

ORIGINAL PAPER

Toll-like receptor 4 agonists adsorbed to aluminium hydroxide adjuvant attenuate ovalbumin-specific allergic airway disease: role of MyD88 adaptor molecule and interleukin-12/interferon- γ axis

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Clinical and Experimental Allergy

Summary

Background Epidemiological and experimental data suggest that bacterial lipopolysaccharides (LPS) can either protect from or exacerbate allergic asthma. Lipopolysaccharides trigger immune responses through toll-like receptor 4 (TLR4) that in turn activates two major signalling pathways via either MyD88 or TRIF adaptor proteins. The LPS is a pro-Type 1 T helper cells (Th1) adjuvant while aluminium hydroxide (alum) is a strong Type 2 T helper cells (Th2) adjuvant, but the effect of the mixing of both adjuvants on the development of lung allergy has not been investigated.

Objective We determined whether natural (LPS) or synthetic (ER-803022) TLR4 agonists adsorbed onto alum adjuvant affect allergen sensitization and development of airway allergic disease. To dissect LPS-induced molecular pathways, we used TLR4-, MyD88-, TRIF-, or IL-12/IFN- γ -deficient mice.

Methods Mice were sensitized with subcutaneous injections of ovalbumin (OVA) with or without TLR4 agonists co-adsorbed onto alum and challenged with intranasally with OVA. The development of allergic lung disease was evaluated 24 h after last OVA challenge.

Results Sensitization with OVA plus LPS co-adsorbed onto alum impaired in dose-dependent manner OVA-induced Th2-mediated allergic responses such as airway eosinophilia, type-2 cytokines secretion, airway hyper-reactivity, mucus hyper production and serum levels of IgE or IgG1 anaphylactic antibodies. Although the levels of IgG2a, Th1-affiliated isotype increased, investigation into the lung-specific effects revealed that LPS did not induce a Th1 pattern of inflammation. Lipopolysaccharides impaired the development of Th2 immunity, signaling via TLR4 and MyD88 molecules and via the IL-12/IFN- γ axis, but not through TRIF pathway. Moreover, the synthetic TLR4 agonists that proved to have a less systemic inflammatory response than LPS also protected against allergic asthma development.

Conclusion Toll-like receptor 4 agonists co-adsorbed with allergen onto alum down-modulate allergic lung disease and prevent the development of polarized T cell-mediated airway inflammation.

Keywords airway hyper-reactivity, allergic airway inflammation, LPS, MyD88, TLR4 signaling
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Introduction

Allergic asthma is a chronic inflammatory lung disease mediated by Type 2 T helper cells (Th2) and is characterized by airway eosinophilia, airway hyper-reactivity (AHR), mucus hyper-secretion and elevated levels of IgE. Epidemiological evidence indicates that endotoxin lipopolysaccharide (LPS); a prototypic cell wall component of

gram-negative bacteria that activates immune cells via the transmembrane toll-like receptor 4 (TLR4) [1, 2] can influence the development of asthma [3–5].

Endotoxin LPS is ubiquitous in the environment and is often present in polluted air as well as in organic or household dusts [6]. Evidence for the role of LPS in modulating Th2 immunity is robust, but the results obtained by different groups of investigators are quite

conflicting [3]. For instance, epidemiological studies claim that endotoxin exposure during childhood could protect against the development of asthma later in life [3, 7, 8] while other studies indicate that endotoxin exposure is a risk factor for asthma [9–11]. Similarly, experimental data obtained with the murine ovalbumin (OVA) model of asthma indicated that exposure to LPS could either protect against asthma or exacerbate it [12–16].

Controversy also exists as to whether TLR4 and MyD88 molecules are required for optimal development of Th2 immunity [13, 15, 17–19]. In addition, different mechanisms have been postulated to operate in LPS-mediated protection or exacerbation of allergic lung disease [12–15].

