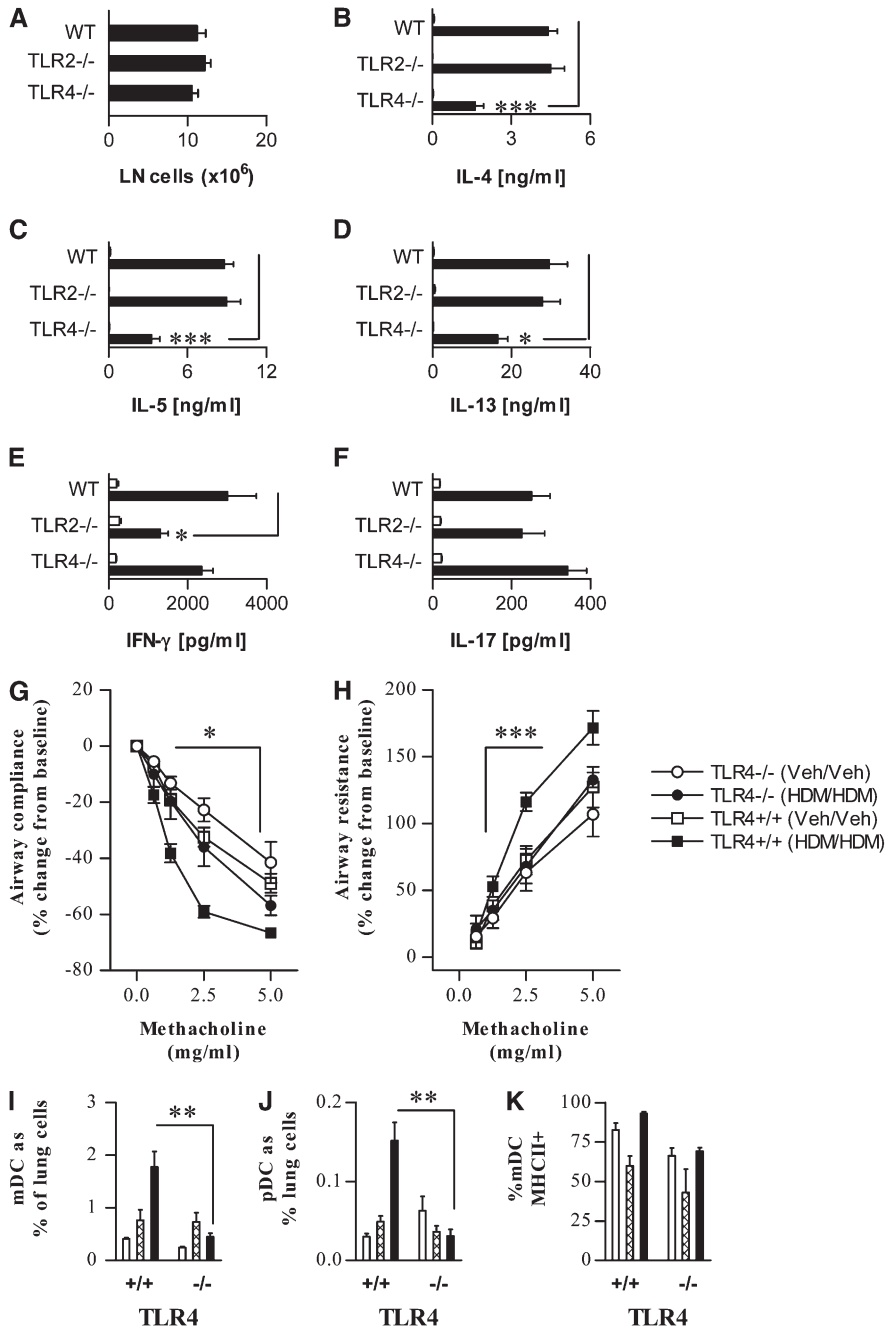

Figure 6. Toll-like receptor-4 (TLR4) deficiency attenuates helper T cell type 2 (Th2), but not Th1 or Th17, responses and confers protection from airway hyperreactivity (AHR). (*A–F*) Draining lymph nodes of antigen-sensitized/antigen-challenged wild-type, TLR2-deficient, and TLR4-deficient mice were excised and cell counts were performed (*A*). Cells were cultured at 1 million per well for 4 days in the absence (*open bars*) or presence (*solid bars*) of house dust mite (HDM) antigen and supernatants were analyzed for (*B*) IL-4, (*C*) IL-5, (*D*) IL-13, (*E*) IFN-γ, and (*F*) IL-17 by ELISA. Negligible cytokine responses were detected in non-HDM–stimulated cultures. (*G* and *H*) Antigen-sensitized/antigen-challenged (*solid symbols*) and vehicle control (*open symbols*) TLR4-sufficient ($^{+/+}$; *squares*) or TLR4-deficient ($^{-/-}$; *circles*) mice were mechanically ventilated and airway compliance (*G*) and resistance (*H*) were determined as described in the caption to Fig. 1. Statistical comparisons (analysis of variance) were made between antigen-sensitized/antigen-challenged TLR4$^{+/+}$ and TLR4$^{-/-}$ mice. (*I* and *J*) Lungs of vehicle control (*open bars*), challenged control (*cross-hatched bars*), and antigen-sensitized/antigen-challenged mice were stained for (*I*) mDC (FSc^{hi}CD11c^{hi}) and (*J*) pDC (FSC^{med}pDCAg^{hi}B220^{+}) populations, and (*K*) percent mDC MHC class II expression. Data represent means ± SEM (four to six mice in each group) and the significance of differences was calculated between wild-type and gene-deficient strains of HDM-sensitized/challenged mice: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. LN = lymph node; mDC = myeloid dendritic cells; MHCII = MHC class II; pDC, plasmacytoid dendritic cells; Veh = vehicle; WT = wild type.

