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Toll-Like Receptor Activation during Cutaneous Allergen Sensitization Blocks Development of Asthma through IFN-Gamma-Dependent Mechanisms

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Toll-like receptors (TLRs) are pattern-recognition receptors that have a pivotal role as primary sensors of microbial products and as initiators of innate and adaptive immune responses. We investigated the role of TLR2, TLR3, and TLR4 activation during cutaneous allergen sensitization in the modulation of allergic asthma. The results show that dermal exposure to TLR4 ligand lipopolysaccharide (LPS) or TLR2 ligand Pam_3Cys suppresses asthmatic responses by reducing airway hyperreactivity, mucus production, Th2-type inflammation in the lungs, and IgE antibodies in serum in a dose-dependent manner. In contrast, TLR3 ligand Poly(I:C) did not protect the mice from asthmatic symptoms but reduced IgE and induced IgG2a in serum. LPS (especially) and Pam_3Cys enhanced the activation of dermal dendritic cell (DCs) by increasing the expression of CD80 and CD86 but decreased DC numbers in draining lymph nodes at early time points. Later, these changes in DCs led to an increased number of $CD8^+$ T cells and enhanced the production of IFN-γ in bronchoalveolar lavage fluid. In conclusion, dermal exposure to LPS during sensitization modulates the asthmatic response by skewing the Th1/Th2 balance toward Th1 by stimulating the production of IFN-γ. These findings support the hygiene hypothesis and pinpoint the importance of dermal microbiome in the development of allergy and asthma.

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INTRODUCTION

Asthma is a heterogeneous inflammatory disease of the lungs characterized by mucus hypersecretion, airway inflammation, bronchial hyperresponsiveness, and Th2 cell infiltration in the lungs (Cohn *et al.*, 2004). It is estimated that as many as 300 million people suffer from asthma globally, and the number is increasing especially in the Western countries (Masoli *et al.*, 2004; von Mutius and Vercelli, 2010). In addition to multiple genetic factors known to be involved in the pathogenesis of asthma, environmental factors have also been hypothesized to contribute to the outcome of asthma. These include, for example, different allergens, air pollutants, respiratory viruses, and endotoxins (Holloway *et al.*, 2010).

The hygiene hypothesis suggests that exposure to microbial infections in early childhood favors the development of Th1-type immunity and protects from the later development of the Th2-type phenotype (Strachan, 1989; Ernst and Cormier, 2000; Bach, 2002; Braun-Fahrländer *et al.*, 2002; Yang and Gao, 2011). Various Toll-like receptors (TLRs) are important mediators of innate immunity that recognize pathogen-associated microbial patterns from microbial and viral products and activate distinct signaling pathways. Activation of the innate immune system through TLRs is also linked to the responses of adaptive immunity and thus may influence the development of allergic diseases such as asthma (Reijmerink *et al.*, 2010; Tesse *et al.*, 2011).

The skin barrier of atopic individuals is often disrupted, and several microbial components and allergens can penetrate easily through the dermis. In the present study, we investigated the role of TLR ligands in the development of asthma by mimicking the natural route of sensitization from the skin to systemic and airway responses. The results show that dermal exposure to both TLR4 ligand lipopolysaccharide (LPS) and TLR2 ligand Pam_3Cys suppresses asthmatic responses, when given at the time of sensitization, by reducing airway hyperreactivity (AHR), Th2-type inflammation, and IgE antibodies in a dose-dependent manner. On the other hand, TLR3 ligand Poly(I:C) was not able to protect the mice from asthmatic parameters in general but reduced IgE and induced IgG2a concentrations in serum. The protecting effect of TLR4 ligand

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Abbreviations: AD, atopic dermatitis; AHR, airway hyperreactivity; DC, dendritic cell; LN, lymph node; LPS, lipopolysaccharide; MCh, metacholine; OVA, ovalbumin; Pam_3Cys, (S)-(2,3-bis(palmitoyloxy)-(2RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser(S)-Lys4-OH, trihydrochloride; poly(I:C), polyinosic-polycytidylic acid; TLR, Toll-like receptor

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LPS was found to be due to an enhanced production of IFN-γ in the airways.

RESULTS

Dermal exposure to TLR ligands decreases allergic inflammation and AHR

Mice were sensitized intradermally with PBS, OVA, and OVA in combination with low (1 μg) or high (10 μg) doses of TLR ligands Pam_3Cys (TLR2), poly(I:C) (TLR3), and LPS (TLR4) once a week for 4 weeks (Figure 1a). Samples were analyzed after three intranasal ovalbumin (OVA) challenges. High doses of LPS or Pam_3Cys significantly attenuated airway responsiveness to metacholine (MCh), whereas Poly(I:C) had only a minor effect on AHR (Figure 1b and Supplementary Figure S1 online). Low doses of TLR ligands had no effect on airway responses (data not shown). The total number of cells and inflammatory cells in bronchoalveolar lavage (BAL) fluid were significantly reduced after LPS and Pam_3Cys treatments, whereas Poly(I:C) had no effect (Figure 1c). The number of eosinophils was significantly reduced with all three ligands (Figure 1d), whereas no significant differences were detected in the number of neutrophils or lymphocytes (data not shown). Very high doses (100 μg) of Poly (I:C) induced the recruitment of significantly higher numbers of eosinophils and lymphocytes in BAL fluid (Supplementary Figure S2 online).

PAS^+ cells were reduced dose dependently with each ligand tested, although the effect showed statistical significance only with LPS (Figure 1e and Supplementary Figure S3 online). In addition, LPS treatment significantly decreased the number of F4/80-positive macrophages in the lungs (Figure 1f and Supplementary Figure S3 online). Instead, the number of $CD3^+$ T cells showed no change with any of the TLR ligands tested (Figure 1g and Supplementary Figure S3 online). All TLR agonists significantly enhanced serum OVA–specific IgG2a at high doses, and the effect was dose dependent (Figure 1h). In addition, both low and high doses of each TLR ligand significantly decreased OVA-specific IgE (Figure 1i).