Another variable, in studies that use OVA as allergen, is the contamination of commercial OVA with LPS [19]. It is now clear that the route, concentration, timing and duration of LPS exposure can determine whether LPS down- or up-regulates Th2-mediated allergic responses [15, 17]. We have previously shown that intravenous (i.v.), but not intranasal (i.n.), LPS administration during allergen challenge inhibits ongoing AHR, eosinophilic inflammation and pulmonary Th2-cytokine production [12]. Others studies have shown that LPS given shortly before allergen/alum sensitization down-regulates lung Th2 responses [20]. It is believed that LPS protects from the development of asthma by stimulating Th1 immunity. However, none of the studies that elicited allergic sensitization with alum have investigated whether LPS shifts the lung-specific immunity towards a Th1 pattern. This is an important point because recent studies in humans and mice clearly indicated that Th1 cells and IFN- γ are critically involved in the development of severe type 1 asthma and AHR [21, 22].

In the present study, we used a new approach to study the effects of natural (LPS) or synthetic (ER-803022) TLR4 agonists [23] on Th2-driven allergic disease. During the sensitization phase, we co-adsorbed TLR4 agonist and allergen (OVA) to alum. Thus, we sensitized animals to OVA with a mixture of pro-Th1 (LPS) and pro-Th2 (alum) adjuvants and monitored the OVA-induced lung immune responses and inflammation.

We demonstrate that LPS suppressed asthma-like responses without shifting the lung inflammation towards a Th1 pattern. To determine the role of downstream signaling pathways of TLR4 activation, we used gene-targeted mice deficient for the adaptor molecule MyD88 or Toll/IL-1R domain-containing adaptor inducing IFN- β -deficient (TRIF) mice. We also investigated the effect of LPS in double deficient mice lacking IL-12 and IFN- γ molecules. We found that suppression of Th2 immunity by LPS required TLR4 and MyD88 signaling. Moreover, the TRIF pathway was dispensable, but IL-12/IFN- γ axis was crucial for the inhibitory effect of LPS. Finally, a synthetic TLR4 agonist (ER-803022 compound) [23, 24] that was found to be without apparent systemic inflammatory

response was still as effective as LPS in preventing airway allergic disease.

Materials and methods

Mice

Six- to eight-week-old female mice on BALB/c background were weight-matched and used throughout this study. TLR4- and MyD88-deficient mice were on BALB/c background whereas TRIF-deficient and IL-12/IFN- γ double knock out (KO) mice were on C57BL/6J background. IL-12/IFN- γ double KO mice were obtained by mating IL-12p40 KO with IFN- γ KO animals originally obtained from Jackson Laboratory. All mouse strains were kept under standard pathogen-free conditions and were bred for many generations in the Institute of Biomedical Sciences breeding unit (São Paulo, Brazil) or in the Transgenose Institute animal breeding facility (Orleans, France). TLR4-, MyD88-, or TRIF-deficient mice were originally provided by S. Akira [25]. Mice were treated according to animal welfare guidelines of both Institutes.

Removal of lipopolysaccharides from ovalbumin

Chicken OVA (Sigma-Aldrich, St. Louis, MO, USA) was diluted in phosphate buffer saline (PBS) (2 mg/mL) and depleted of the endotoxin activity [measured by Limulus amoebocyte lysate (LAL) QCL-1000 kit from BioWhittaker, Walkersville, MD, USA], using two to four cycles of Triton X-114 extractions, as described by Aida and Pabst [26]. OVA concentration was determined by BCA kit assay (Pierce Biotechnology Inc., Rockford, IL, USA) and adjusted to 2 mg/mL. The endotoxin level of purified OVA was below the limit of detection in the LAL QCL-1000 kit (< 0.1 EU).

Alum gel preparation

Alum ($\text{Al}(\text{OH})_3$) gel was prepared by precipitating 0.184 M of ammonium aluminium sulphate dodecahydrate ($\text{AlH}_4\text{NO}_8\text{S}_2 \cdot 12\text{H}_2\text{O}$) with an excess of 1 N NaOH, roughly 2.5 v/1 v. After precipitation, $\text{Al}(\text{OH})_3$ was suspended in water (Milli Q, Ontario, Canada) and washed five times at 3000 r.p.m. for 15 min. The final precipitate was dissolved in water and the concentration $\text{Al}(\text{OH})_3$ was calculated by determining the dry weight of 1 mL solution. For sensitization, alum gel stock solution (50 mg/mL) was dissolved in 10 mL PBS containing OVA (10 $\mu\text{g}/\text{mL}$) and TLR4 agonists at different concentrations that were co-adsorbed to alum in a multi-tube rotator for 30 min.

