

TLR4 Signaling Attenuates Ongoing Allergic Inflammation¹

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The relationship between LPS exposure and allergic asthma is poorly understood. Epidemiologic studies in humans have found that exposure to LPS can protect, have no effect, or exacerbate allergic asthma. Similarly, LPS has had variable effects on allergic pulmonary inflammation in the mouse, depending on the model used. In the present study, we studied the effect of very low doses of LPS in models of both short-term and long-term allergen challenge. When challenged with allergen for short periods, wild-type and *tlr4*-deficient mice had similar responses. However, when challenged for periods of 1 wk or longer, *tlr4*-deficient mice developed dramatically increased airway eosinophils, serum IgE, and Th2 cytokines compared with similarly challenged, genetically matched C57BL/6 mice. The relative attenuation of allergic responses seen in C57BL/6 mice was dependent on bone marrow-derived cell-specific expression of *tlr4*, and was not associated with an increase in Th1 responses. The number of dendritic cells in lungs of challenged *tlr4*-deficient mice was significantly increased compared with those in challenged C57BL/6 mice. No differences were seen in the abilities of naive C57BL/6 and *tlr4*-deficient mice to develop allergen-specific tolerance after exposure to similar preparations of OVA, suggesting that tolerance and regulation of existing inflammation develop through different mechanisms. The attenuation of eosinophilic inflammation in C57BL/6 mice was abolished when these mice were challenged with OVA supplemented with additional LPS. Together, these findings show that low doses of endotoxin can have regulatory effects on allergic inflammation, particularly in the setting of ongoing allergen exposure. *The Journal of Immunology*, 2006, 176: 5856–5862.

Asthma remains a major cause of morbidity in developed nations and is a leading cause of hospitalization (1). Despite the widespread prevalence of this disease, the genetic and environmental determinants that contribute to progression of asthma remain poorly understood. One example of such an environmental agent is LPS, or endotoxin. This bacterial cell wall product is commonly found in dust from many domestic and occupational sources (2). Several studies have found that endotoxin can exacerbate existing asthma (3–5), whereas other studies have shown that exposure to endotoxin in childhood can protect against developing asthma later in life (6), but even this finding is controversial (3, 7). The latter finding is part of a larger body of epidemiologic evidence associating exposure to pathogens or their products early in life with protection against developing asthma or atopy in adult life (8). The molecular and cellular mechanism underlying such a relationship has not been established. It has been suggested that pathogens or their products elicit Th1 immune responses, which down-regulate the Th2 cells, a hallmark of allergic asthma. However, recent studies suggest that a simple change in

the ratio of Th1 and Th2 cytokines does not account for the ability of pathogens to protect against the progression of asthma (8, 9).

Animal models of asthma are frequently used to explore mechanisms of allergic responses. In the most widely used mouse model of asthma, animals are first sensitized by an i.p. injection of OVA complexed with aluminum hydroxide (alum). OVA challenge of sensitized mice results in many of the responses seen in asthmatic individuals, including eosinophilic inflammation, production of Th2 cytokines, increases in serum IgE, and airway hyperreactivity. However, there is considerable variation in procedures that different laboratories use to challenge sensitized mice. In some studies, allergen exposures are restricted to a single day (10), or continue for 3 days, 5 days, 7 days, 10 days, and even as long as 6 wk (11). Other differences include the route of challenge, which can be by intratracheal instillation, oropharyngeal application, or inhalation of OVA-based aerosol. In view of these procedural differences among various laboratories, it is perhaps not surprising that endotoxin has been reported to both increase (12–15) and decrease (16–22) the intensity of allergic responses, and a comprehensive understanding of the mechanisms by which endotoxin affects allergic responses has remained elusive.

The goals of the present study were 2-fold. First, we sought to determine whether very low levels of endotoxin, closer to those found in ambient environments, would impact pulmonary allergic responses in an experimental setting. Second, we investigated whether the duration of allergen exposure affected the impact of endotoxin on allergic responses. Because low levels of endotoxin are present in commercially available OVA (16), we studied allergic responses to this protein in C57BL/6J mice and genetically matched mice lacking the TLR4 (*tlr4*), which is required for signaling responses to endotoxin (23). These studies revealed that wild-type and *tlr4*-deficient mice had similar responses to OVA containing low levels of endotoxin after short-term allergen exposures. However, *tlr4*-deficient mice had dramatically enhanced pulmonary allergic responses compared with C57BL/6 mice when

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challenged with OVA for prolonged periods. These findings demonstrate that in the presence of very low levels of endotoxin, *tlr4*-dependent signaling attenuates ongoing allergic responses in the lung.

Materials and Methods

Mice

The *tlr4*^{-/-} and *tlr2*^{-/-} mice were provided by Dr. S. Akira (Osaka University, Osaka, Japan) (24), and backcrossed for 8–10 generations onto a C57BL/6J background. Age- and gender-matched C57BL/6J mice were purchased from The Jackson Laboratory. Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Duke University Medical Center, and were conducted in accordance with the standards established by the U.S. Animal Welfare Acts.