TLR ligands decrease Th2 cytokines in the lung and in draining lymph nodes (LNs)

Dermal administration of LPS and Pam_3Cys decreased the expression of Th2-type cytokines IL-4, IL-5, and IL-13 as well

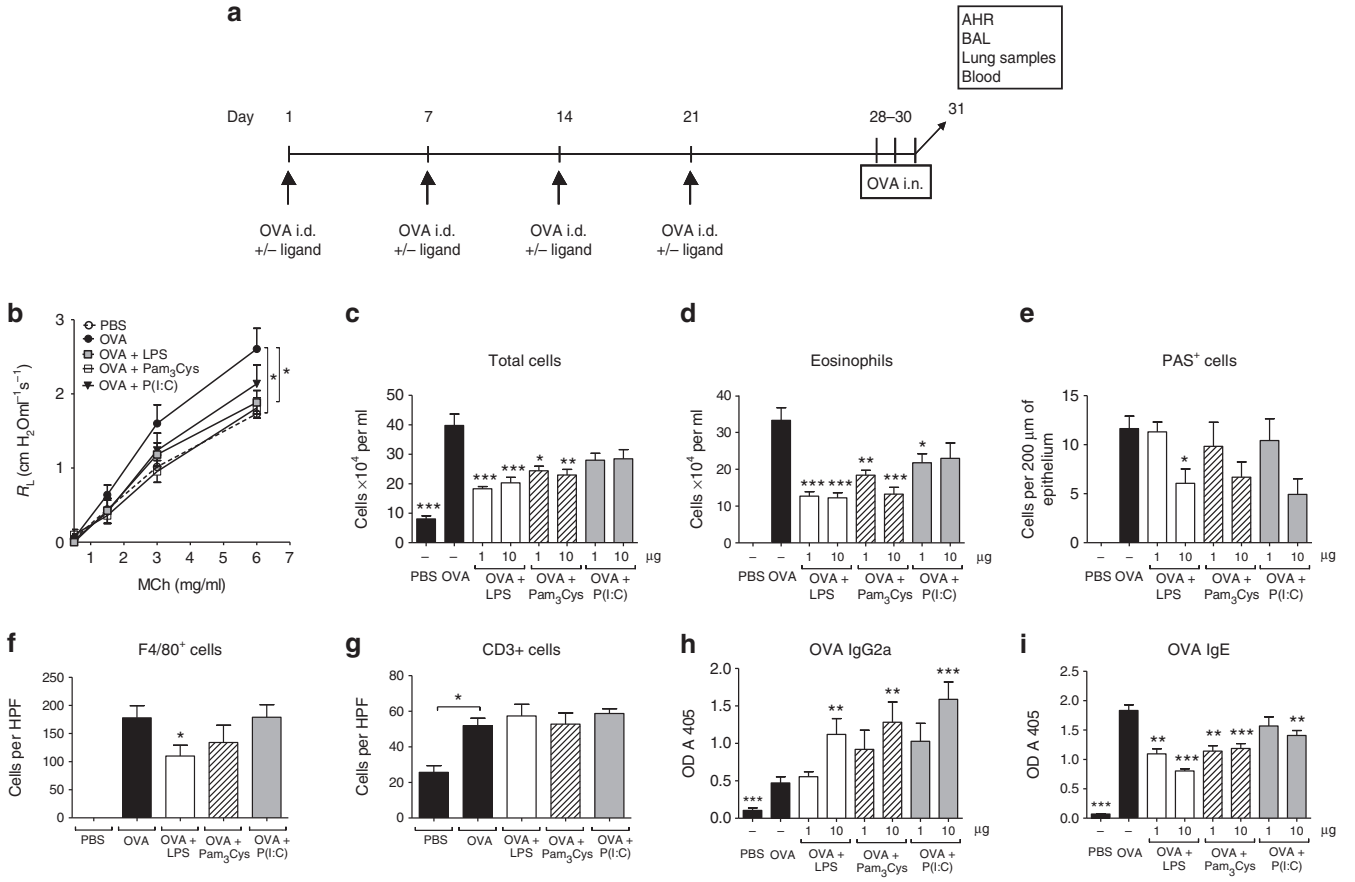

Figure 1. The effects of intradermal (i.d.) administration of Toll-like receptor (TLR) ligands on the lung inflammatory response. (**a**) The study protocol. (**b**) Lung resistance (R_L) and (**c**, **d**) the total number of cells and eosinophils in bronchoalveolar lavage (BAL) fluid. (**e**) Periodic acid-Schiff (PAS) staining of mucus-producing cells. Results are indicated as the average of PAS^+ cells per 200 μm of bronchus surface. Number of immunohistochemically stained (**f**) $F4/80^+$ antigen-presenting cells (APCs) and (**g**) $CD3^+$ cells in the lungs counted as an average from five randomly selected areas. TLR ligands upregulated (**h**) ovalbumin (OVA)-specific IgG2a and downregulated (**i**) OVA-specific IgE antibodies in the serum, as measured with ELISA. Results are presented as absorbance units (405 nm). **P*, 0.05; ***P*, 0.01; ****P*, 0.001. $n = 8$ mice per group. AHR, airway hyperreactivity; HPF, high power field; i.n., intranasally; LPS, lipopolysaccharide; OD, optical density; PBS, phosphate-buffered saline.

as that of pro-Th2-type IL-33 in lung tissue (Figure 2a and b). The most drastic reductions were seen with both doses of LPS and with 10 μg of Pam_3Cys. The expression of IL-13 was significantly lower at the protein level in OVA-stimulated LNs after LPS or Pam_3Cys treatment (Supplementary Figure S4 online). On the contrary, Poly(I:C) treatment at three different doses (1, 10, and 100 μg) showed no change in the expression of Th2-type cytokines in the lung (Figure 2a, Supplementary Figure S2 online).