Ovalbumin/alum sensitization with toll-like receptor 4 agonists and ovalbumin challenge

To test the effect of TLR4 agonists on OVA sensitization, we used LPS from *Escherichia coli* 0111:B4 (Sigma-

Aldrich) as natural TLR4 ligand and as synthetic lipid A analogue we used ER-803022 (Eisai Research Institute of Boston-Andover, MA, USA), hereafter referred to as ER. We found that 1 ng/mL of LPS has 5 EU as measured by the Limulus amoebocyte lysate (LAL QCL-1000 kit). The endotoxin activity of our stock solution of ER (2 mg/mL) was < 0.1 EU. Mice were sensitized on days 0 and 7 by subcutaneous (s.c.) injection (0.4 mL total volume) in the nape of the neck with 4 µg OVA adsorbed to 2.0 mg of alum or with OVA mixed with different concentrations of LPS (1 ng–10 µg) or with 10 µg of ER co-adsorbed to alum. On days 14 and 21, mice were challenged i.n. with 10 µg of OVA in 50 µL of PBS. This type of protocol allows us to measure almost all allergic parameters such as AHR, eosinophils, T cells and type-2 cytokine production 24 h after second OVA challenge, because the first OVA challenge recruits memory T cells to the lung that respond promptly upon the second OVA challenge.

Determination of airway responsiveness

Airway responsiveness to increasing doses of inhaled methacholine (3, 6, 12 and 25 mg/mL) in conscious unrestrained mice was determined using a single-chamber, whole-body plethysmograph (Buxco Electronics Inc., Wilmington, NC, USA), as previously described [27, 28]. After each nebulization with methacholine, recordings were taken for 5 min. The Penh values measured during each 5 min sequence were averaged and expressed for each methacholine concentration. Although the non-invasive method is controversial, it was established that when the animals are challenged once or twice with OVA via the airway route, the Penh measurements correlate with lung resistance and compliance [29].

Bronchoalveolar lavage fluid

Mice were deeply anaesthetized by an intraperitoneal injection of 4 mg/g body weight of chloral hydrate (Lab-synth, São Paulo, Brazil) and blood samples from the retro orbital plexus were collected for serum antibody determinations. The trachea was cannulated, and lungs were lavaged twice with 0.5 and 1.0 mL PBS. Total and differential cell counts of bronchoalveolar lavage (BAL) fluid were determined by haemocytometer and cytopsin preparation stained with Instant-Prov (Newprov, Paraná, Brazil).

Lung draining lymph node cultures

Cells from mediastinal lymph node (mLN) were adjusted to 5×10^6 cells/mL and cultured in 24-well plates in medium consisting of RPMI supplemented with 10% fetal calf serum and 100 U/mL penicillin/streptomycin (1 mL cell suspension per well) for 48 h with Con A (5 µg/mL) at 37 °C in a humidified incubator with 5% CO₂. Supernatants were assayed for cytokine content by ELISA.

Cytokine measurements

The levels of cytokines (IL-4, IL-5, IL-13 and IFN-γ) in the BAL fluid or in supernatants were assayed by sandwich kit ELISA according to the manufacturer's suggestion (Pharmingen, San Diego, CA, USA) as previously described [30]. For IL-13 determinations, the pairs were 38 213.11 and biotinylated goat polyclonal anti-IL-13 from R&D Systems (Minneapolis, MN, USA). Values are expressed as pg/mL deduced from standards run in parallel with recombinant cytokines. The limit of detection was 10 pg/mL for IFN-γ, IL-4 and IL-5, and 31 pg/mL for IL-13.

Measurement of total immunoglobulin E and ovalbumin-specific immunoglobulin G1, immunoglobulin G2a and immunoglobulin E antibodies