OVA sensitization and challenge

Mice were sensitized on days 0 and 7 by i.p. injections of 10 μ g of OVA (Sigma-Aldrich) complexed with aluminum hydroxide (Pierce). Beginning on day 14, mice were exposed daily for 20 min to an aerosol of 1% OVA (Sigma-Aldrich) generated using an Ultra-Neb99 (DeVilbiss Healthcare). The number of daily allergen exposures ranged from 1 to 21. Animals were harvested 24 h after the last aerosol challenge.

Analysis of airway inflammation, airway hyperresponsiveness, and serum IgE

Whole lung lavage and cell differentials were determined, as previously described (25). A luminex instrument (Bio-Rad) was used to evaluate protein concentrations of IL-2, IL-5, and IFN- γ (Bio-Rad). ELISA kit was used to evaluate IL-13 (R&D Systems) and IFN- β (PBL Biomedical). Serum IgE and IgG2a levels were detected by ELISA (BD Pharmingen). Unrestrained whole-body plethysmography (Buxco Electronics) was conducted, and measurements were obtained at baseline and after stimulation with inhaled methacholine (0, 5, 10, and 25 mg/ml), as previously described (25).

Analysis and removal of endotoxin from OVA

Levels of endotoxin in OVA were measured by the *limulus* amebocyte lysate assay (BioWhittaker), and were $\sim$ 70 EU/mg. In some experiments, endotoxin in the OVA was reduced with Detoxi-Gel (Pierce). In other experiments, the OVA was supplemented with additional endotoxin (Sigma-Aldrich), and generated aerosols with this mixture. Aerosol was sampled from Hinner chamber by drawing 80 L of air through a filter and measuring *limulus* amebocyte lysate activity. The level of endotoxin in the aerosols include: air (0 μ g/m³), endotoxin-reduced OVA (0 μ g/m³), commercially available Sigma-Aldrich OVA (0.11 μ g/m³), and contaminated OVA (0.18 μ g/m³).

Generation of bone marrow chimeric mice

Bone marrow chimeric mice were created, as previously described (26). Briefly, 4×10^6 bone marrow cells from donor mice were transferred into lethally irradiated (10.5 Gy) recipient mice. Donor marrow engraftment was assessed by flow cytometric analysis of peripheral blood leukocytes 6 wk after the transplant, including: CD3⁺/CD4⁺ T cells, 83.4–99.4%; CD3⁺/CD8⁺ T cells, 77.8–99.9%; and B cells, 98.2–99.3%.

Flow cytometric analysis

To prepare parenchymal dendritic cells (DCs),⁴ lungs were perfused with HBSS/collagenase/DNase, minced, and digested at 37°C for 30 min. Lung tissue from five animals per group was pooled. Samples were then transferred to Ca-free HBSS/EDTA and layered over a 17% metrizamide solution and centrifuged at $700 \times g$. Cells at the interface were recovered, counted, blocked (CD16/CD32 (Fc γ RIII/II), murine IgG, rat IgG), and stained with mAbs, including: CD11c, CD11b, I-A/I-E, Gr-1, CD3, CD19, CD40, CD80, CD86, B220, ICOS, IL-10, and glucocorticoid-induced TNFR. For airway DCs, 1×10^6 lavaged cells from lung lavage were resuspended in 100 μ l of sterile endotoxin-free PBS with 0.1% sodium azide. Mediastinal lymph nodes were prepared similarly to parenchymal DCs. Cells were analyzed using FACSVantage SE (BD Biosciences) and counts were calculated automatically by FlowJo software (Tree Star).

Statistics

Data are expressed as mean $\pm$ SEM. Significant differences between groups were identified by ANOVA. Individual comparisons between groups were confirmed by Student's *t* test, unless otherwise stated. A two-tailed *p* value of <0.05 was considered statistically significant.

Results

TLR4 negatively regulates responses to ongoing allergen challenge

To gain insight into mechanisms by which TLR4 affects allergic responses in the lung, we first compared pulmonary allergic responses in *tlr4*-deficient mice with those of genetically matched C57BL/6J mice in an acute model of allergen challenge (10). Sensitized and challenged *tlr4*-deficient mice had slightly more airway eosinophils than similarly sensitized and challenged C57BL/6J mice, but in most experiments this difference did not reach statistical significance (Fig. 1A). These data show that *tlr4* does not markedly impact sensitization to OVA, or initial responses to the aerosolized protein.