When analyzing Th1 cytokines, LPS was the only ligand eliciting a significant increase in IFN-γ mRNA in the lung tissue (Figure 2c). The expression of IL-12 showed no change with any of the three TLR ligands (data not shown).

Regulatory T cells are not involved in the attenuation of asthmatic responses

Involvement of regulatory T cells (Tregs) in mediating tolerance and suppressive function in asthma has been well characterized (Lloyd and Hawrylowicz, 2009). Mice treated with OVA and LPS exhibited significantly lower amounts of Foxp3 mRNA in lung tissue when compared with mice treated with OVA alone, whereas other TLR ligands had no effect (Figure 3a). Expression of IL-10 was downregulated after dermal exposure to both doses of LPS and to the high dose of Pam_3Cys (Figure 3b). All TLR ligands also significantly reduced CTLA-4, a negative regulator of immune response, in the lung tissue. The most drastic reduction was observed with LPS ($P<0.001$) when compared with either Pam_3Cys ($P>0.01$) or Poly(I:C) ($P<0.05$) (Figure 3c). Instead, a small but significant increase in the expression of CD40 and major histocompatibility complex II (MHCII) on lung $CD11b^+$ cells was detected (Supplementary Figure S5 online).

Cutaneous exposure to TLR ligands modifies the function of dendritic cells (DCs) during sensitization

To understand the early events in dermal sensitization, we analyzed the function and number of DCs in draining LNs 24 hours after administration of OVA-Alexa647. Activated DCs ($CD11c^+OVA^+$) showed 20% enhanced cell adhesion and cell proliferation (CD40), 32–42% enhanced costimulation (CD80, CD86), and 17% enhanced antigen presentation (MHCII) when compared with resting DCs ($CD11c^+OVA^-$) (Supplementary Figure S6 online). Among OVA^+ DCs, dermal exposure to both LPS and Pam_3Cys reduced the total number of OVA^+ $CD11c^+$ DCs in draining LNs in a dose-dependent manner (Figure 4a). LPS enhanced the expression of activation markers CD80 and CD86, whereas no differences in MHCII were detected with any of the TLR ligands (Figure 4b–d).

Next, we conducted a kinetic study using dermal injections of OVA and OVA with 10 μg of LPS. After testing at all time points (2–48 hours), the percentage of OVA^+ DCs in the draining LNs of the skin was found to be lower in mice receiving OVA and LPS when compared with the group given OVA alone (Figure 4e). In contrast, the expression levels of surface activation markers, CD80 and CD86, were clearly enhanced in mice given OVA and LPS, and the effect lasted from 2 to 48 hours (Figure 4f and g). No differences were detected in the expression of MHCII at any time point (Figure 4h).

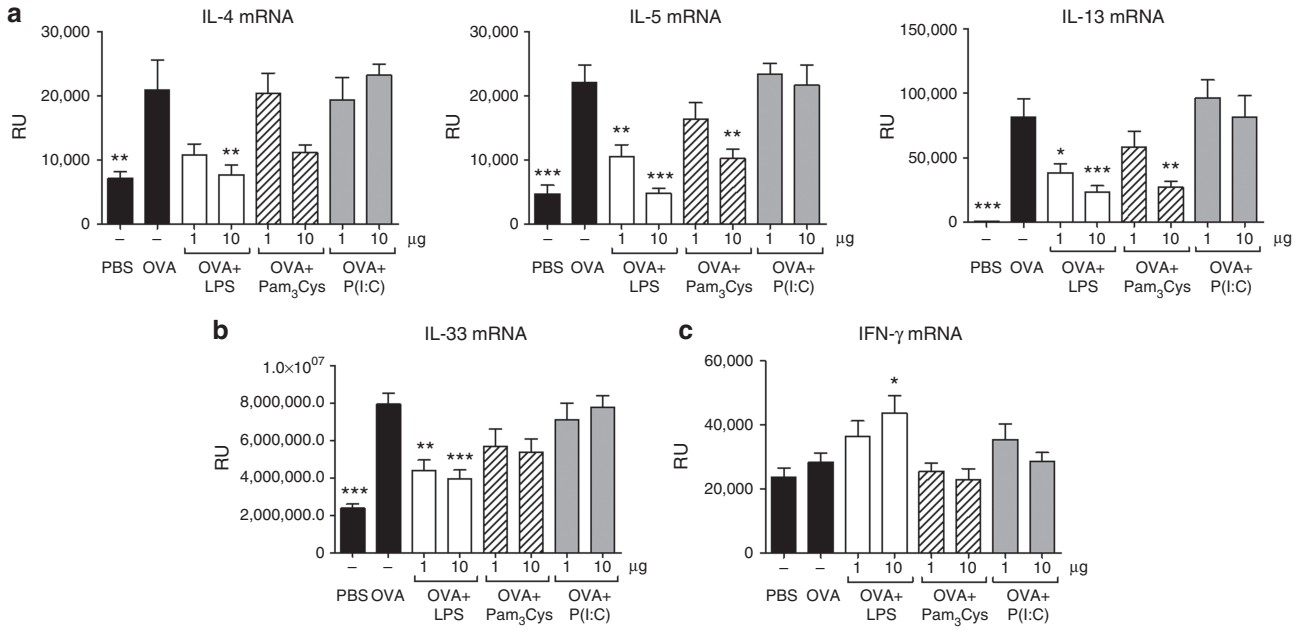

Figure 2. The expression of Th1, Th2, and proinflammatory cytokines at the RNA level in the lung after Toll-like receptor (TLR) ligand exposures. (**a**) Relative amounts of Th2-type cytokine RNAs IL-4, IL-5, and IL-13, and (**b**) the amount of pro-Th2-type cytokine IL-33 in lung tissue. (**c**) The amount of mRNA of Th1-type IFN-γ in the lungs. All cytokines are determined by reverse transcriptase–PCR as relative units (RU). *P, 0.05; **P, 0.01; ***P, 0.001. $n=8$ mice per group. LPS, lipopolysaccharide; OVA, ovalbumin; PBS, phosphate-buffered saline.