Total IgE and OVA-specific antibodies were assayed by sandwich ELISA as previously described [30]. Briefly, serum samples were titrated for optimal dilutions for testing different isotypes. For total IgE, we used 1/100 dilution and for OVA-specific IgE, IgG1 and IgG2a, we used, respectively, 1/10, 1/10 000 and 1/100 dilutions. For total IgE determinations, plates were coated overnight at 4 °C with 0.2 µg/mL of unlabelled rat anti-mouse ε heavy chain monoclonal antibody (clone 23G3 from Southern Biotechnology, Birmingham, AL, USA) as a capture antibody. Serum samples were added and bound IgE was revealed with biotin-labelled antibody (same clone) followed by extrAvidin Peroxidase conjugate (Sigma-Aldrich). The levels of IgE were deduced from IgE standard run in parallel. For OVA-specific IgE determinations, plates were coated with anti-IgE and subsequently biotin-labelled OVA was added to the wells. The bound OVA-biotin was revealed and OVA-specific IgE levels of samples were deduced from an internal standard arbitrarily assigned as 1000 U. For OVA-specific IgG1 and IgG2a antibodies, serum samples were plated on 96-wells previously coated with OVA (2 µg/well). The bound antibodies were revealed with goat anti-mouse IgG1 or IgG2a followed by peroxidase-labelled rabbit anti-goat antibodies (all from Southern). The concentrations of each OVA-specific isotype was estimated by comparison with IgG1 and IgG2a standards run in parallel as previously described [30].

Measurement of immunoglobulin G1 and immunoglobulin E anaphylactic antibodies

The anaphylactic activity of IgG1 and IgE antibodies was evaluated by passive cutaneous anaphylactic reaction (PCA), respectively, in mice and rat as previously described [27, 31]. Briefly, for determination of anaphylactic IgG1 antibodies, the serum samples were first incubated for 1 h at 56 °C to inactivate IgE antibodies. Mice were shaved 24 h before performing the PCA reaction and injected dermally (50 µL) with three serial dilutions on each side of the dorsal skin. After 2 h, they were

challenged i.v. with 250 µg of OVA + 0.25% of Evans blue solution (0.5 mL). For the IgE titration, rats were shaved and injected with 100 µL of four serial dilutions of non-inactivated serum in four sides of the dorsal skin and 24 h later the animals were injected with 250 µg of OVA + 0.25% of Evans blue (1 mL) and 30 min later the PCA reaction determined. All tests were made in pooled sera and in triplicate and the PCA titres were expressed as the reciprocal of the highest dilution that gave a lesion of > 5 mm in diameter. The detection threshold of the technique was established at 1 : 5 dilution.

Histological analyses

After BAL collection, lungs were perfused via the right ventricle with 10 mL of PBS to remove residual blood, immersed in 10% phosphate-buffered formalin for 24 h, and then in 70% ethanol until embedded in paraffin. Lung sections with 5 µm were stained with hematoxylin/eosin for evaluation of peribronchial and perivascular lung inflammation or with periodic acid-Schiff (PAS)/haematoxylin for the evaluation of mucus production as described earlier [27]. Briefly, a quantitative digital morphometric analysis was performed using the application program Metamorph 6.0 (Universal Images Corporation, Downingtown, PA, USA). The circumference area of bronchi and the PAS-stained area were electronically measured and the mucus index was determined by the following formula: (PAS-stained area/bronchial circumference area) × 100.

Tumour necrosis factor-α and nitric oxide and determinations

TNF-α was determined by the standard L929 cytotoxic assay as described earlier [32]. Nitric oxide production was estimated converting nitrate to nitrite with nitrate reductase and nicotinamide adenine dinucleotide phosphate (NADPH; Boehringer Mannheim, Mannheim, Germany) for 6 h at 37 °C as described earlier [12]. Values represent sample data against standard curve.

Statistical analysis

ANOVA was used to determine the levels of difference between all groups. Comparisons of all pairs were performed by Tukey-Kramer honest significant difference test. Values for all measurements are expressed as mean ± SEMs, and the *P*-values for significance were set to 0.05.

Results

Co-adsorption of lipopolysaccharide and allergen to alum suppresses dose-dependently the development of airway allergic disease

Previous studies indicated that depending on the dose, LPS can enhance or suppress Th2 immunity [13, 15]. Thus,

