

Cardiovascular, Pulmonary and Renal Pathology

Antigen and Lipopolysaccharide Play Synergistic Roles in the Effector Phase of Airway Inflammation in Mice

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T helper (Th) cells play major roles in orchestrating asthmatic airway inflammation, but the molecular mechanisms controlling Th-cell recruitment to the airways remain incompletely defined. Innate immunity contributes importantly to the recruitment of effector T cells into sites of inflammation. To understand better the role of innate immune signals in the development of airway inflammation, we used a murine model in which lipopolysaccharide (LPS) contaminating the antigen is thought to trigger Toll-like receptor 4 (TLR4). To investigate the importance of the TLR4-signaling pathway in induction of lung inflammation, we compared recruitment of adoptively transferred ovalbumin-specific Th1 and Th2 cells in wild-type and TLR4 mutant (TLR4m) mice after intranasal or aerosol challenge. Intranasal challenge of TLR4m mice with ovalbumin resulted in decreased recruitment of Th1 and Th2 cells compared with that of wild-type mice. The numbers of Th1 and Th2 cells recruited to the airways of TLR4m mice were less profoundly reduced after aerosol ovalbumin challenge. Comparing the effects of altering the dose of ovalbumin with that of LPS suggested that both contribute to the magnitude of the response in wild-type mice. Our findings demonstrate the importance of both antigen and endotoxin acting in a synergistic manner in the development of airway inflammation. (*Am J Pathol* 2006, 168:1425–1434; DOI: 10.2353/ajpath.2006.050986)

Airway inflammation is central in the pathogenesis of asthma. The mouse model, which shares many pathological and immunological features with human asthma, has increasingly been used to investigate the nature of asthmatic airway inflammation. This is because of the large

number of serological, cellular, and genetic tools available to investigate the mechanisms controlling immune and inflammatory responses in mice. Although many of the detailed molecular mechanisms that underlie the asthmatic phenotype have not been identified, T helper 2 (Th2) cells are believed to be essential for the development of allergic airway inflammation and consequent airway obstruction.¹ These cells are prevalent in lung infiltrates and airway exudates after experimental airway antigen challenge in sensitized mice.² Many of the characteristics of allergic inflammation can also be attributed to Th2 cell-derived cytokines. For example, interleukin (IL)-5 and IL-13 have been shown to be critical in the development of eosinophilia and airway hyperreactivity (AHR), respectively, observed in murine models of antigen-induced airway inflammation.^{3–5} Similarly, IL-4 is essential for Th2-cell differentiation from naïve CD4⁺ precursor T cells as well as for development of the allergic response.^{6,7}

Although the dominant immune effector functions associated with asthmatic inflammation are Th2 in character, we have observed that Th2 cells are poorly competent to initiate a tissue inflammatory response on their own.⁸ Specifically, we found that when Th2 cells were adoptively transferred into a naïve mouse, aerosol airway challenge did not induce recruitment of the transferred Th2 cells into the airways. Interestingly, when Th1 and Th2 cells were transferred together into a naïve host, airway challenge induced the recruitment of both Th1 and Th2 cells into the airway, resulting in the accumulation of large numbers of eosinophils as well. Thus, whereas Th2 cells alone were poorly competent to initiate a tissue inflammatory response, they were fully competent to enter the tissue if the environment was prepared, in this case by recruitment of Th1 cells. Once in the lungs,

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these cells were also fully competent to drive the local inflammatory response to that of a Th2 character. In related studies, we found that other inflammatory signals, including local treatment with lipopolysaccharide (LPS) or local activation of mast cells via an IgE-dependent mechanism, were able to recruit Th2 cells into the lungs and airways.⁹

Respiratory infections have been linked both to the development of susceptibility to asthma and to the expression of asthmatic inflammation, implicating contributions of signals from Toll-like receptors (TLRs) in this Th2-driven airway disease.¹⁰ Of particular interest in this regard is TLR4, which can be activated by LPS, a cell wall component of gram-negative bacteria that is ubiquitous in the environment.¹¹ LPS contaminates most environmental antigens, including household dust.¹² After binding LPS or other ligands, TLR4 together with the accessory proteins CD14 and LPS-binding protein can activate cells, enhancing their expression of inflammatory cytokines, chemokines, and adhesion molecules.¹¹ Endotoxin has the potential to contribute to the development of asthma in several different ways; studies indicate its potential either to suppress the development of asthma through induction of counterregulatory Th1 cells or to exacerbate asthma severity, presumably through its pro-inflammatory activities.^{1,10,13} It has been shown that activation of TLR4 can modulate the induction of Th2 responses in a dose-dependent fashion.¹⁴ Furthermore, very high doses of LPS (25 $\mu\text{g}/\text{mouse}$) instilled into the airway activate recruitment of Th2 cells into the lungs, supporting robust Th2-type airway inflammation.⁹ Using a different mouse model of asthma, Lundy et al¹⁵ showed that challenge with very high doses of LPS (15 $\mu\text{g}/\text{challenge}$ repeated for a total of four times) significantly inhibits AHR and eosinophilic airway inflammation in previously sensitized mice, whereas challenge with low doses of LPS (0.3 $\mu\text{g}/\text{challenge}$ repeated for a total of four times) shows only a minimal effect on AHR and the recruitment of eosinophils. It was also shown that instillation of exogenous tumor necrosis factor (TNF) into the airway could overcome deficient signaling through the TLR4 pathways to generate effector Th2 cells.¹⁶ Additional investigation is needed to define the role of TLR4 in mobilization of the airway inflammatory responses characteristic of acute asthma, because the amounts of LPS previously used greatly exceed the amounts likely to be encountered in most cases of human asthma.

To understand the specific role of TLR4 and antigen in stimulating airway inflammation, we used adoptive transfer of *in vitro*-cultured chicken ovalbumin (OVA)-specific Th1 and Th2 cells, followed by either aerosol or intranasal antigen challenge. We found a strong correlation between antigen dose and degree of eosinophilic inflammation after intranasal challenge. Signals delivered by TLR4 exerted a greater impact on the recruitment of Th2 cells and the development of inflammation after intranasal (i.n.) challenge compared with after aerosol challenge. We also found that exogenous TNF administered intranasally bypassed the requirement for TLR4 sig-

naling to induce CD4 T-cell recruitment and airway inflammation.