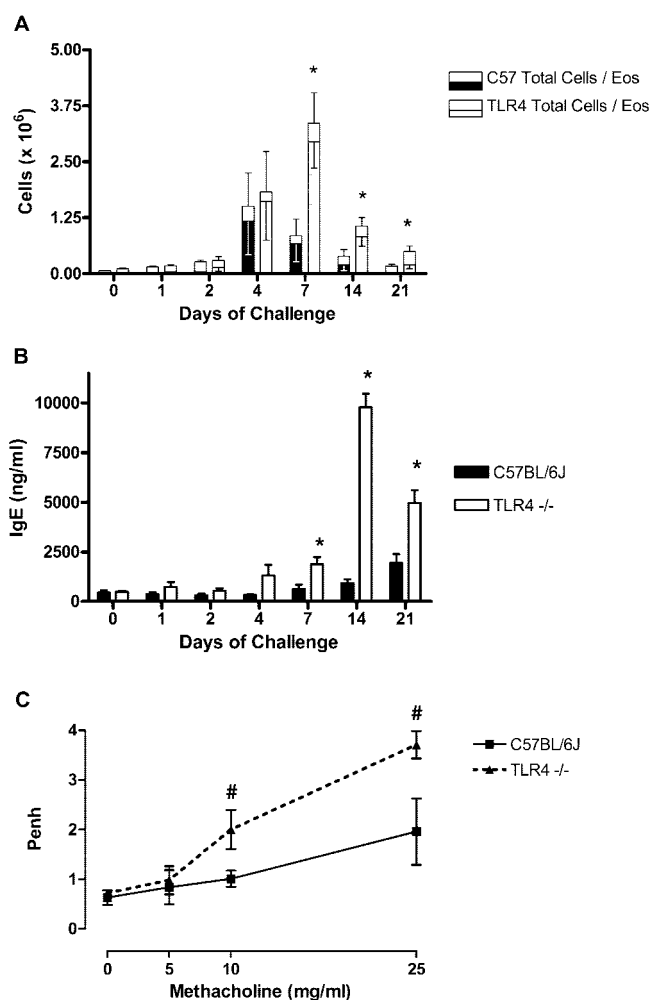


FIGURE 1. *tlr4* signaling attenuates ongoing pulmonary allergic responses. Mice were sensitized twice with OVA, then challenged daily with aerosols of this allergen and harvested 24 h after the last aerosol challenge. Mice were harvested at various points during this challenge, and allergic responses were determined by analysis of airway eosinophils (A), serum IgE (B), and airway hyperresponsiveness (C). Note that differences in airway cells between *tlr4*-deficient and C57BL/6 mice become apparent beginning after $\sim$ 1 wk of daily challenges (*, $p < 0.01$), and that airway hyperreactivity is different between the two strains after 21 days of challenge (#, $p < 0.05$).

⁴ Abbreviations used in this paper: DC, dendritic cell; Treg, regulatory T cell.

Allergens are not normally encountered by asthmatics on a single day, but rather on a continuing basis, and it is in this context that exposure to endotoxin has been shown to protect against progression of asthma. We therefore conducted a series of experiments in which wild-type and *tlr4*-deficient mice were exposed to various numbers of aerosol-based daily challenges. As the number of daily challenges increased, dramatic differences between C57BL/6 and *tlr4*-deficient mice became more apparent. In C57BL/6 mice, total cells and eosinophilic inflammation in the airway peaked after 4 days of challenge, and declined thereafter. By contrast, in *tlr4*-deficient mice, the inflammation continued to intensify until they had undergone exposures for 7 consecutive days. At this time, the inflammation in *tlr4*-deficient mice finally began to subside, but remained significantly elevated compared with C57BL/6 mice for the duration of the experiment. Even after 21 days of exposures, when the inflammation of both strains had subsided substantially, airway eosinophils in *tlr4*-deficient mice remained elevated 16-fold over C57BL/6 mice. This increased allergic inflammation in *tlr4*-deficient mice demonstrates that *tlr4* signaling leads to the attenuation of ongoing allergic responses in the lung. This finding is consistent with the wealth of epidemiologic data showing that ongoing exposure to low levels of endotoxin protects against asthma.

In addition to eosinophil recruitment, Ig class switching from IgG to IgE and increases in airway hyperresponsiveness are other well-described hallmarks of allergic inflammation. We therefore measured serum levels of wild-type and *tlr4*-deficient mice after various periods of daily allergen challenges. Challenged *tlr4*-deficient mice had dramatic increases in serum IgE compared with wild-type mice, particularly after 1 wk of challenges (Fig. 1B). Moreover, ongoing challenged C3H/HeJ mice, which have a naturally occurring mutation in *tlr4*, also had significantly increased allergic responses compared with similarly challenged C3H/OuJ mice (data not shown). By contrast, responses of *tlr2*-deficient mice did not differ significantly from those of C57BL/6 mice (data not shown). In addition, we performed studies of airway physiology using noninvasive whole body plethysmography (Buxco's enhanced pause or Penh). No differences between the two strains were observed during the first 2 wk of exposures, although *tlr4*-deficient mice had increased hyperreactivity after 21 days of daily allergen challenge (Fig. 1C). Although airway hyperreactivity is generally associated with eosinophilic inflammation, this association is not always observed (27). Taken together, these findings demonstrate that *tlr4* is specifically required to attenuate inflammatory responses to ongoing aeroallergen challenge, but not for airway hyperreactivity.