we first performed experiments to determine whether different doses of LPS, ranging from 1 ng to 10 µg, co-adsorbed with OVA to alum could up- or down-regulate lung allergic responses. Mice were sensitized on days 0 and 7 by s.c. injections of LPS-free OVA plus increasing concentrations of LPS co-adsorbed to alum. On days 14 and 21, mice were challenged i.n. with OVA and 24 h later, the experiments were performed. Total cell counts in BAL fluid revealed that mice sensitized with purified LPS-free OVA plus alum and challenged twice with OVA (OVA group) showed more than 10-fold increase in the number of cells (> 2 million) when compared with control group (Fig. 1a). The cellular influx with LPS at 1 ng was similar to that of OVA group, indicating that at this dose LPS (equivalent to 5 EU) did not modify the inflammatory response (Fig. 1a). However, groups of animals that received increasing doses showed a significant inhibition of total cell recruitment (Fig. 1a). Co-adsorption of 0.01 µg (low dose) or 10 µg (high dose) of LPS with OVA to alum inhibited in a dose-dependent manner the development of airway eosinophilia without increasing other cells types (Fig. 1b). Thus, it appears that LPS did not shift the airway inflammation towards a Th1 pattern as we did not find an increase in the number of macrophages, lymphocytes or neutrophils in LPS group as would be expected for Th1-mediated airway inflammation [21, 33, 34]. Next, we determined the levels of Th2- or Th1-affiliated cytokines in the BAL fluid. As shown in Fig. 1c, animals that received LPS had significant dose-related reductions in Th2-associated cytokines when compared with OVA group. IFN-γ production was very low and similar in all groups tested, including control animals and the group that received a high dose of LPS (Fig. 1c). To address this issue more specifically, we cultured lung draining mediastinal lymph nodes cells with Con A and measured IFN-γ and IL-5 production. We found that IFN-γ production was similar in the OVA/LPS groups when compared with OVA group while IL-5 production was suppressed in OVA/LPS groups (Fig. 1d). The above results indicate that the presence of LPS during OVA sensitization decreases airway Th2-allergic responses, without inducing increased IFN-γ production.

Co-adsorption of lipopolysaccharide and allergen to alum prevents the development of Type 2 T helper cells-mediated lung pathology

Because asthma pathology is associated with AHR and mucus hyper-production, we determined AHR to increasing doses of methacholine and goblet cell metaplasia in our model. We found that in contrast to animals sensitized with OVA, the OVA/LPS groups did not develop AHR (Fig. 2a). Also, exposure to LPS during sensitization inhibited in a dose-dependent manner mucus hyper production (Fig. 2b). Next, we analysed lung inflammation as assessed