Materials and Methods

Mice

BALB/c and C.C3H-Tlr4Lps-d (TLR4m) mice (Jackson Laboratory, Bar Harbor, ME) were used between 6 and 12 weeks of age. DO11.10 mice (DO), which are transgenic for a T cell receptor (TCR) that recognizes the chicken ovalbumin OVA peptide (323–339) presented by I-A^d, were the generous gift of Kenneth Murphy (Washington University, St. Louis, MO). Thy1.1⁺ DO mice were a gift of Osami Kanagawa (Washington University). All mice were kept in microisolator cages in the specific pathogen-free Animal Resources Program facility at the University of Alabama at Birmingham. They were fed and watered *ad libitum*, and all experiments were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Preparation of Differentiated T Helper Cells

OVA-specific Th1 and Th2 cells were generated by 2 rounds of *in vitro* culture of DO spleen cells with irradiated syngeneic spleen antigen-presenting cells, antigen, and cytokines as described previously.⁸ The T-cell culture medium was Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 2×10^{-5} mol/L 2-mercaptoethanol, 1 mmol/L sodium pyruvate, 100 $\mu\text{mol}/\text{L}$ nonessential amino acids, 10 $\mu\text{g}/\text{ml}$ penicillin, 10 $\mu\text{g}/\text{ml}$ streptomycin, and 2 mmol/L L-glutamine (all from Gibco-BRL Life Sciences, Grand Island, NY). For the first round, splenocytes from the DO mice were made into a single cell suspension using a disposable mesh (Becton Dickinson, Franklin Lakes, NJ) and cultured at $5 \times 10^6/\text{ml}$ with 0.3 mmol/L OVA_{323–339} peptide. After 3 days, the cells were split, and IL-2 (40 U/ml; PharMingen, San Diego, CA) was added. Differentiation to yield Th2 cells was induced by culture with recombinant IL-4 (40 ng/ml; R&D Systems, Minneapolis, MN) and 10 $\mu\text{g}/\text{ml}$ anti-IL-12 hybridoma supernatant (TOSH; generously provided by Emil R. Unanue, Washington University), whereas differentiation to Th1 cells was induced by adding 10 ng/ml IL-12 (PharMingen) and 10 $\mu\text{g}/\text{ml}$ of the anti-IL-4 monoclonal antibody 11B11 (kindly provided by William E. Paul, National Institutes of Health, Bethesda, MD). Seven days after culture initiation, cells were washed and frozen. For the second round of culture, frozen cells were thawed, washed, and plated at approximately 5×10^5 cells/ml with fresh irradiated BALB/c splenocytes as antigen presenting cell together with the OVA peptide and the differentiating cytokines as in round 1. Cells were split at day 3 with fresh IL-2. After culture for 6 hours with phorbol 12-myristate 13-acetate (10 ng/ml), ionomycin (1 $\mu\text{mol}/\text{L}$), and monensin (2 $\mu\text{mol}/\text{L}$) at either day 6 or 7 into the second round of culture, approximately 50% of the CD4⁺ cells in the Th1 cell culture were interferon- γ (IFN- γ)⁺/

IL-4⁻, and 40% of the cells in the Th2 cell culture were IFN- γ ⁻/IL-4⁺ as shown by intracellular staining. Less than 0.5% of the cells in the Th1 cultures were IFN- γ ⁻/IL-4⁺, and less than 0.5% of the cells in Th2 cultures were IFN- γ ⁺/IL-4⁻. Cells were used for adoptive transfer 7 days after the second stimulation.

Determination of Th1 and Th2 Recruitment: Adoptive Transfer and Challenge

Differentiated, resting DO Th cells were transferred intravenously to naïve recipient mice. The following day, antigen challenge was performed twice, 6 hours apart. Aerosol challenges were performed as described previously using 1% OVA in sterile phosphate-buffered saline (PBS) (Gibco).⁸ For i.n. challenge, different concentrations of OVA in 30 μ l of sterile PBS were administered twice, 6 hours apart, to mice anesthetized briefly with isoflurane (Schering-Plough Animal Health Corp., Union, NJ).

Reagents Used for Airway Challenge

OVA (grade V) and *Salmonella typhosa* lipopolysaccharide were purchased from Sigma (St. Louis, MO). Lipopolysaccharide from *Salmonella abortus equi* S-form was purchased from Alexis Corp. (San Diego, CA). Stock solutions of OVA were prepared at 10 mg/ml (1% w/v) in pyrogen-free PBS. The amount of endotoxin contaminating this OVA stock solution was approximately 8 ng/ml as measured by limulus amebocyte assay (BioWhittaker, Walkersville, MD). Using this assay, we were able to detect as little as 0.5 ng/ml endotoxin contamination. No endotoxin was detectable in sterile PBS using this method. Recombinant mouse TNF was purchased from R&D Systems and mixed with the OVA solutions used for airway challenges to make a final concentration that provided 1 μ g per challenge per mouse. When added, TNF administration was done only at the first challenge.

Analysis of Airway and Lung Inflammation

Mice were sacrificed 3 days after challenge by lethal injection using 50 μ l of a mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml), and their airways were assessed for inflammation. The trachea was cannulated, and airway inflammatory cells were obtained by bronchoalveolar lavage (BAL) with four 0.8-ml aliquots of ice-cold 2% fetal bovine serum in PBS. Red blood cells were lysed using 8.3 g/L ammonium chloride and 10 mmol/L Tris-HCl, and nucleated cells were counted using a hemacytometer. Eosinophil counts were determined using cytospin (Shandon Cytospin 3; Shandon Inc., Pittsburgh, PA) preparations of the BAL cells followed by Diff-quick staining (Dade Behring Inc., Deerfield, IL). Other BAL cells were identified by antibody staining and fluorescence activated cell sorter (FACS) analysis. The transferred Th1 and Th2 cells were tracked using fluorescein isothiocyanate (FITC)-labeled clonotypic antibody for the

DO TCR, KJ1-26-FITC (Caltag, Burlingame, CA). Other antibodies and reagents used were anti-Thy1.1-PE, anti-Thy1.1-FITC, anti-CD69-biotin, anti-CD4-PE, anti-CD4-APC, and streptavidin-APC (PharMingen). Fluorescently labeled cells were detected using a FACSCalibur (Becton Dickinson) with CELLQuest software (version 3.3; Becton Dickinson). For histological studies, lungs were fixed with 10% formalin, and then the tissues were embedded in paraffin. Sections (5 μ m) were stained with periodic acid-Schiff and hematoxylin for light microscopic examination.