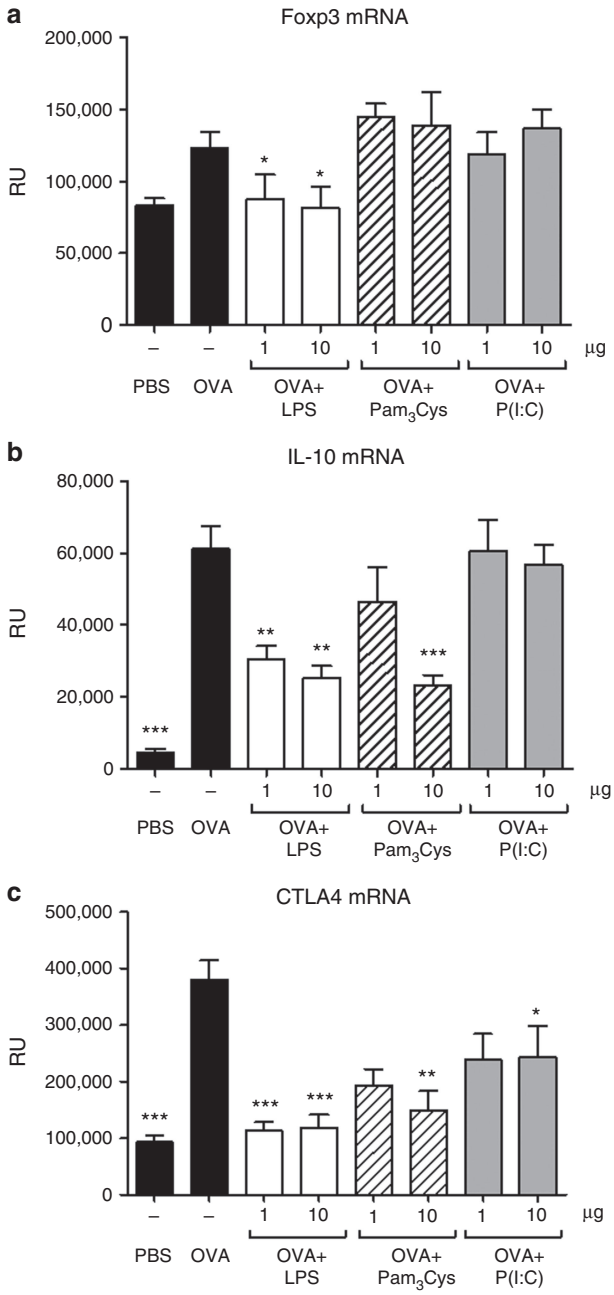

Figure 3. The effect of Toll-like receptor (TLR) ligands on lung regulatory factors at the RNA levels after 4 weeks of sensitization. The relative expressions of (**a**) transcription factor Foxp3, (**b**) cytokine IL-10, and (**c**) an inhibitory molecule CTLA-4 were determined by reverse transcriptase–PCR. **P*, 0.05; ***P*, 0.01; ****P*, 0.001. $n = 8$ mice per group. LPS, lipopolysaccharide; OVA, ovalbumin; PBS, phosphate-buffered saline.

IFN-γ is central to the protective effects of LPS

Dermal exposure of mice to OVA and LPS or Pam_3Cys had no effect on the number of $CD4^+$ T cells in BAL fluid (Figure 5a); however, a slight but insignificant enhancement in the production of IFN-γ and IL-13 from $CD4^+$ T cells in the OVA and LPS group was detected (Figure 5b and c). Interestingly, the number of $CD8^+$ T cells and their IFN-γ production were significantly enhanced after dermal exposure of mice to OVA and LPS (Figure 5d and e) along with a slight but non-significant increase in the total number of T cells in BAL fluid (Supplementary Figure S7 online). In addition, a slight increase was observed in the expression of CD69 of $CD8^+$ T cells (Figure 5f) in BAL fluid.

To assess the role of $CD8^+$ T cells in mediating the effect of LPS, we depleted the mice of $CD8^+$ T cells with anti-mouse CD8 mAb injections before OVA challenge. Instead of reversing the protective effect of LPS, the depletion of $CD8^+$ T cells resulted in further reduction in Th2-type cytokines in the lung tissue and in the numbers of eosinophils in the BAL fluid compared with mice treated with OVA (Supplementary Figure S8 online).

To investigate the role of IFN-γ in mediating the effect of LPS, we treated the mice with anti-mouse IFN-γ mAb injections before the OVA airway challenge. Neutralization of IFN-γ resulted in significantly reduced IFN-γ mRNA levels in the lung tissue (Figure 6a), along with increased Th2-type cytokines IL-5 and IL-13 (Figure 6b and c), and increased numbers of eosinophils in the BAL fluid (Figure 6d), compared with mice treated with OVA + LPS or with control IgG. Anti-IFN-γ mAb treatment also induced an influx of neutrophils (Figure 6e) and lymphocytes in BAL fluid (Figure 6f) but had no effect on mucus production or airway reactivity (data not shown).

DISCUSSION

Recent PARSIFAL and GABRIELA studies show that children who live on farms have lower prevalence of asthma and atopy and are exposed to a greater variety of environmental microorganisms compared with children in the reference group (Ege *et al.*, 2011). Atopic dermatitis (AD) may precede asthma during childhood according to the ''atopic march'' (Spergel, 2005), which suggests that cutaneous sensitization and skin inflammation are important for the development of asthma later in life. In addition, mutations in the *filaggrin* gene, which has an important role in skin barrier function, have been shown to associate with asthma in AD patients (Batchelor *et al.*, 2010). Experimental sensitization is most efficient when the antigen is administered through skin instead of intraperitoneally (Lehto *et al.*, 2005). Microbial components are recognized by TLRs that initiate inflammatory responses (Iwasaki and Medzhitov, 2004). In this study, we investigated the role of three TLR ligands, LPS, Pam_3Cys, and Poly(I:C), in the development of asthma by mimicking the natural route of sensitization from skin to asthmatic symptoms. We show that dermal administration of TLR4 ligand LPS during allergen sensitization protected the mice from asthma and airway inflammation by reducing AHR, BAL cell number, eosinophilia, mucus formation, and systemic IgE concentration. TLR2 agonist Pam_3Cys also effectively reduced Th2-type inflammatory responses, whereas TLR3 ligand Poly(I:C) had a minimal effect on local asthmatic parameters.