compared with antigen-sensitized/antigen-challenged wild-type mice. However, the attenuated AHR observed in TLR4-deficient mice remained significantly elevated as compared with MyD88-deficient mice ($P < 0.05$ and $P < 0.01$; compliance and resistance, respectively).

We next assessed whether the reduced AAI in TLR4-deficient mice was associated with fewer mDCs in the lung. Notably, mDC recruitment was significantly reduced in the absence of TLR4 in antigen-sensitized/antigen-challenged mice (Figure 6I), as was the recruitment of pDCs (Figure 6J). To examine whether APC function was affected in the absence of TLR4, we measured the expression of major histocompatibility complex (MHC) class II, and of the costimulatory molecules CD86 and inducible costimulator ligand (ICOS-L) on lung mDCs. MHC class II expression was mildly lower on mDCs from TLR4-deficient mice compared with wild-type mice although this was apparent in both naive and HDM-exposed mice (Figure 6K). However, there was a reduced percentage of ICOS-L^{+} mDCs in the lungs of wild-type mice but not TLR4-deficient mice (data not shown).

Requirement for TLRs in mDC Migration during Primary HDM Exposure

To determine whether the attenuated AAI and Th2 cytokine production in MyD88- and TLR4-deficient mice were the result of a defect in sensitization we examined APC migration and/or maturation to the DLN during primary HDM exposure (Figure 1A, Day 3). HDM immunization of wild-type mice induced a greater than sixfold increase in the numbers of mDCs in the DLN (Figure 7A). By contrast, the numbers of pDCs were not significantly different between vehicle controls and HDM-sensitized mice (Figure 7B). As a proportion of DLN cells, mDCs were significantly lower in MyD88-deficient mice as compared with MyD88-sufficient mice sensitized with HDM