Statistical Analyses

Statistical analyses were performed by two-tailed equal variant Student's *t*-test. A *P* value below 0.05 was considered statistically significant.

Results

Differing Inflammatory Responses in the Airway After Aerosol and Intranasal Challenge

In previously published studies analyzing airway inflammation after specific antigen challenge of sensitized mice, both aerosol and intranasal administration of antigen have been used; however, the differences between the two routes of antigen challenge have not been well defined. To investigate potential differences in the recruitment of CD4⁺ T cells to the airways after aerosol or i.n. antigen challenge, we used an experimental protocol that focuses primarily on the tissue recruitment portion of the inflammatory process.⁸ We generated polarized populations of OVA-reactive Th1 and Th2 cells from DO Thy1.2⁺ and Thy1.1⁺ mice. Th2 cells (10⁷) with or without 10⁷ Th1 cells were transferred intravenously into naïve BALB/c recipient mice. The next day, one group of mice was challenged by i.n. administration of 30 μ l of 1% OVA in PBS, whereas the other group was challenged by 30 minutes of exposure to an aerosol of OVA, also 1% in PBS. Second doses of antigen were administered using the same techniques 6 hours later. Two control groups received mock adoptive cell transfers with intravenous injection of PBS alone, followed by challenge with i.n. or aerosol antigen. On the third day after challenge, BAL was performed, and cells were counted and analyzed for eosinophil recruitment by cytospin preparation (Figure 1). Mice that received either PBS or Th2 cells followed by aerosol challenge did not show detectable airway inflammation, whereas mice that received a mixture of Th1 and Th2 cells followed by aerosol challenge developed eosinophilic airway inflammation as described previously.⁸ Compared with these groups, mice challenged i.n. using 1% OVA in 30 μ l of PBS developed easily detectable amounts of eosinophilic inflammation in both the animals that received Th2 cells alone and those that received a mixture of Th1 and Th2 cells. We concluded that the quality and quantity of the allergic immune response is importantly influenced by the route used for antigen administration at the time of challenge.

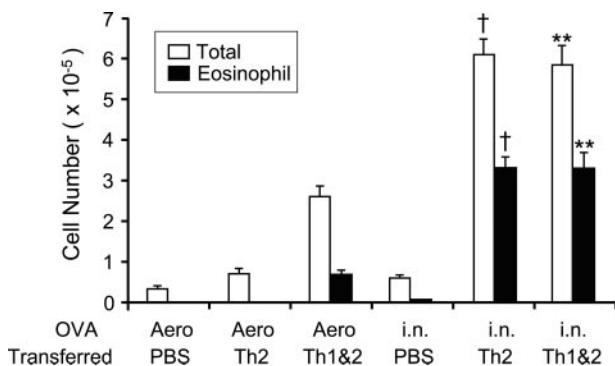


Figure 1. Aerosol and intranasal challenges yield different airway inflammatory responses. Th1 and Th2 cells were grown *in vitro* and then were transferred intravenously to naïve BALB/c mice. Mice were challenged using either 1% OVA aerosol (Aero) or 1% OVA in 30 μ l of PBS instilled i.n. Total cells (open bars) and eosinophils (closed bars) recovered in BAL fluid were measured at day 3 after the challenge by cytospin preparation. (mean \pm SEM, $n = 3$; $**P < 0.005$ and $^{\dagger}P < 0.00001$, respectively, compared with mice challenged with OVA aerosol) These data are representative of three independent experiments.

Airway Inflammation Is Sensitive to the Dose of Intranasal Antigen Challenge

Because our initial experiments showed that i.n. challenge with 1% OVA induced a greater magnitude of airway inflammation compared with challenge with 1% OVA aerosol, we reasoned that the different inflammatory responses after these different methods of antigen challenge might be a result of different amounts of antigen delivered. To address this, we analyzed the airway inflammatory responses after i.n. challenge with various dilutions of OVA. We compared the responses in mice that received either Th2 cells alone or a mixture of Th1 and Th2 cells. As shown in Figure 2, airway inflammation 3 days after OVA challenge diminished as the concentration of the challenge antigen was reduced. Mice challenged with concentrations of 0.01% OVA or higher developed more vigorous airway inflammation than mice challenged with aerosol OVA (data not shown). After intranasal challenge with 0.003% OVA, the numbers of total cells and eosinophils in BAL were similar to those observed in mice challenged with aerosol 1% OVA. At this dose of i.n. antigen, the animals did not develop eosinophilic inflammation when

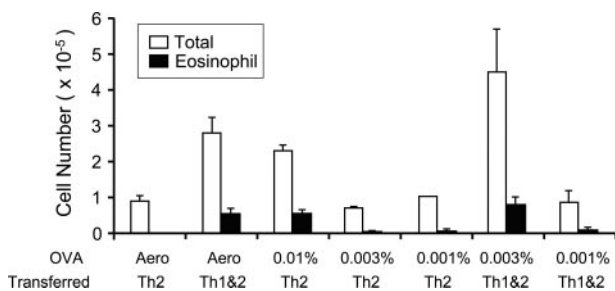


Figure 2. Titration of i.n. antigen dose. DO Th1 and Th2 cells were generated *in vitro* and then transferred adoptively into naïve mice. On the following day, these mice were challenged using different doses of i.n. OVA in 30 μ l of PBS or 1% OVA by aerosol (Aero.). BAL cells were harvested 3 days after the challenge. Eosinophils were counted using cytospin preparations, followed by Diff-quick staining. Data shown are mean \pm SEM, $n = 3$. We obtained similar results in four different sets of experiments.

they had been treated with adoptive transfer of Th2 cells only. On the other hand, mice that received adoptive transfer of Th1 and Th2 cells together showed recruitment of eosinophils and other inflammatory cells in response to the 0.003% dose of i.n. OVA. The inflammatory response observed after challenge with 0.003% i.n. OVA mirrored results observed after aerosol challenge with 1% OVA. We concluded that the quality of the allergic inflammatory response is dependent on the dose of antigen used for challenge. We subsequently used i.n. challenge with 0.03% (designated "high-dose") and 0.003% (designated "low-dose") OVA to induce Th1 cell-independent and Th1 cell-dependent inflammation, respectively.