The *tlr4*-dependent regulation of allergic inflammation is dose dependent

Analysis of the commercially obtained OVA used to generate aerosols revealed that a 1% solution of this product contained ~70 EU/mg endotoxin, a finding that is consistent with previous studies (16). We attempted to remove this contaminating endotoxin through the use of a purification kit, but were only able to reduce levels to ~25 EU/mg. This preparation of OVA was used to generate aerosols for challenge of sensitized C57BL/6 and *tlr4*-deficient mice for 10 consecutive days. Challenged *tlr4*-deficient mice had significantly increased inflammation and serum IgE compared with challenged C57BL/6 mice (Fig. 2). This result demonstrates that in C57BL/6 mice, aerosols of OVA containing as little as 25 EU/mg are sufficient to attenuate inflammatory responses to ongoing allergen challenge.

We next investigated the consequences of increasing the endotoxin contamination to levels above those found in the commer-

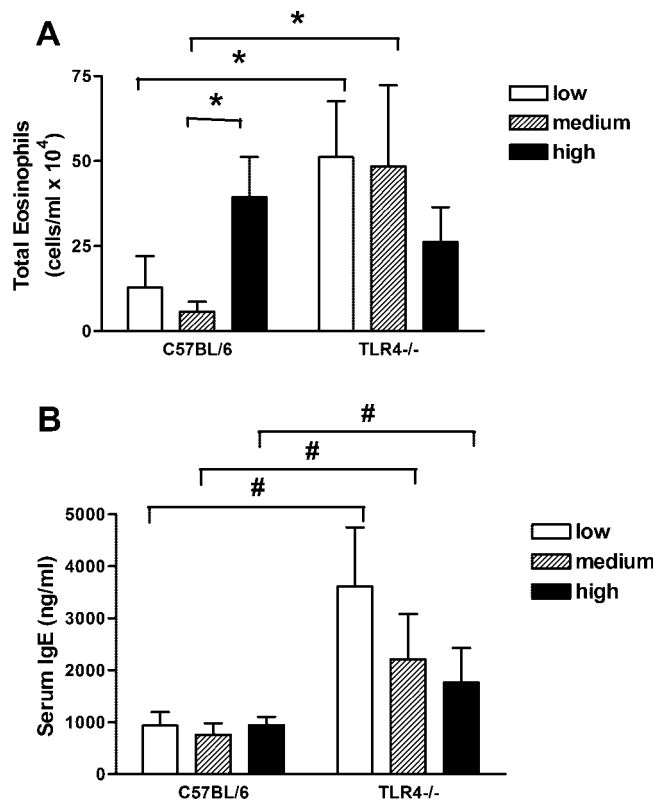


FIGURE 2. Dose-dependent effect of endotoxin on allergic inflammation. Mice were sensitized twice with OVA, then challenged for 10 consecutive days with aerosols of OVA containing different levels of endotoxin: low (25 EU/mg), medium (70 EU/mg), and high (375 EU/mg). Following this challenge, mice were analyzed for airway eosinophils (A) and serum IgE (B) (*, $p < 0.01$ and #, $p < 0.05$).

cially obtained OVA. To do this, we added an additional 300 EU of LPS/mg OVA, bringing the final concentration of LPS to 375 EU/mg, and compared responses of mice challenged with this preparation with mice challenged with OVA containing lower levels of endotoxin. C57BL/6 mice challenged with OVA containing high levels of endotoxin had significantly more airway eosinophils than their counterparts challenged with OVA containing either 25 or 75 EU endotoxin/mg OVA (Fig. 2A). IgE levels were less affected by endotoxin levels than were airway eosinophils, as *tlr4*-deficient mice had increased levels of this Ig isotype at each concentration of endotoxin (Fig. 2B).

Tolerance to inhaled OVA is *tlr4* independent

Mice exposed to OVA aerosols before their sensitization become tolerant to that Ag and do not develop allergic responses when subsequently challenged (28). Our finding that *tlr4* is required to attenuate ongoing allergic inflammation during ongoing allergen challenge suggested that this receptor might also be required to develop Ag-specific immune tolerance. To test this possibility, we exposed both wild-type and *tlr4*-deficient mice to an aerosol of 1% OVA before sensitizing and challenging these animals as before. This treatment caused both C57BL/6 and *tlr4*-deficient mice to become tolerant to OVA and unable to respond to subsequent sensitization and challenge (data not shown). To determine whether differences between these groups would become apparent at reduced concentrations of OVA, we used a 0.001% solution of this protein, and exposed animals for as little as 3 min. Again, *tlr4*-deficient and C57BL/6 mice became tolerant to OVA (Fig. 3).