Allergic asthma is usually linked to enhanced Th2 responses, including increased number of eosinophils, mucus overproduction, and AHR. In addition, Th2-type cytokines, such as IL-4, IL-5, and IL-13, contribute to the induction and maintenance of airway inflammation and asthmatic response.

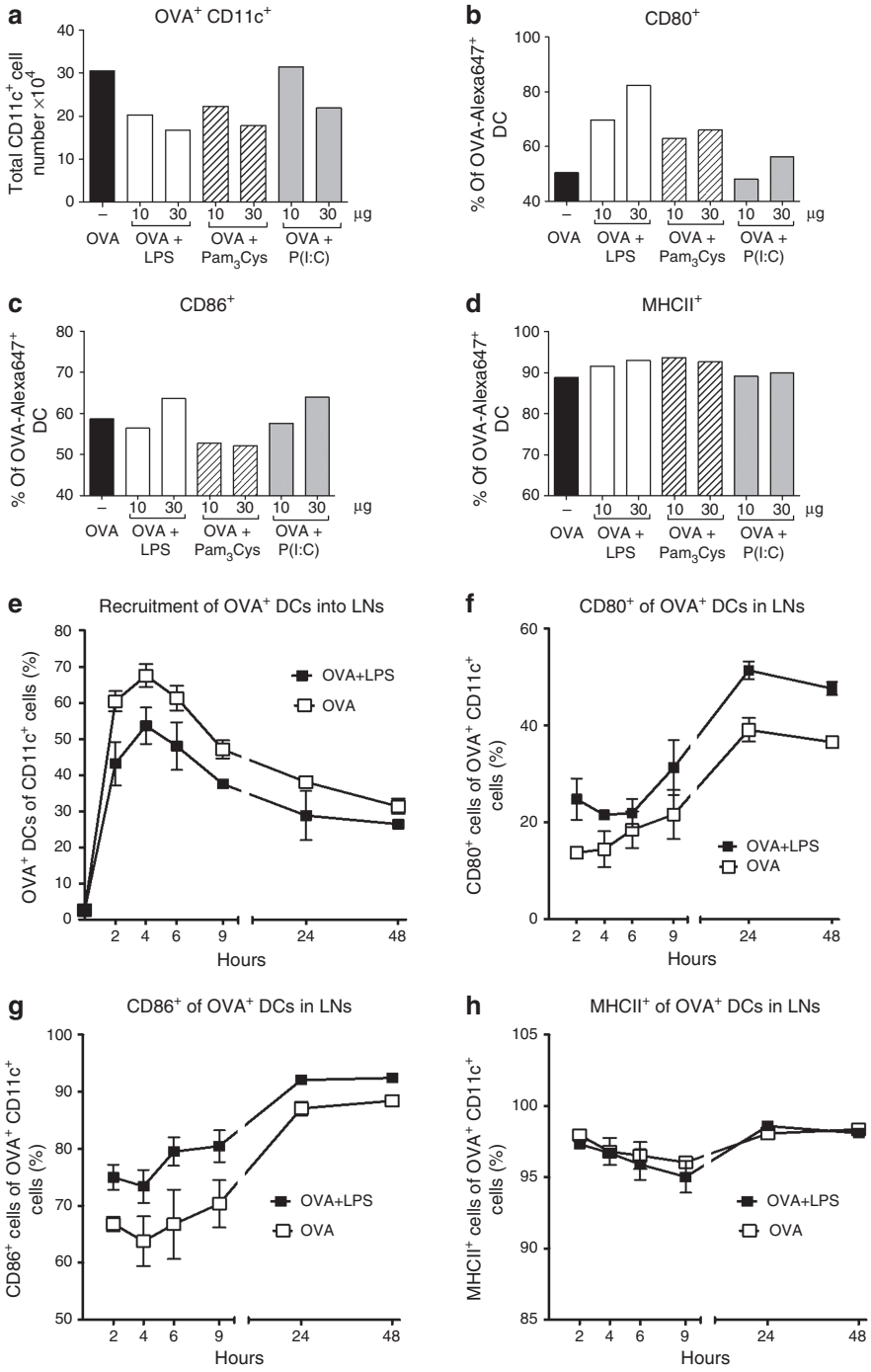

Figure 4. The recruitment and activation status of dendritic cells (DCs) in draining lymph nodes (LNs). Ovalbumin (OVA)-Alexa647 was intradermally administered alone or with 10 µg or 30 µg of Toll-like receptor (TLR) ligands, and the draining LNs were collected 24 hours after injection. (**a**) The total number of OVA^+ $CD11c^+$ DCs and their activation status were measured as the surface expression of (**b**) CD80, (**c**) CD86, and (**d**) major histocompatibility complex II (MHCII) by flow cytometry (pool of three mice). (**e**–**h**) The same parameters were studied in a kinetic study, in which LN cells were collected from local draining LNs 0, 2, 4, 6, 9, 24, or 48 hours after the intradermal injection. **P*, 0.05; ***P*, 0.01; ****P*, 0.001. $n = 3$–5 mice per time point. LPS, lipopolysaccharide.