TLR4 Participates Importantly in the Recruitment of Th1 and Th2 Cells After Intranasal Challenge

We previously reported that i.n. instillation of lipopolysaccharide resulted in recruitment of Th2 cells to the lungs and airways.⁹ Because detectable quantities of LPS contaminate the solutions of OVA used here and the amount of contaminating endotoxin may be similar to the amount that humans inhale daily, we tested whether this endotoxin might contribute to the airway inflammation observed in either the high- or low-dose challenge models using wild-type and TLR4 mutant mice. To use adoptive transfer of Th cells from the I-A^d-restricted DO TCR transgenic strain, we used mice in which the C3H/HeJ TLR4 mutation had been backcrossed onto the BALB/c background (TLR4m). Both TLR4m and age-matched wild-type mice received *in vitro*-differentiated Th2 cells or a mixture of Th1 and Th2 cells, followed by intranasal challenge with 0.03% or 0.003% OVA. Wild-type recipient mice developed the pattern of eosinophilic airway inflammation we had previously observed; however, TLR4m mice had reduced numbers of eosinophils and total leukocytes in their airways 3 days after the challenge (Figure 3a). Further analysis of the BAL cells by flow cytometry demonstrated that, compared with results in control mice, the numbers of transgenic Th2 cells were significantly reduced in TLR4m mice that had been treated with adoptive transfer of Th2 cells only and were then challenged with high-dose i.n. OVA (Figure 3b). In addition, Th1 cell recruitment into the airways of TLR4m mice was also low when both Th1 and Th2 cells were transferred and i.n. challenge was with 0.003% OVA (Figure 3b). To assess the degree of parenchymal lung inflammation that was associated with the airway inflammatory response observed in these mice, we fixed and stained the lungs of mice 3 days after antigen challenge as described in Materials and Methods. Both wild-type and TLR4m mice showed no evidence of inflammation after mock cell transfer using PBS alone and challenge with the high dose of OVA (Figure 4, a and d). Wild-type mice that received DO Th2 cells or a mixture of DO Th1 and Th2 cells followed by challenge

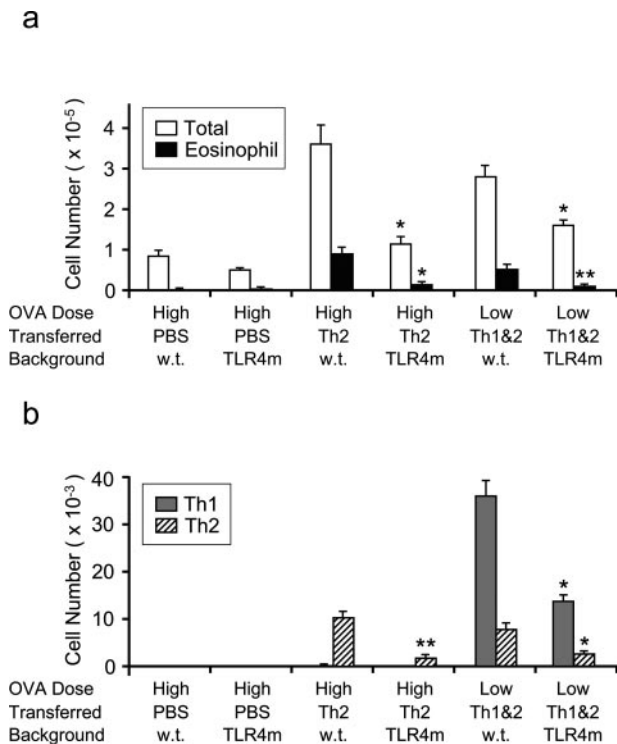


Figure 3. A crucial role for TLR4 in airway inflammation after intranasal OVA challenge. *In vitro*-cultured Th1 and Th2 cells were transferred into either wild-type or TLR4m mice. Mice were challenged with the indicated doses of i.n. OVA on the following day, and BALs were performed 3 days after the challenge. **a:** Total cells (open bar) and eosinophils (closed bar) were counted by hematocytometer and cytopsin preparation. **b:** Recruited Th1 (gray bars) and Th2 (hatched bars) cells were counted by flow cytometry (mean \pm SEM, $n = 5$; * $P < 0.05$ and ** $P < 0.005$ compared with wild-type control). Similar results were obtained in four additional experiments.

with high or low doses of OVA, respectively, developed very mild parenchymal inflammation. Substantial portions of the lung, including essentially all of the alveolar tissue, showed no inflammation. Mixed cellular infiltrates were detected around medium-sized blood vessels and their associated conducting airways, with the infiltrates consisting of both mononuclear and polymorphonuclear cells. The airways in many of the areas of mild inflammation showed associated increases in mucus production (Figure 4, b and c). Under similar conditions of Th2 cell transfer and airway antigen challenge, TLR4m mice showed minimal tissue inflammation but occasional airways with very modestly increased mucus production (Figure 4e). In contrast, when TLR4m mice were treated with adoptive transfer of a mixture of Th1 and Th2 cells followed by intranasal challenge with the low dose of OVA, we detected only very low levels of parenchymal leukocytes and no evidence of increased mucus production. Together, analyses of infiltrating cells recovered by BAL and of tissue inflammation assessed by histology show that the TLR4 signaling pathway plays a key role in support of the recruitment of both Th1 and Th2 cells after intranasal airway antigen challenge. Our data demonstrate further that under conditions of acute airway antigen challenge, substantial luminal airway inflammation develops before the accumulation of large parenchymal infiltrates. Whether this dissociation of parenchymal and luminal inflammation is entirely the result of the acute time course or is partly the result of the absence of sensitized CD8⁺ T cells and sensitized B cells in this model remains to be investigated.