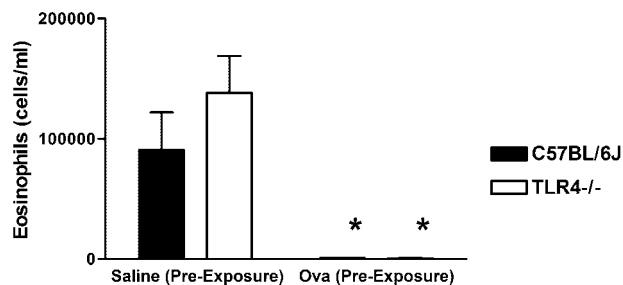


FIGURE 3. Tolerance to Ag is independent of TLR4. Exposure to Ag before sensitization can induce robust tolerance. Animals were exposed to a 1000-fold dilution of 1% OVA for 3 min 1 wk before immunization. These very low levels of Ag pre-exposure are sufficient to induce tolerance in both C57BL/6 and *tlr4*-deficient animals, when compared with naive animals not pre-exposed to Ag (*, $p < 0.05$). TLR4-dependent differences in tolerance were not observed in this assay.

These data show that unlike the regulation of existing allergic responses, induction of tolerance by aerosols before sensitization is not dependent on *tlr4* signaling. Therefore, the molecular mechanisms involved in conferring tolerance to inhaled allergens are most likely distinct, at least in part, from those that attenuate ongoing allergic responses.

*Increased allergic responses in *tlr4*-deficient mice do not result from decreased Th1 cytokines*

Signaling from TLRs, including *tlr4*, leads to the production of Th1-associated cytokines such as IFN- γ (29). It has been suggested that endotoxin-stimulated production of these Th1 cytokines might decrease Th2 cytokine production and thereby protect against the development of asthma. To test whether this mechanism might underlie the increased allergic responses in ongoing challenged *tlr4*-deficient mice, we measured airway levels of Th1 and Th2 cytokines. Compared with C57BL/6 mice, *tlr4*-deficient mice had increased level of the Th2 cytokine IL-5, particularly during the first week of challenge, as would be expected for mice having increased airway eosinophils and serum IgE (Fig. 4A). However, the level of the Th1 cytokine, IFN- γ , was not correspondingly decreased between challenged C57BL/6 and *tlr4*-deficient mice (Fig. 4B). Moreover, serum levels of OVA-specific IgG2a, the Ig isotype associated with Th1 cytokine production (30), were dramatically increased in *tlr4*-deficient mice compared with similarly challenged C57BL/6 mice (Fig. 4C). Taken together, these data demonstrate that Th1 responses in *tlr4*-deficient mice are at least as robust as those of C57BL/6 mice. Therefore, a reduction in Th1 cytokine production does not account for the increased Th2-associated allergic inflammation seen in ongoing challenged *tlr4*-deficient mice.

*Bone marrow-derived *tlr4* regulates allergic inflammation*

The *tlr4* is expressed on many cell types that might participate in attenuating allergic inflammation. In particular, both hemopoietic and nonhemopoietic cells have been shown previously to require *tlr4* expression for inflammatory responses to endotoxin, depending on the route of endotoxin administration (26, 31–34). To determine which of these cellular compartments requires *tlr4* expression for attenuation of allergic inflammation, we generated bone marrow chimeric mice using C57BL/6 and *tlr4*-deficient mice. Because maximum differences between these two parental strains for eosinophilic inflammation and IgE levels were seen at 7 and 14 days, respectively, we challenged the limited number of chimeric animals at 10 days, midway between these two time points. Irra-