IL-5 is known to have a pivotal role as a major maturation and differentiation factor for eosinophils (Takatsu *et al.*, 2009), whereas IL-13 has been shown to directly contract airway smooth muscle cells and thus enhance the development of AHR (Kuperman *et al.*, 2002; Akbari *et al.*, 2003). In our model, dermal exposure to LPS, especially, and also to Pam_3Cys, significantly reduced the production of IL-4, IL-5, and IL-13 in the lungs and that of IL-13 in draining LNs. We therefore hypothesize that IL-13, which is highly produced by lung eosinophils, is related to the significant reduction of

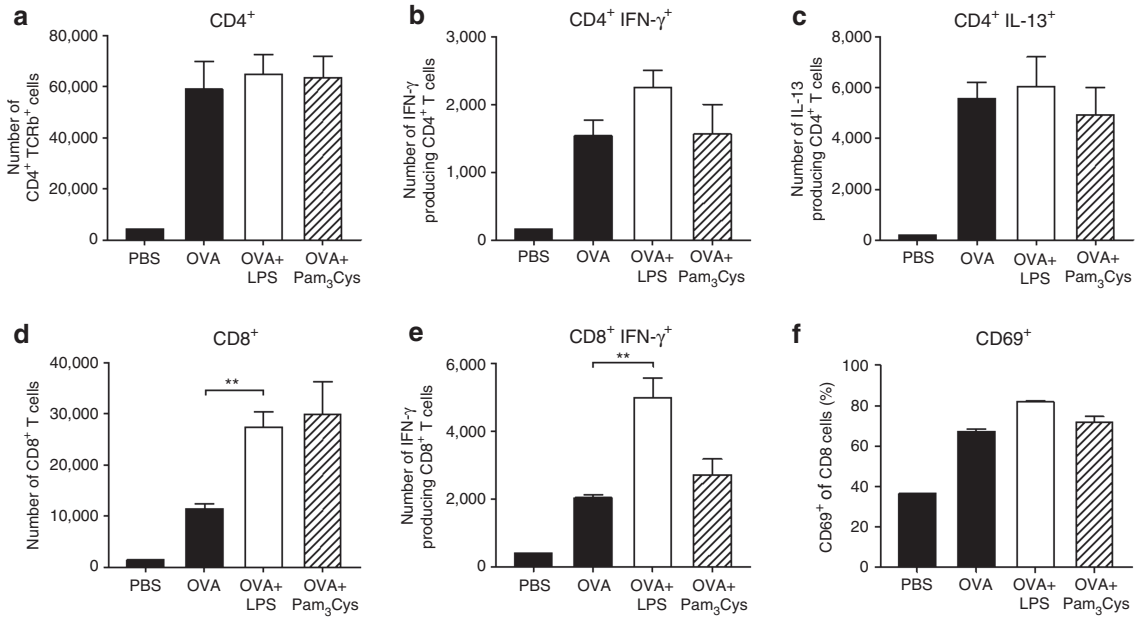

Figure 5. T-cell phenotyping of bronchoalveolar lavage (BAL) fluid cells by FACS. BAL fluids were collected after 4 weeks of sensitization with ovalbumin (OVA) or OVA and Toll-like receptor (TLR) ligands. The (**a**) total number of $CD4^+$ T cells and their (**b**) IFN-γ and (**c**) IL-13 production were measured by flow cytometry. (**d**) The number of $CD8^+$ T cells and their (**e**) IFN-γ production as well as their activation status were determined by the expression of (**f**) CD69. *P, 0.05; **P, 0.01; ***P, 0.001. $n = 3$, each representing a pool of BAL fluid from three mice. LPS, lipopolysaccharide; PBS, phosphate-buffered saline.