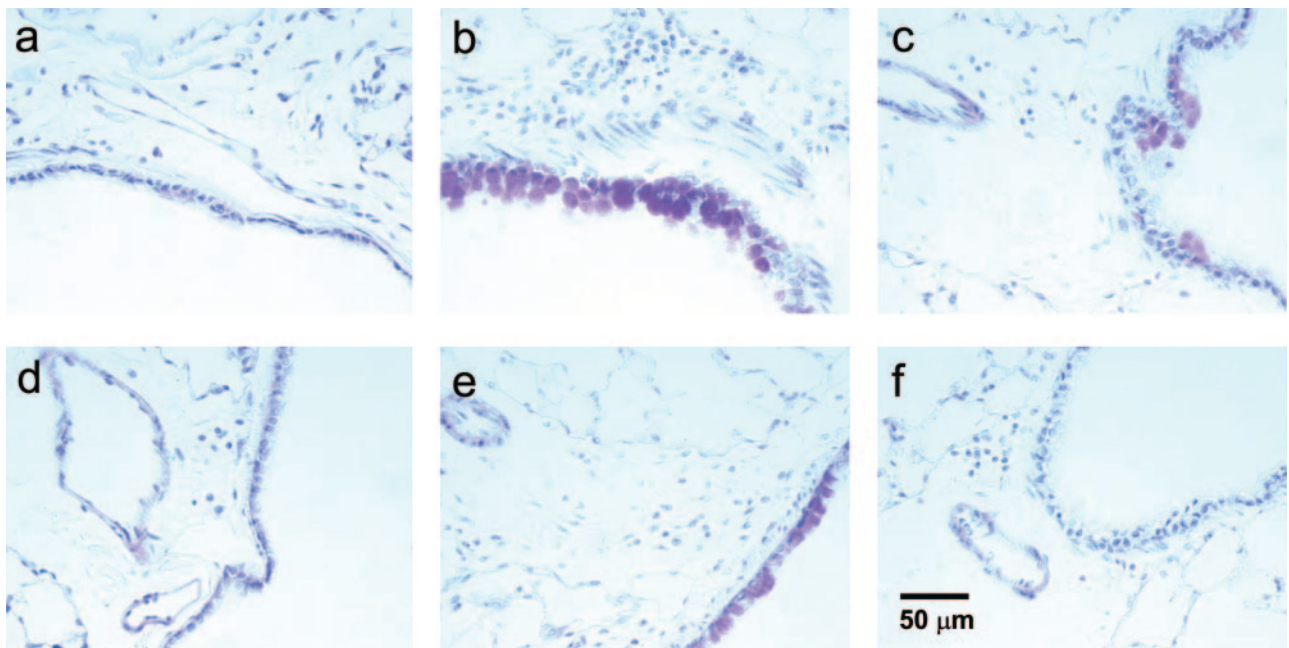


Figure 4. Airway antigen challenge after adoptive Th-cell transfer induces modest, TLR4-dependent lung parenchymal inflammation. Th2 cells (b and e) or a mixture of Th1 and Th2 cells (c and f) were transferred intravenously to naïve wild-type (a–c) or TLR4m (d–f) mice. PBS was used for the mock cell transfer (a and d). Mice were challenged the following day with either the low-dose (Th1+Th2 cells) or the high-dose (Th2 cells only) OVA protocol. Tissues were harvested 3 days after challenge, fixed with buffered formalin, and 5- μ m sections were analyzed by periodic acid-Schiff and hematoxylin staining. Original magnification, $\times 400$.

Neutrophil Influx Was Significantly Decreased in TLR4m Mice After *i.n.* OVA Challenge

We have consistently observed the recruitment of neutrophils into the lungs and airways shortly after antigen challenge (data not shown). Because LPS can bind to TLR4 and then induce the mobilization of neutrophils after instillation into the airways,¹⁷ we next tested whether *i.n.* OVA induced the recruitment of neutrophils into the airways in an endotoxin-dependent fashion. We challenged naïve mice with 1% OVA *i.n.* and counted the numbers of neutrophils recovered by BAL using cytopsin preparations. Because the influx of neutrophil in the airway peaks 9 to 12 hours after OVA challenge (data not shown), we harvested BAL cells 12 hours after the challenge. Compared with wild-type mice, the recruitment of neutrophils into the airway of TLR4m mice was nearly completely abolished (Figure 5a). The numbers of neutrophils recruited did not change with the addition of adoptively transferred T cells (data not shown), indicating that this neutrophil influx represents part of the innate immune response. Because in our initial experiments, mice were challenged with both high and low doses of OVA to induce airway inflammation, we addressed whether these doses were sufficient to recruit significant numbers of neutrophils into the airways. Twelve hours after either high- or low-dose OVA challenge, both groups of mice showed similar mild neutrophilic airway inflammation (Figure 5b). Our data show that in this model, neutrophil influx is dependent on the TLR4 signaling pathway and that OVA challenge triggers both innate and adaptive immune responses.

Increasing the Amount of LPS in the Low-Dose OVA Challenge Solution Resulted in the Recruitment of More Th2 Cells to the Airways but Did Not Increase the Intensity of the Total Airway Cell Recruitment Response Compared with Mice Challenged with the Low Dose of OVA

Because our previously published data indicated that instillation of LPS into the airway induced the local recruitment of Th2 cells⁹ and our data here showed that signaling through TLR4 is required for the normal inflammatory response to *i.n.* antigen, we questioned whether the difference in the amount of contaminating endotoxin was responsible for the different inflammatory responses seen after high- and low-dose *i.n.* OVA challenge. We first measured the amount of LPS in the stock OVA solution by using the limulus ameocyte assay. The 1% stock solution of OVA contained approximately 8 ng LPS/ml. Thus, in the 30- μ l aliquot used for *i.n.* dosing, there was a total of 240 pg of LPS. The high-dose regimen (0.03% OVA), in contrast, delivers 7.2 pg of endotoxin per challenge, and the low-dose regimen (0.003% OVA) delivers 0.72 pg of endotoxin per challenge. Using this information, we compared the airway inflammatory response in mice