(Figure 7A). In concert with the reduced increase in lymph node cellularity (data not shown), this suggested that MyD88 is critically important for mDC migration and Th2 differentiation after primary exposure to HDM. However, this association was not apparent in TLR4-deficient mice, in which Th2 cytokine production was diminished whereas the number of mDCs in the DLN was not significantly different from that of wild-type mice. In view of these findings we investigated the effect of HDM immunization on the surface expression of MHC class II and the costimulatory molecules CD80 and OX40L, both associated with Th2 differentiation (30, 31). We were unable to evaluate DC maturation in vehicle control mice because of insufficient mDCs in these DLN. Nevertheless, in response to HDM immunization, the percentage of MHC class II–positive mDCs was significantly lower in TLR2- and TLR4-deficient mice as compared with wild-type controls (Figure 7C). CD80 expression was mildly lower in TLR4- and MyD88-deficient mice (data not shown). Importantly, the frequency of mDCs expressing the Th2-inducing costimulatory molecule OX40L was significantly lower in TLR4- and MyD88-deficient mice as compared with wild-type or TLR2-deficient mice (Figure 7D).

Finally, because DC migration to the DLN is not crucial for T-cell priming to occur in the lung (32), we addressed whether mDCs in the lung were also differentially activated in the absence of individual TLRs. In vehicle control mice, the expression of MHC class II on mDCs was low, consistent with the finding of others (32). In response to HDM immunization, MHC class II was up-regulated on wild-type and TLR2-deficient mice, but not on TLR4- or MyD88-deficient mice (Figure 7E). Taken together, these data suggest that inhalational exposure to HDM induced the maturation of mDCs in the lung and the DLN, and that this was dependent on a TLR4–MyD88-mediated signaling cascade.