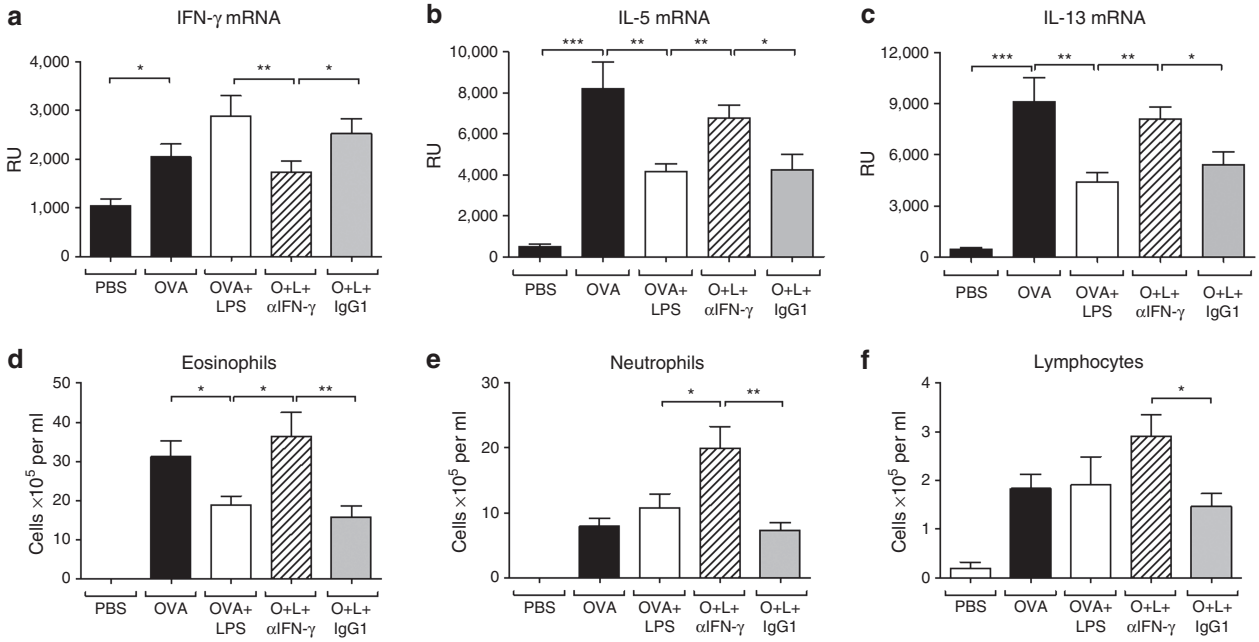

Figure 6. The effect of IFN-γ neutralization on Th1/Th2-type cytokines and airway inflammation. Mice were sensitized as shown in Figure 1a by ovalbumin (OVA) with 10 μg of LPS. On day 27, the mice were treated intraperitoneally (i.p.) with 200 μg anti-mouse IFN-γ mAb or control IgG1, and intranasally (i.n). on days 28, 29, and 30 together with OVA. The relative amount of (**a**) Th1-type cytokine IFN-γ and Th2-type (**b**) IL-5 and (**c**) IL-13 mRNAs was measured at the RNA level in lung tissue by reverse transcriptase–PCR on day 31. The amount of (**d**) eosinophils, (**e**) neutrophils, and (**f**) lymphocytes in bronchoalveolar lavage fluids counted from May-Grünwald Giemsa (MGG)-stained slides. *P, 0.05; **P, 0.01; ***P, 0.001. $n = 8$ mice per group. LPS, lipopolysaccharide; PBS, phosphate-buffered saline.

eosinophils by LPS or Pam_3Cys treatment. This result is supported by decreased mucus production in concert with reduced production of IL-4 and IL-5. Further analysis showed that expression levels of regulatory transcription factor Foxp3, cytokine IL-10, and inhibitory marker CTLA-4 were decreased especially after LPS treatment. Therefore, our findings strongly

suggest that the inhibition of allergic responses could not be a result of an induced amount or function of regulatory T cells but instead because of a decreased amount of eosinophils in the lungs.

Endotoxin tolerance (ET) or TLR tolerance is a phenomenon, where repeated systemic application of LPS or other TLR ligands can induce a state of immune unresponsiveness (Broad *et al.*, 2007; Biswas and Lopez-Collazo, 2009). Matsushita *et al.* (2010) suggest that the downregulated expression of MHC class II and costimulatory molecules, CD86 and CD40, on DCs and monocytes is a good biomarker for ET *in vivo*. However, we saw a small but significant increase instead of a reduction in the expression of CD40 and MHCII on lung $CD11c^+$ and $CD11b^+$ cells after 4 weeks of sensitization and elicitation. This advocates that TLR tolerance does not explain the decreased asthma symptoms in this model.

TLR ligands are potent activators of DCs resulting in the initiation of adaptive immunity. In our study, dermal administration of TLR ligands during allergen sensitization decreased the number of antigen-bearing DCs in LNs but enhanced their activation status. DCs that engulfed OVA or OVA and LPS had similar kinetics, and they traveled into draining LNs peaking by 4 hours after antigen administration. However, their number was decreased in the LPS-treated groups at all time points tested, maybe because of a decreased intrinsic migratory ability as described by Granucci *et al.* (1999). In addition, LPS is shown to accelerate the maturation and redistribution of DCs followed by a marked decrease in DC numbers, unless a signal is received from Ag-specific T cells (De Smedt *et al.*, 1996, 1998). DCs that survived through the OVA and LPS treatment in our study maintained a high antigen presentation capacity (MHCII), and activation by a highly increased expression of CD80 and a less increased expression of CD86. CD86 is linked to Th2 development, whereas CD80 might be a more Th1-type or neutral costimulatory molecule (Kuchroo *et al.*, 1995; Moser and Murphy, 2000).

Exposure to household endotoxins during childhood is inversely related to allergen sensitization (Gereda *et al.*, 2000) and to the development of allergic asthma (Braun-Fahrländer *et al.*, 2002). The mechanisms that drive immunity after endotoxin or LPS exposure are multifactorial and partly unknown. LPS can support the development and function of the major T-cell subsets depending on the situation, which has led to a re-evaluation of its effects on adaptive immunity (McAleer and Vella, 2010). In our study, when comparing the OVA and LPS group with the OVA group after 4 weeks of sensitization, the number of $CD8^+$ T cells in BAL fluid was seen to have increased significantly, whereas the number of $CD4^+$ T cells had remained unchanged. T cells in OVA- and LPS-treated mice produced significantly more IFN-γ because of increased numbers of $CD8^+$ T cells, and these cells were also more active as indicated by the expression of CD69. However, *in vivo* depletion of $CD8^+$ T cells did not reverse the protective effect of LPS but instead led to further decreased numbers of eosinophils and reduction of Th2-type cytokines in the lung, likely a consequence of depleting the entire compartment of CD8+ T cells, including Th2-like CD8+ T cells, which are known to mediate airway inflammation and AHR (Miyahara *et al.*, 2004; Stock *et al.*, 2004). Nevertheless, *in vivo* neutralization of IFN-γ demonstrated a clear dependence of the protective effect of LPS on IFN-γ.

To the best of our knowledge, no other report has studied the early events of LPS directly *in vivo* in similar study settings. In one study, low-dose LPS-pulsed bone marrow–derived DCs caused massive eosinophilia and enhanced Th2 cytokine production in mice, whereas high-dose LPS-induced Th17-type immune responses and LPS-free OVA did not induce allergic symptoms at all (Peters *et al.*, 2010). Depending on the local microenvironment, T cells might be polarized from Th2 to Th1, Th17, or to Treg cells. Treatment with TLR agonists during the challenge before or after allergen exposure has produced quite different outcomes (Duechs *et al.*, 2011). We have previously shown enhanced Th1 polarization after dermal TLR9 ligand CpG exposure during allergen sensitization. (Haapakoski *et al.*, 2011). Here we show that identical exposure regimes of different TLR ligands exhibit various effects on the Th2 inflammatory response. TLR2 and TLR4 agonists induced protective effects, whereas TLR3 agonist poly I:C had no effect at low concentrations and augmented the Th2-type response at high concentrations. The different outcomes might be the result of the distinct signaling pathways used by the TLRs, with TLR3 utilizing exclusively the TRIF-dependent pathway and TLR2 and TLR4 signal utilizing the MyD88-dependent pathways as well (Akira *et al.*, 2006). The various responses mediated by TLRs likely evolved to fine-tune adaptive immunity, and different dose, time, and administration routes elicit qualitatively distinct signals through TLRs, influencing T-cell polarization.