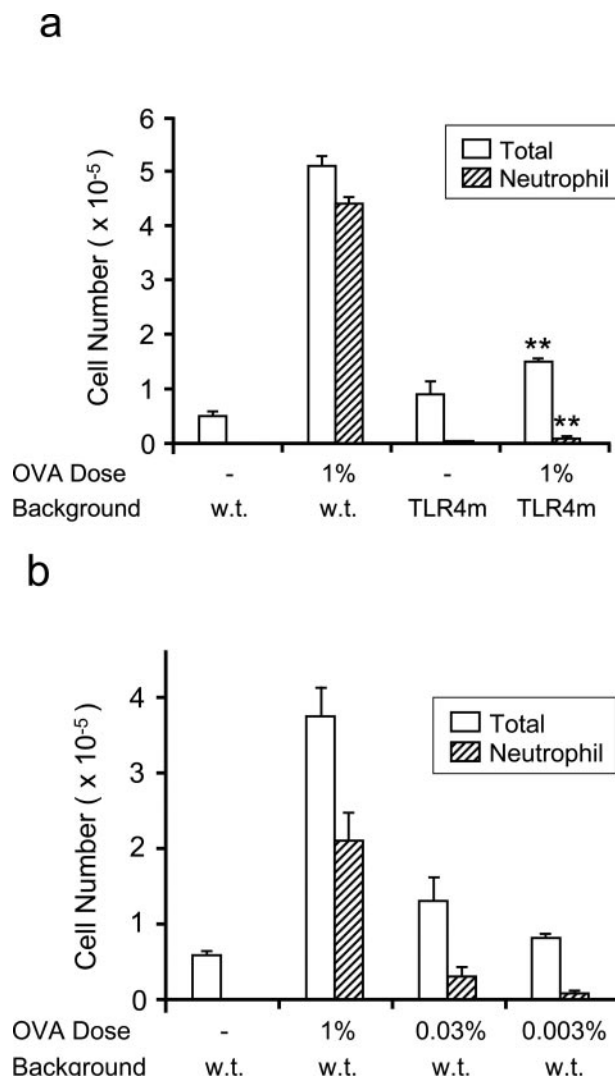


Figure 5. Neutrophil influx shortly after *i.n.* OVA challenge. **a:** Reduced numbers of neutrophils in TLR4m mice after *i.n.* challenge with 1% OVA. **b:** Mice were challenged with either high or low dose of OVA intranasally. BAL cells were harvested 12 hours after *i.n.* OVA challenge. Cells were counted using cytopsin preparations. Shown are total cells (open bars) and neutrophils (closed bars) (mean \pm SEM, $n = 3$; ** $P < 0.005$, compared with wild-type control).

treated with adoptive transfer of 10^7 DO Th2 cells before challenge with 30 μ l of 0.003% unsupplemented OVA (0.72 pg of LPS), 30 μ l of 0.003% OVA supplemented with 6.48 pg of LPS (7.2 pg of LPS total), or 30 μ l of 0.03% unsupplemented OVA (7.2 pg of LPS). Three days later, airway inflammation was assessed by BAL. The mice that received *in vitro*-differentiated Th2 cells followed by the high-dose regimen of *i.n.* OVA challenge (0.03%) showed the expected airway inflammation with recruitment of both DO Th2 cells and eosinophils, whereas those that were challenged with the low-dose regimen of *i.n.* OVA (0.003%) showed few recruited DO Th2 cells and very few eosinophils (Figure 6, a and b). Mice that received Th2 cells followed by *i.n.* challenge with 0.003% OVA supplemented with sufficient LPS to equal the amount present in the high-dose challenge showed recruitment

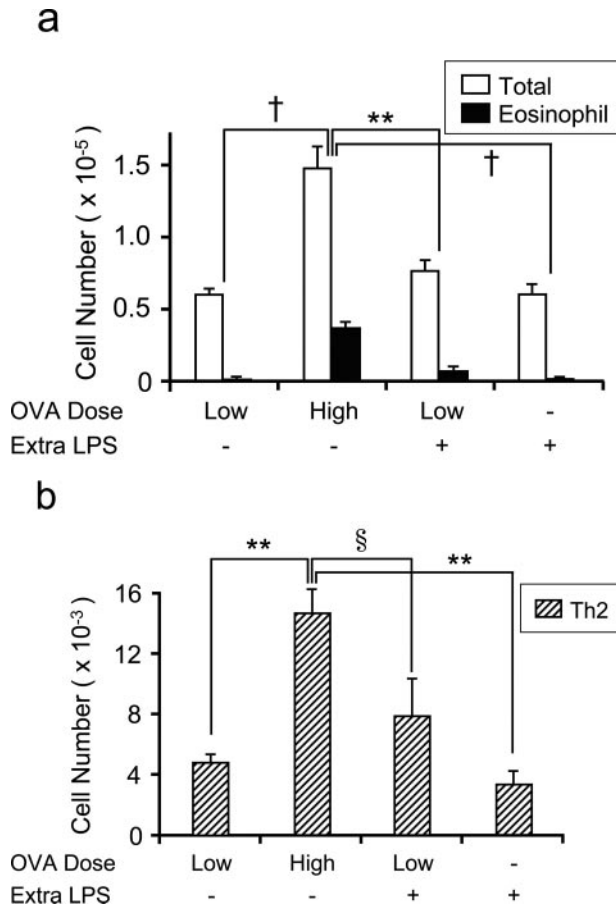


Figure 6. No changes in the quality of airway inflammation after airway challenge with OVA plus supplemental LPS. Supplemental LPS (6.48 pg of LPS) was added to 30 μ l of low-dose OVA solution and used to challenge mice that received *in vitro*-cultured Th2 cells. The total amounts of LPS in each OVA challenge solution were verified using the limulus amoebocyte assay. **a:** Inflammatory cells were harvested from the airways 3 days after the challenge, and total cells (open bars) and eosinophils (closed bars) were determined by cytospin preparations. **b:** Recruited Th2 cells were counted using flow cytometry (mean \pm SEM, $n = 5$; ** $P < 0.005$, † $P < 0.0001$, and § $P = 0.069$, respectively, compared with challenge with high-dose OVA). This experiment is representative of five additional experiments.

of eosinophils and total inflammatory cells at a level similar to that detected with the low-dose challenge. Interestingly, the number of recruited Th2 cells into airways increased when mice were challenged with low-dose OVA plus supplemental LPS but not to the level observed in mice challenged with high-dose OVA. Because we do not know the microbial sources of the LPS that contaminates in our OVA preparation, we tested two different types of LPS from two different types of gram-negative bacteria purchased from two different sources, and the data showed identical results (data not shown). These data suggest that increasing the amount of LPS in the challenge antigen results in recruitment of higher numbers of Th2 cells but that low-dose OVA plus supplemental LPS supports the development of the higher level of airway inflammation observed with the high-dose OVA challenge.