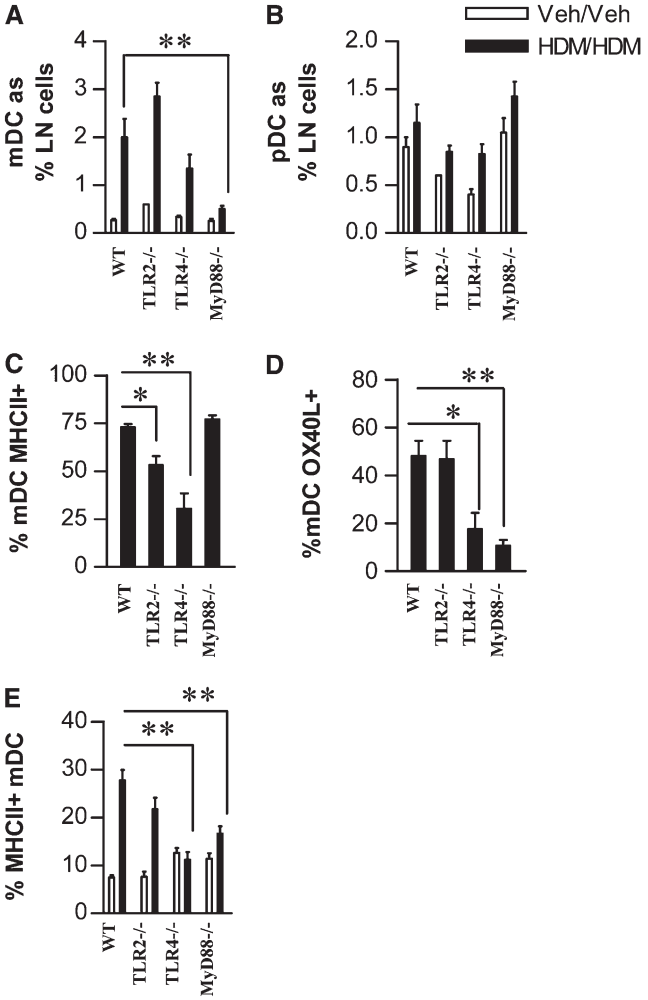

Figure 7. Dendritic cell (DC) maturation is attenuated in Toll-like receptor-4 (TLR4) and MyD88-deficient mice. (*A–E*) Wild-type and TLR2-, TLR4-, and MyD88-deficient mice were sensitized with house dust mite (HDM) or vehicle on three consecutive days (Days 0–2) and draining lymph node and lungs were excised 24 hours later (Day 3). Single-cell preparations were stained and (*A*) mDC ($FSc^{hi}CD11c^{hi}$) and (*B*) pDC ($FSC^{med}pDCAg^{hi}B220^{+}$) populations were expressed as a percentage of draining lymph node cells. Surface expression of (*C*) MHC class II and (*D*) OX40L are presented as percent positive mDCs. (*E*) mDCs in the lungs were stained for surface expression of MHC class II and are presented as percent positive mDCs and total cell numbers. Data represent means ± SEM (four to six mice in each group). The significance of differences was calculated between wild-type and gene-deficient strains of HDM-sensitized/challenged mice: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. LN = lymph node; mDC = myeloid dendritic cells; MHCII = MHC class II; pDC, plasmacytoid dendritic cells; Veh = vehicle; WT = wild type.

DISCUSSION

Activation of innate pattern recognition receptors on DCs and other accessory cells provides the critical instructions for appropriate programming of the adaptive immune response. Thus, in general, the recognition of molecular motifs associated with bacteria and viruses induces the production of IL-12 to facilitate the expansion of antigen-specific $CD4^{+}$ Th1 cells (33). However, such a paradigm is less well defined for the promotion of Th2 responses and the newly recognized subtype of Th17 cells. To address whether Th2 responses are TLR dependent we developed an experimental model of allergic asthma to the clinically relevant allergen house dust mite. We demonstrated that inhalational allergen exposure induced Th2 differentiation in the local DLN and promoted many of the hallmark features of asthma; and that these responses, with the exception of GCH, were dependent on the Toll/IL-1 receptor domain adaptor molecule MyD88. Although our HDM preparations contained TLR2 and TLR4 activity, which both signal through MyD88, we found that mice deficient in TLR2 generated a robust allergic phenotype, whereas the absence of TLR4 led to a protective response. Thus the absence of TLR4 or MyD88 attenuated HDM-induced eosinophilia, Th2 responses, and AHR, suggesting that these features were triggered through a convergent TLR4–MyD88 pathway.

The contribution of TLRs to the development of Th2 responses remains contentious (20–23, 34). This likely reflects the diverse range of antigens, exogenous adjuvants, and timing of exposure, as well as the phenomenon of site-specific innate immunity (24). The activation of TLR2 has broadly been linked to the induction of Th2 responses, although notably, the majority of these studies assessed DC and T cell cytokine profiles *in vitro* or induced AAI in response to coimmunization subcutaneously or intraperitoneally with the synthetic TLR2 ligand Pam-3-cys and OVA (12–14). By contrast, our study focused on direct sensitization without the confounding use of adjuvant or artificial spiking with microbial molecules. Importantly, Eisenbarth and colleagues have elegantly demonstrated that the route of immunization critically affects the requirement for the activation of PRRs in the development of experimental asthma (11). Our findings are consistent with and extend studies in which both antigen sensitization and challenge occurred via the nasal mucosa (11, 24), and concur with the notion that low-dose, contaminating levels of LPS (about 50 ng per sensitiza-

tion) promote the development of Th2 responses. In a similar model we have shown that coadministration of high-dose LPS during HDM sensitization can attenuate Th2 responses (data not shown), supporting the paradigm that exposure levels may dictate protective or aberrant T-cell responses and thus the development of atopy and allergic diseases (35–37). The concept that endotoxin and thus Toll/IL-1 receptor signaling is necessary for Th2 responses is also supported by Ng and colleagues, who demonstrated that house dust collected from carpeted bedrooms induced Th2 responses in an MyD88-dependent manner (38). However, of greater interest to us was the observation that only the absence of MyD88, and not TLR2 or TLR4, suppressed lymph node hyperplasia and mDC migration to the DLN, suggesting that the allergic response was not simply initiated through a linear TLR4–MyD88 pathway. Thus, an immunologic response was apparent in TLR4-deficient mice, in which the TLR2 pathway remains intact, in contrast to MyD88-deficient mice, in which TLR2 signaling is ablated.