According to the hygiene hypothesis, the increase in atopy and asthma is related to improved hygiene status and reduced Th1-type immune responses in the industrialized world (Yang and Gao, 2011). Endotoxins and other bacterial derivatives present in the environment are suggested to have a role in the process by affecting DC maturation and T-cell polarization by favoring the development of protective immunity. Our results show that allergen sensitization through skin with LPS and Pam_3Cys, but not with Poly(I:C), decreases Th2-type allergic responses and airway reactivity. TLR4 ligand LPS was the only ligand to shift the balance toward Th1-type immunity by inducing IFN-γ production from $CD8^+$ T cells and by reducing Th2-type cytokines. The suppressive effect was strictly dependent on IFN-γ. These findings shed light on the interaction of innate immunity-activating microbial components and the dermal route of sensitization during allergic inflammation. Our results emphasize the importance of restoring the skin barrier function and also highlight the role of cross-talk between the dermal microbiome and the host in the development of allergies.

MATERIALS AND METHODS

Mice

Female Balb/c mice (NOVA-SCB AB, Sollentuna, Sweden) aged 6–8 weeks were maintained on OVA-free diets and water *ad libitum*. All animal experiments were approved by The Social and Health Care Department of the State Provincial Office of Southern Finland.

TLR ligands

Ultra Pure *E. coli* LPS (strain 0111:B4, #06B24-MT) and High Molecular Weight Poly(I:C) (endotoxin level <1.25 EU/ml, #09L02-MT) were purchased from InvivoGen (San Diego, CA). Pam_3Cys (PamCys-SKKKK, L2000) was purchased from EMC microcollections GmbH (Tübingen, Germany).

Treatment protocols

Mice were anesthetized and their backs were shaved and tape-stripped three times before the injections to introduce the skin injury. The mice were sensitized weekly intradermally with 50 μg of OVA in 100 μl, or with PBS only for 4 weeks. Groups of mice were treated with 1 μg or 10 μg of LPS concurrent with 50 μg of OVA (OVA and LPS groups), with 1 μg or 10 μg of Pam_3Cys (OVA and Pam_3Cys groups), or with 1 μg, 10 μg, or 100 μg of Poly(I:C) (OVA and Poly(I:C) groups). On days 28, 29, and 30 all mice were challenged with intranasal OVA (50 μg OVA in 50 μl of PBS).

For kinetic studies, the backs of the mice were shaved and tape stripped three times, and they were treated either with 50 μg of OVA-Alexa647 (Invivogen, San Diego, CA) alone or in combination with 10 μg of each TLR ligand intradermally. After different time points (0, 2, 4, 6, 9, 24, and 48 hours), the mice were killed and the draining LNs were collected for FACS analysis.

CD8 T cells were depleted by 200 μg of anti-mouse CD8 mAb intraperitoneally 4, 2, and 1 day before the 3-day OVA elicitation period. Control mice were treated with the same amount of rat IgG2. IFN-γ was neutralized by 200 μg of anti-mouse IFN-γ mAb or control IgG1 mAb intraperitoneally on day 27 (1 day before the OVA airway challenge) and again three times intranasally (50 μg of anti-IFN-γ Ab or IgG1 together with 50 μg of OVA) on days 28, 29, and 30.

Determination of airway reactivity to methacholine

Airway resistance and compliance were measured from sensitized and challenged mice as previously described (Haapakoski *et al.*, 2011).

BAL and lung histology

BAL inflammatory cells and lung histology were handled and counted as previously described (Haapakoski *et al.*, 2011).

RT-PCR analysis

Total RNA extractions with Trisure Reagent (Bioline, London, UK), complementary DNA synthesis, and real-time PCR (RT-PCR) were performed as previously described (Lehto *et al.*, 2003).

Measurement of cytokines by ELISA and flow cytometry

The cytokine levels of IL-13 were measured by ELISA from BAL fluids and from supernatants of cultured LN and from splenocytes according to the manufacturer's instructions (R&D Systems, Minneapolis, MN for IL-13). The detection limit was 8 $pg\,ml^{-1}$.

Flow cytometric analyses

BAL cells were washed once with PBS/2% FBS; Fc receptors were blocked with anti-mouse CD16/32 (eBioscience, San Diego, CA); and cells were stained with anti-TCRβ, anti-CD3, anti-CD4, and anti-CD8 antibodies (BD Pharmingen, San Diego, CA). For intracellular cytokines, BAL cells were stimulated at 37 °C for 4 hours with phorbol myristate acetate (20 $ng\,ml^{-1}$) and ionomycin (1 $mg\,ml^{-1}$) (Sigma, St. Louis, MO), including brefeldin A (Sigma). After washing, Fc blocking, and surface staining, cells were permeabilized using the Fix and Perm kit (Caltag, Burlingame, CA) and stained with anti-IFN-γ (BD Pharmingen) and anti-IL-13 (eBioscience).

For kinetic studies, brachial LNs were removed, teased with glass slides, and filtered through a 70-μm cell strainer; thereafter, the total number of cells was counted. Single-cell suspensions were Fc blocked, stained with mAbs for CD11c, CD11b, MHCII, CD40, CD80, and CD86 (eBioscience), and run on a FACSCantoII instrument (Becton Dickinson, Franklin Lakes, NJ). Data were processed using FlowJo Software (Tree Star, Ashland, OR).

Measurement of serum antibodies

OVA-specific IgE and IgG2a antibodies were measured from serum using ELISA as previously described (Lehto *et al.*, 2003).

Statistics

Single-group comparisons were made using the nonparametric Mann–Whitney *U*-test. Results are expressed as mean ± SEM and *P*-values of <0.05 were considered statistically significant. Statistical analyses were performed using GrapPadPrism (version 4 GraphPad Software, La Jolla, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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