Exogenously Administered TNF Bypasses the Inflammatory Defect in TLR4m Mice

TLR4m mice manifest a major defect in innate immune responsiveness. Because lifelong deficiencies of single gene products in mutant mice can lead to compensatory changes in expression of other genes or to structural or regulatory alterations that might preclude development of a normal immune response, we tested whether the reduced airway inflammatory responses seen after *i.n.* OVA challenge in TLR4m mice were solely due to the lack of acute TLR4-mediated signals. Because TNF is a potent pro-inflammatory cytokine produced in both Th1 and Th2 type immune responses,¹⁸ and because it serves as a downstream signal in the TLR4 activation pathway,¹¹ we used this cytokine to mimic TLR4 signals in these mutant mice. Thus, we tested whether *i.n.* instillation of TNF (1 μ g per 30- μ l challenge) could restore Th2 cell and eosinophil recruitment in the TLR4m mice. As shown in Figure 7a, when TLR4m mice that had been treated with adoptive transfer of OVA-specific Th1 and Th2 cells were challenged *i.n.* with the low dose of OVA supplemented with TNF, they developed eosinophilic airway inflammation equal to that seen in wild-type recipients of Th1 and Th2 cells that had been challenged with OVA alone. In addition, when TNF was added at the time of *i.n.* OVA challenge, wild-type mice developed more vigorous inflammation than wild-type mice challenged with OVA alone. Not only were TLR4m mice able to develop normal eosinophilic inflammation, but they also manifested recovered recruitment of the adoptively transferred Th2 cells (Figure 7b). In a similar set of experiments in which mice received adoptive transfer of only Th2 cells, *i.n.* administration of TNF restored normal levels of total airway inflammation and eosinophil recruitment after high-dose OVA challenge of TLR4m mice (Figure 7c). Analysis of BAL cells by flow cytometry showed that exogenous TNF enhanced the recruitment of adoptively transferred OVA-specific Th2 cells in both the low- and high-dose antigen challenge models (Figure 7d). We also found that exogenous TNF was able to induce pulmonary neutrophilia in TLR4m mice (data not shown). These data suggest, therefore, that TNF can overcome defective inflammatory cell recruitment in TLR4m mice by restoring the recruitment of Th2 cells. This demonstrates further that lifelong deficiency of TLR4 does not cause an absolute inability to generate Th2-driven airway inflammation but rather results in an inability to respond appropriately to airway antigen challenge.

Discussion

In an effort to clarify innate immune mechanisms that contribute to the development of airway inflammation after antigen challenge, we focused on the effector phase of the inflammatory response. We developed an experimental protocol in which the sensitization phase can be circumvented by the adoptive transfer of previously activated and differentiated antigen-specific CD4⁺ T cells. Using this model, we have been able to investigate the