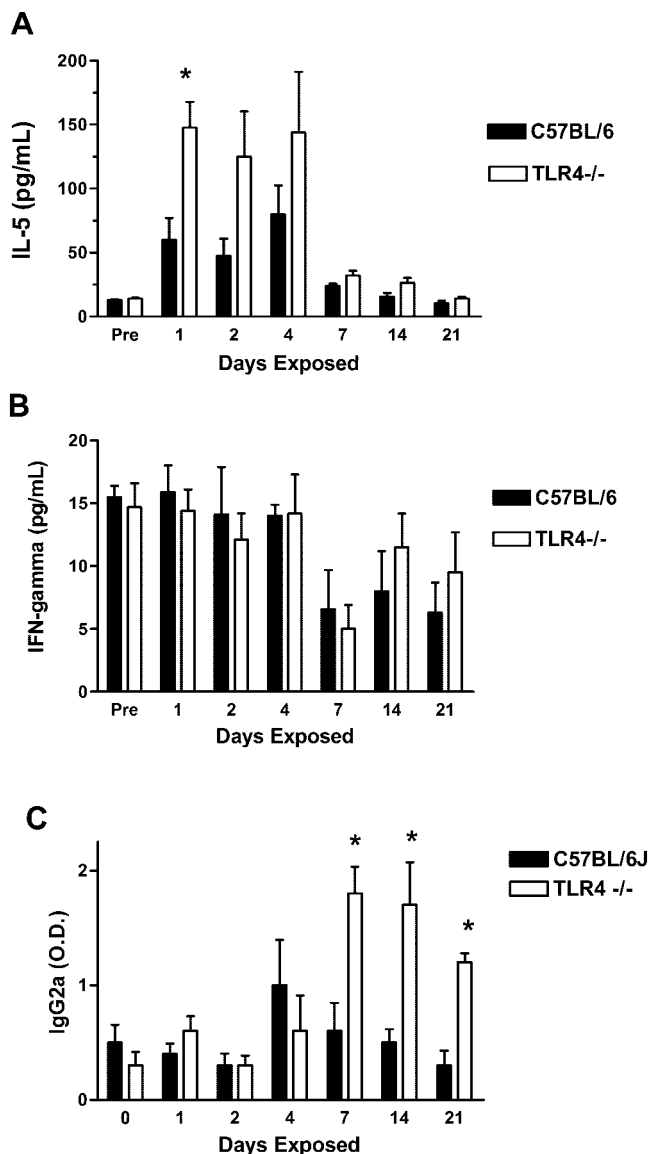


FIGURE 4. Increased allergic responses in *tlr4*-deficient mice are not associated with decreased Th1 cytokines. Sensitized mice were exposed to daily OVA aerosols for various lengths of time, and their lungs were lavaged for measurements of airway Th1 (IFN- γ) and Th2 (IL-5) cytokines. Serum was also analyzed to determine levels of OVA-specific IgG2a. Significant differences between genotypes are denoted by an asterisk (*, $p < 0.05$).

diated C57BL/6 and *tlr4*-deficient mice receiving C57BL/6 bone marrow developed relatively modest levels of eosinophilic inflammation and serum IgE after 10 consecutive days of OVA challenge (Fig. 5). By contrast, *tlr4*-deficient mice and C57BL/6 mice receiving *tlr4*-deficient bone marrow had high levels of eosinophilic inflammation and serum IgE, similar to those seen in nonirradiated, *tlr4*-deficient mice. These data demonstrate that a cell type derived from the bone marrow requires *tlr4* expression to limit allergic responses to ongoing allergen challenge.

*Increased DCs in allergen-challenged *tlr4*-deficient mice*

Within the hemopoietic compartment, there are several cell types whose immunoregulatory function might be affected by *tlr4*. Specifically, *tlr4* expression has been reported in regulatory T cells (Treg) (35), macrophages (36), and DCs (37). We therefore used flow cytometry to determine the abundance of these cell types after

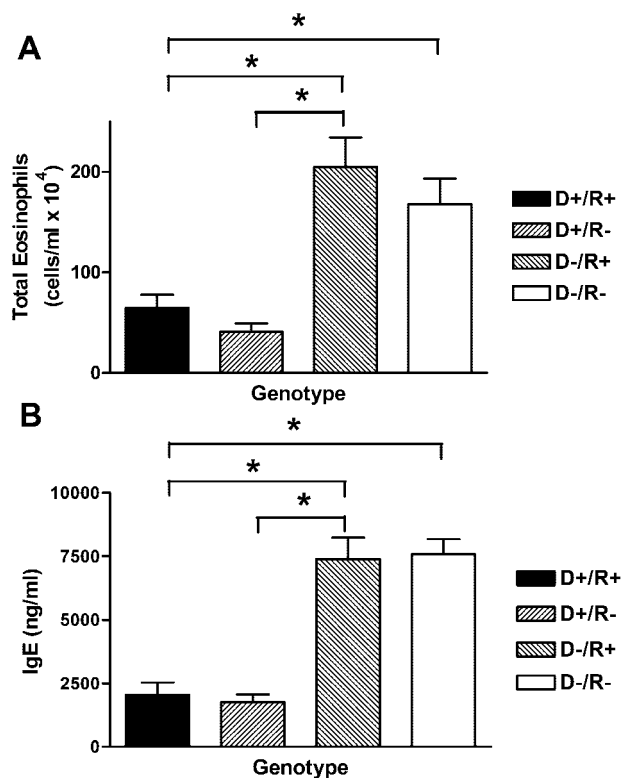


FIGURE 5. Bone marrow-derived *tlr4* is required to regulate allergic responses in the lung. Bone marrow chimeric mice were created using donor (D) mice expressing *tlr4* (D⁺), or deficient in *tlr4* (D⁻). These cells were transferred into irradiated recipient (R) mice expressing *tlr4* (R⁺), or deficient in *tlr4* (R⁻). Six weeks later, animals were sensitized to OVA, and exposed to 10 days of aerosolized OVA and analyzed for airway eosinophils (A) or serum IgE (B). All mice receiving D⁺ cells were able to attenuate allergic responses, whereas mice receiving D⁻ cells were not, regardless of the recipient's genotype (*, $p < 0.01$).