Notably we found that wild-type sensitized and challenged mice produced IL-17 on recall stimulation with HDM. Although IL-17 has been found to be increased in the sputum of patients with asthma as compared with healthy control subjects (39), it is not typically associated with allergic asthma. Indeed, IL-17 was reported to attenuate established features of experimental asthma (40), although the role of Th17 responses in asthma remains contentious; with more recent examinations of the IL-23/IL-17 axis demonstrating both attenuation and promotion of allergic airway disease in mice (41–43). Consistent with our findings, Krishnamoorthy and colleagues also demonstrated that intranasal HDM immunization of mice provoked both Th2 and Th17 responses (44). In our model HDM-specific IL-17 production was diminished in MyD88-deficient mice but was not dependent on TLR4, unlike the production of Th2 cytokines. Indeed, in the mediastinal lymph nodes, IL-17 transcripts were markedly higher in TLR4-deficient mice and associated with elevated IL-1β, IL-6, and IL-23 (data not shown). These responses were absent in double TLR2/TLR4-deficient mice, suggesting that TLR2 ligands within the HDM extract promote a Th17-like response (data not shown). Of note, Krishnamoorthy and colleagues depleted endotoxin from the HDM extract before sensitization, consistent with the concept that HDM-specific Th17 responses are TLR4 independent. Indeed, we would speculate that removal of endotoxin from the HDM antigen enhanced the TLR2 signaling pathway and the development of a Th17 response. Collectively, these data suggest that either another PRR that uses MyD88 (e.g., TLR2) is activated to promote IL-17, or alternatively that the production of an IL-1 family member, such as IL-1, IL-18, or IL-33, which also signal through MyD88, is an upstream requirement for Th17 differentiation. In this context, it is noteworthy that the induction of IL-17 production by human naive and memory T cells was shown to be dependent on the release of IL-1β from activated DCs (6, 45), although in the mouse IL-6 and transforming growth factor-β are proposed to mediate Th17 polarization. Intriguingly, the generation of neutrophilic responses has been described as a function of Th17 cells (46) and allergic responses are often characterized by neutrophils (47, 48). Indeed, neutrophils may contribute to pathogenesis, particularly in severe disease and in noneosinophilic (neutrophil-mediated) asthma (49, 50). Notably, subjects with noneosinophilic asthma have significantly higher TLR2 gene expression in sputum and a greater prevalence of airway bacterial colonization than do subjects with eosinophilic asthma (51). Unlike the canonical OVA-induced model of asthma, neutrophils were prevalent in our model and were dependent on MyD88 but not TLR4, identical to the pattern of IL-17 production, suggesting an association between these two responses. Furthermore, although HDM-induced AHR was reduced in TLR4-deficient mice, the attenuation was not as great as that observed in the absence of MyD88, suggesting a residual component that may be neutrophil mediated. Further work will be required to determine whether TLR2-mediated recognition of bacteria promotes the development of an aberrant Th17 response that underlies the development of neutrophilic asthma.