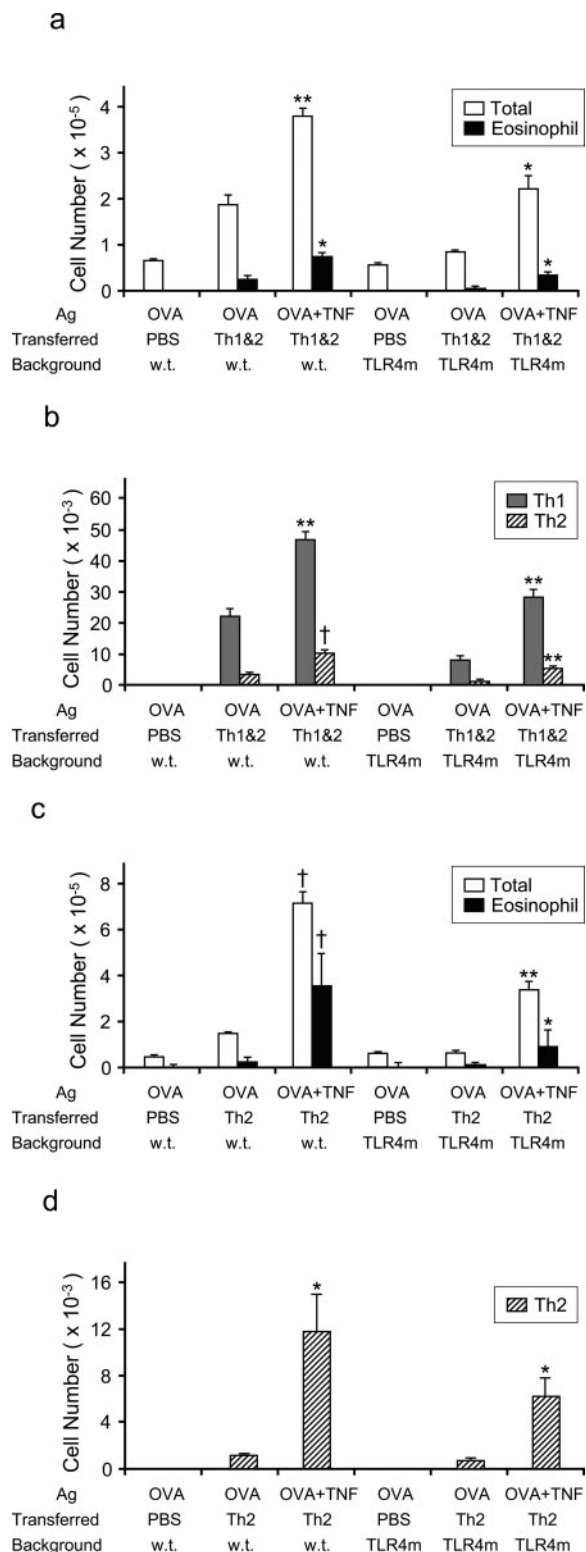


Figure 7. Intranasal instillation of TNF restores airway inflammation in TLR4m mice challenged with i.n. OVA. Th2 cells (10^7) (**a** and **b**) or a mixture of Th1 and Th2 cells (**c** and **d**) were transferred to either wild-type or TLR4m mice. These mice were challenged using either low-dose OVA or low-dose OVA plus 1 μ g of TNF and were analyzed 3 days later. **a** and **c**: Total cells (open bars) and eosinophils (closed bars) are shown. **b**: Transferred Th1 cells (gray bars) and Th2 cells (hatched bars) recovered in BAL are shown. **d**: Th2 cells recruited into the airways are shown (mean \pm SEM, $n = 4$; * $P < 0.05$ and ** $P < 0.005$ compared with wild-type control). Data shown are representative of three independent experiments.

impact of innate immune stimuli in the recruitment of inflammatory cells to the airways. Our first evidence for a contribution of innate immune mechanisms to the antigen-induced airway inflammatory response came from a comparison of the inflammation induced by either aerosol or intranasal OVA challenges. Differences in the quality of airway inflammation were due not only to the amount of antigen delivered by these two challenge methods but also to signals delivered through pathways activated by TLR4 signaling. We previously showed that a high dose of LPS can induce the recruitment of adoptively transferred Th1 and Th2 cells in this model. In the current study, we extend our understanding of the role of endotoxin by experiments in which the doses of endotoxin contaminating the challenge antigen are at lower, physiologically relevant levels. Activation of TLR4 was also critical for the development of the neutrophilic inflammation seen at early time points after antigen challenge (Figure 5). Interestingly, exogenously administered TNF was able to overcome the deficiency of TLR4 signaling, suggesting that TNF signaling may be an event downstream of TLR4 signal transduction.

In previously published studies, TLR4m mice have been used to investigate the role of endotoxin in the development of Th2 type airway immune responses.^{14,19} In these studies, two different routes of antigen administration (i.n. and intraperitoneal) were used to elicit systemic sensitization. Both reports showed that TLR4m mice exhibited blunted airway inflammatory responses, regardless of the route of sensitization used. In addition, dendritic cells (DCs) were shown to be key regulatory cells in the sensitization phase of the response, with DCs requiring activation by LPS to participate effectively in systemic sensitization. In other studies, it has been suggested that DCs are the major cell type that presents antigen to T cells in the lung, whereas alveolar macrophages have little ability to induce T-cell proliferation.²⁰ With this in mind, it is possible that DCs in the lungs of TLR4m mice may not mature fully after intranasal antigen challenge. Consequently, they may be unable either to recruit adoptively transferred T cells or to activate these recruited T cells after treatment with airway antigen.

Neutrophils have recently been increasingly recognized to have immune regulatory function. Especially, neutrophils can modulate the subsequent trafficking of other leukocytes. Notably, Hurst et al²¹ have shown that neutrophils release soluble IL-6 receptor into sites of inflammation, where soluble IL-6 receptor-dependent signaling can switch inflammatory cells from a neutrophil-predominant exudate to one that consists primarily of monocytes and T cells. In other studies, Tacchini-Cottier et al²² demonstrated a key role for neutrophils in Th2-driven immunity, showing that neutrophil-depleted mice had deficient Th2 type responses and that this correlated with increased susceptibility to infection with *Leishmania major*. Although asthma differs in many ways from infectious diseases, we propose that neutrophils may similarly play an important role in recruiting Th1 and Th2 cells to the allergen-challenged airway. We hypothesize that the inability of TLR4m mice to express a fully developed allergic airway inflammatory response may at least in part

be due to their lack of endotoxin-induced neutrophil recruitment into the lungs and airways and the subsequent downstream defects that result from this lack of neutrophil response.

After LPS binding, signal transduction from TLR4 depends on interactions with additional cellular signaling molecules for an endotoxin response to occur.²³ The major endotoxin response is dependent on myeloid differentiation factor 88 (MyD88) and leads to the activation of nuclear factor- κ B (NF- κ B). This activated NF- κ B induces the expression of a pro-inflammatory response, including production of the cytokines TNF and IL-18. Other responses to LPS rely on the TIR domain-containing adapter-inducing interferon- β protein. Its activation leads to the activation of NF- κ B and to the induction of cytokines, chemokines, and other transcription factors. Because exogenously administered TNF was able to rescue the deficient Th-cell recruitment in TLR4m mice, it is most likely that the recruitment of Th cells depends on the MyD88 signaling pathway. If so, this underscores the importance of MyD88 in allergic airway inflammation, because prior studies showed that it was also required for the sensitization of Th cells for allergic responses to intranasal antigen.²⁴

LPS in asthma has been described as a two-edged sword. The hygiene hypothesis suggests that exposure to endotoxin early in life can reduce the risk of later atopy.²⁵ However, a correlation has also been seen between asthma severity and endotoxin levels in household dust, identifying endotoxin as a key risk factor for asthma in atopic children.^{26,27} Suggestive evidence that endotoxin might be more important in inducing asthmatic symptoms than previously thought is provided by a study demonstrating that acute inhalation of LPS by asthmatic patients induced airway hyperresponsiveness.²⁸ Additional progress in understanding the role of endotoxin in the expression of airway inflammatory responses would be facilitated by the development of technologies for accurate determination of the quantities of endotoxin that reach the lower airway in both natural and experimental challenge models. Given the extremely low levels of endotoxin that can elicit significant biological effects and the diversity of microbial structures that possess endotoxin activity, this remains a major technical hurdle. Given that it is virtually impossible to avoid exposure to environmental endotoxins, it has been suggested that therapeutic targeting of LPS binding protein, also called LBP, might serve to better control disease in asthmatic individuals.²⁹ These studies, taken together with our current study, suggest that the time of exposure and exposure levels are critical for determining the ultimate response to endotoxin taken up via the airways.

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