various days of challenge. With regard to Treg cells, no differences were found in the number of CD25-expressing cells in thoracic lymph nodes of C57BL/6 and *tlr4*-deficient mice. In addition, the CD25-expressing cells of both genotypes expressed similar levels of the glucocorticoid-induced TNFR and IL-10, two molecules associated with the activities of Treg cells. Analysis of DCs in the lung and draining lymph nodes did not reveal differences in the cell surface levels of CD40, CD80, CD86, B220, ICOS, or MHC class II Ag (data not shown). However, after 1 wk of OVA exposures, the number of classical, CD11c⁺CD11b⁺ DCs expressing high levels of MHC class II was dramatically increased in the lung parenchyma of *tlr4*-deficient mice compared with similarly challenged C57BL/6 mice (Fig. 6). This difference coincides with the time at which *tlr4*-deficient mice begin to develop dramatic increases in pulmonary eosinophils and serum IgE compared with challenged C57BL/6 mice.

Discussion

The experiments described in this work were begun to study the effect of low doses of endotoxin on allergic responses, and to determine whether the duration of allergen exposure affected the impact of endotoxin on allergic responses. Wild-type and *tlr4*-deficient mice had similar responses to short-term challenges with OVA containing low levels of LPS, but *tlr4*-deficient mice developed progressively increased allergic responses compared with wild-type mice as the duration of exposure to allergen challenges increased. This result, although unexpected, is consistent with ep-

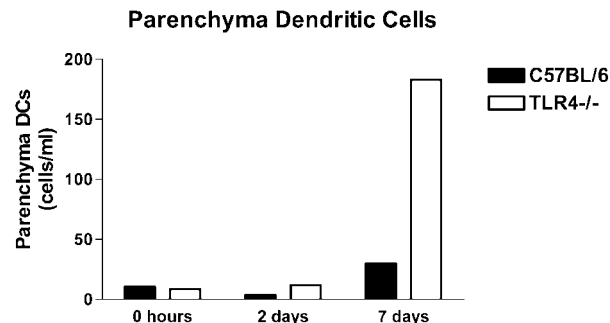


FIGURE 6. Ongoing challenged *tlr4*-deficient mice have increased numbers of pulmonary DCs. Groups of five sensitized mice were challenged for 1, 2, or 4 consecutive days of a 20-min OVA exposure. Lungs from animals within a group were pooled and analyzed by flow cytometry. By comparison with C57BL/6 mice, *tlr4*-deficient mice developed large increases in pulmonary DCs (MHCII high, CD11c high, CD11b positive, Gr-1 negative). One representative experiment of three is shown.

idemiologic data showing that ongoing exposure to low levels of endotoxin can protect against allergic asthma, and suggests that *tlr4* functions in an anti-inflammatory pathway that limits the extent of allergic responses to continued allergen challenge. Taken together with data showing that *tlr4* promotes sensitization to allergens, our data provide an explanation as to why epidemiologic studies have appeared contradictory; with some studies showing that asthma is exacerbated by endotoxin (3–5) and other studies indicating that asthma is diminished by this agent (6).

In contrast to the protective effect of low levels of endotoxin, adding higher doses of endotoxin to OVA increased the magnitude of allergic responses in the lung. Taken together, these data demonstrate that the level of endotoxin associated with an allergen is critically important to the magnitude of ensuing allergic responses. The most likely explanation for these findings is that the proinflammatory effects of high levels of endotoxin override the attenuating affect of low levels of endotoxin. Interestingly, epidemiologic studies also suggest that low, but not high, levels of endotoxin can attenuate asthma. It is conceivable, therefore, that some epidemiologic studies have failed to reveal an overall association between endotoxin and asthma because of the opposing actions of low and high levels of endotoxin.

C3H/HeJ mice, which have a naturally occurring mutation in *tlr4*, are reported by Dabbagh et al. (38) to have diminished allergic responses following three intranasal challenges with OVA. In contrast, we found that in our model, C3H/HeJ mice had increased allergic inflammation compared with control C3H/OuJ mice following prolonged challenges with aerosolized allergen. No significant differences between C3H/HeJ and C3H/OuJ mice were seen after only a single aerosol challenge. These responses of *tlr4*-deficient C3H/HeJ mice in our model were therefore very similar to those of *tlr4*-deficient gene-targeted mice. Thus, differences in the procedures used to challenge the mice most likely account for the apparent discrepancy between our findings and those of Dabbagh et al. Although we have not compared responses of mice challenged with an intranasal application with those challenged multiple times with an OVA-based aerosol, it is noteworthy that intranasal administration of OVA containing low doses of endotoxin can sensitize naive mice (12), whereas exposure of naive mice to an aerosol of OVA can block responses in mice that are subsequently sensitized and challenged (28).