Another striking feature of our model was the innate allergic lung inflammation exemplified by the GCH and eosinophilia that was apparent in antigen-challenged only mice, that is, after primary exposure with HDM. Thus, in contrast to OVA challenge of nonsensitized mice, primary exposure to HDM induced an inflammatory response. It remains to be established whether these responses, often regarded as features of Th2 immunity, are T cell and IL-13 dependent in this phase. With regard to primary T-cell responses, it was shown that Th2 differentiation occurs in mice sensitized with the occupational allergen papain within 3 days, although the IL-4–producing T cells were detected in the DLN in this study (52). Alternatively, intranasal immunization with fungus-associated proteinase was found to induce IL-4 production and STAT6-independent eosinophilic airway inflammation in T-cell–deficient mice within 18 hours (53). Thus, early expression of non-T-cell-derived Th2-associated cytokines may rapidly promote AAI. These data may explain why GCH was not reduced in antigen-sensitized/antigen-challenged MyD88-deficient mice even though the infiltration of $CD4^+$ T cells to the lung and the production of Th2 cytokines were reduced. Intriguingly, the allergic phenotype promoted by both fungus-associated proteinase and papain required intact proteinase activity whereas neither antigen was MyD88 dependent. In contrast, although HDM also contains both cysteine and serine protease activity, we observed that the allergic phenotype, with the exception of GCH, was MyD88 dependent whereas the *in vitro* activation of TLR2 and TLR4 was not because of its cysteine protease activity. However, we cannot exclude the possibility that the serine protease activity of HDM may lead to TLR activation *in vivo*. This is particularly important in light of a report demonstrating that proteases can cleave the neutrophil receptor CXCR8 to yield an endogenous danger signal that can activate airway epithelial TLR2 (29). In the present study we did not seek to determine the nature of the TLR ligands within the HDM extract, but it is possible that the PRR-activating molecular motifs are mite derived or expressed by commensal microbes including protozoa, bacteria or fungi that can reside within the intestinal contents of the mites (54–57). Further studies will be required to determine whether alterations in the type of mite-associated commensals would differentially affect the generation of adaptive responses in humans. An additional concept is provided by the finding that *Der p 2*, a major allergen of HDM, has structural homology to the TLR complex protein MD-2, facilitating the activation of TLR4 (58). Thus, allergenicity may be promoted by proteins that potentiate the activation of pattern recognition receptors by microbially associated molecular patterns.

DCs are necessary for both the sensitization and challenge phase of allergic asthma (59). Although myeloid DCs are proposed to mediate the proinflammatory response associated with allergic inflammation, whereas pDCs are proposed to favor tolerogenic responses (27, 28), both subtypes were increased in the lungs of antigen-sensitized/antigen-challenged wild-type mice, and both populations were reduced by the absence of either MyD88 or TLR4. Analysis of DC subtypes in secondary lymphoid tissue after immunization revealed that the absence of specific TLRs and associated development of Th responses

(data not shown) were related to the migration and maturation state of DCs in the DLN. Thus, the abrogated infiltration of mDCs into the DLN of sensitized MyD88-deficient mice was associated with a marked reduction in AAI and Th2 cytokine production, and attenuated AHR to methacholine. In contrast, the absence of TLR4 did not restrict the immigration of mDCs to the DLN, suggesting that another MyD88-dependent PRR compensated for this deficiency. This observation was consistent with the ability of the TLR4-deficient mice to generate a T-cell response, now characterized by a predominant Th17 phenotype. The association of OX40L-expressing mDCs and the generation of Th2 responses is consistent with a role for this costimulatory molecule in the development of allergic responses, although importantly we did not directly address whether this molecule was critical for the differential allergic responses observed between factor-deficient mice. The IL-7-like Th2-inducing cytokine thymic stromal lymphopoietin, the expression of which is increased by epithelia at sites of allergic inflammation, has been shown to up-regulate the expression of OX40L on mDCs (30). However, whether HDM exposure up-regulates thymic stromal lymphopoietin through a TLR4–MyD88 signaling pathway to promote allergic inflammation is unknown.

In conclusion, the present data demonstrate that Th2 differentiation and the development of associated AAI and AHR to the clinically relevant allergen house dust mite is dependent on the Toll/IL-1 receptor adaptor protein MyD88. This adaptor is used by multiple TLR pathways, but we identified the activation of TLR4 as the most likely candidate to promote the allergic response. However, the absence of TLR4 did not attenuate the development of allergen-specific Th17 responses, suggesting that the balance of danger signals or the activation of different combinations of PRR may promote distinct immune phenotypes. In this context, polymorphisms related to innate receptor signaling, TLR desensitization (as a result of infection or up-regulation of negative regulators or TIR signaling [60, 61]), or the gut flora of animal-derived allergens may all contribute toward protection from, or development of, allergic asthma.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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