We cannot formally exclude the possibility that endogenous ligands of *tlr4*, or a contaminant in the OVA other than endotoxin, contribute to the diminished allergic responses seen in C57BL/6J

mice compared with *tlr4*-deficient mice. We were unable to directly test this hypothesis because we were unable to completely remove endotoxin from our OVA. However, it is noteworthy that a different group of investigators using aseptic techniques to prepare OVA directly from chicken eggs found that inflammatory responses to LPS-free OVA are markedly increased compared with commercially obtained OVA (16). This would be the expected result if low levels of contaminating endotoxin account for negative regulation of allergic inflammation in wild-type mice. Whatever the relevant ligand(s), *tlr4* signaling clearly leads to attenuation of ongoing inflammation in this model of ongoing allergen challenge. Therefore, this model can be used to determine whether low doses of endotoxin activate signaling pathways that are distinct from those pathways activated by high doses.

It has been suggested previously that the protective effect of pathogen infection seen in epidemiologic studies might result from TLR-stimulated production of Th1 cytokines, which would in turn inhibit Th2 cytokine production (39). Although *tlr* ligands can induce Th1 cytokine production in DCs, there is no direct evidence that these molecules diminish allergic responses (40–42). Moreover, several other studies suggest that an alternative mechanism might be responsible for the protective effect of pathogen exposure on asthma (41, 43). In our experiments, Th2 cytokine production in the lung was increased in *tlr4*-deficient mice compared with wild-type mice, but we did not see a corresponding decrease in Th1 cytokines. In fact, serum levels of the Th1-associated Ig isotype, IgG2a, were increased in *tlr4*-deficient mice in concert with IgE. While it remains possible that Th1 cytokine production in tissues other than the lung, such as the spleen, lymph nodes, or bone marrow, is different between wild-type and *tlr4*-deficient mice, our data support the notion that an increase in Th1 cytokines does not account for the lower levels of Th2 cytokines and other allergic responses seen in wild-type mice compared with *tlr4*-deficient mice.

The dramatic increase in the number of pulmonary DCs suggests that these cells might be related to increased allergic inflammation in *tlr4*-deficient mice. Although there is little evidence that Th2 cytokine production in DCs is a consequence of *tlr4* signaling, DCs are nonetheless critical to allergic responses, not only for the sensitization phase, but also during the challenge phase (44). It is possible that low levels of endotoxin somehow attenuate the recruitment of DC precursors to the lung following allergen challenge, thereby limiting the magnitude of responses to that Ag. However, we cannot exclude the alternative possibility that the increase in pulmonary DCs seen in *tlr4*-deficient mice is secondary to the increased inflammation in these animals.

The reduced capacity of *tlr4*-deficient mice to regulate ongoing inflammation prompted us to study their ability to undergo Ag-specific tolerance when exposed to an aerosol of this protein before being sensitized. However, the abilities of wild-type and *tlr4*-deficient mice to undergo Ag-specific tolerance were indistinguishable, suggesting that the mechanisms leading to tolerance are different than those regulating ongoing inflammation.

Interestingly, another group of investigators found that naive mice exposed intranasally to endotoxin-containing OVA became sensitized to this allergen, in a *tlr4*-dependent manner (12). These findings demonstrate that the levels of endotoxin, or its activity, are critical for intranasal sensitization, as well as for regulating existing inflammation. However, these investigators found that *tlr4* was not required for sensitization by i.p. injections of alum-complexed allergen. Although direct comparisons have not been made, we estimate that the low dose of endotoxin (0.1 μ g) used in those experiments (12) contains $\sim$ 300 EU, which is roughly equivalent to the high dose used in our OVA challenges in which wild-type

mice did not have attenuated inflammation compared with *tlr4*-deficient mice. Alternatively, there could be important differences in tolerance dependent on the mode of pre-exposure. It is possible that an aerosol exposure of Ag increased gut mucosal Ag exposure, leading to tolerance in lieu of sensitization to Ag. Additional experiments will be required to identify which experimental variation has the greatest impact on sensitization and generation of allergen-specific tolerance.

Taken together, the findings presented in this study demonstrate that *tlr4* signaling regulates ongoing inflammatory responses to ongoing allergic challenge. This result is consistent with the epidemiologic finding that low doses of endotoxin, particularly in childhood, protect against developing asthma later in life. The *tlr4*-dependent attenuation of inflammation seen in C57BL/6 mice is not associated with an increase in Th1 cytokine production, an observation that is consistent with emerging models of allergic regulation in humans (45). Moreover, our finding that high doses of endotoxin overcome the anti-inflammatory effect is also consistent with the lack of inhibition or exacerbation of asthma seen with exposure to high levels of endotoxin in humans. The similarities between our experimental findings and previously described epidemiologic data suggest that this animal model will prove useful in further elucidating the underlying mechanism underlying the regulation of allergic inflammation. Such an improved understanding should facilitate the development of novel therapies to treat asthma by augmenting natural regulatory mechanisms.

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Disclosures

The authors have no financial conflict of interest.

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