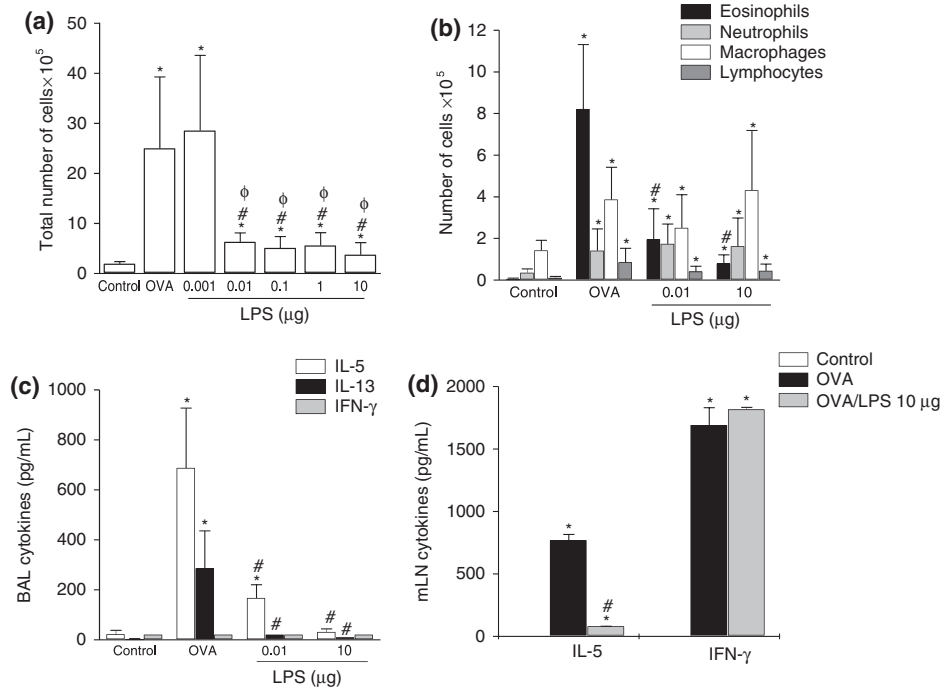


Fig. 1. Lipopolysaccharide (LPS) prevents the development of airway allergic inflammation. Groups of BALB/c mice were sensitized twice with ovalbumin (OVA) (OVA group) or OVA containing increasing concentrations of LPS (OVA/LPS groups) co-adsorbed to alum and challenged twice with LPS-free OVA. Control group consisted of non-manipulated animals. The experiments were performed 24 h after the last OVA challenge. Bronchoalveolar lavage (BAL) total cell counts (a), BAL differential cell counts (b), cytokines in BAL fluid (c). Cytokines in supernatants of Con A stimulated mediastinal lymph node cells (d). Results are expressed as mean $\pm$ SEM for groups of five mice and are representative of two experiments. *Significant difference ($P < 0.05$) when compared with control group. #Significant difference ($P < 0.05$) when compared with OVA group. [†]Significant difference ($P < 0.05$) when compared with LPS 0.001 μ g.

by cellular infiltrates around medium bronchi or vessels and we found that LPS decreased lung inflammation in a dose-dependent manner (Fig. 2c). It is important to note that the group that received LPS at a dose of 10 μ g was almost without peribronchovascular cellular infiltrates (Fig. 2c). Altogether, our results indicate that the presence of LPS during OVA/alum sensitization decreases pulmonary Th2-allergic responses without an apparent immune deviation towards Th1 asthma phenotype.

Co-adsorption of lipopolysaccharide and allergen to alum suppresses dose-dependently anaphylactic antibodies but increases ovalbumin-specific immunoglobulin G2a levels

We next determined whether sensitization to allergen in the presence of different concentrations of LPS could affect allergic isotype pattern. Therefore, we measured serum levels IgE and anaphylactic IgG1 antibodies as markers of Th2 activities. We also measured IgG2a antibodies as a marker of Th1 response [27, 35]. The OVA group showed a significant increase of total IgE as well as OVA-specific IgE and IgG1 anaphylactic antibodies when compared with control group (Figs 3a–c). The OVA group also showed a discrete but significant increase in OVA-

specific IgG2a antibodies (Fig. 3d). In the OVA/LPS groups, we found that LPS at 10 μ g but not 0.01 μ g decreased the production of total and OVA-specific IgE antibodies measured by ELISA and PCA, respectively (Figs 3a and b). The anaphylactic IgG1 production was suppressed by both doses of LPS while the production of OVA-specific IgG2a, a Th1-affiliated immunoglobulin, showed a dose-dependent increase (Figs 3c and d). We conclude that contrary to what we found in the lung, the systemic humoral response of OVA/LPS groups showed a more Th1-affiliated pattern than the OVA group.

Inhibition of Type 2 T helper cells-driven airway allergic responses by lipopolysaccharide is TLR4 and MyD88 dependent

In order to assure specificity and determine downstream signaling pathways of TLR4 activation responsible for the LPS-induced inhibition of allergic lung responses, we performed experiments in gene-targeted BALB/c mice deficient for TLR4 molecule or the adaptor molecule MyD88. As expected, LPS inhibited airway inflammation in wild type (WT) mice but not in TLR4-deficient mice as assessed by the total number of cells and the number of eosinophils present in the BAL (Figs 4a and b). We also

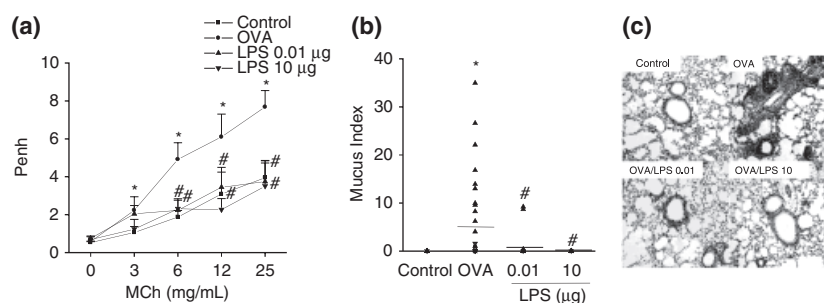


Fig. 2. Lipopolysaccharide (LPS) prevents the development of airway pathology. Groups of BALB/c mice were sensitized with ovalbumin (OVA) (OVA group) or OVA containing increasing concentrations of LPS (OVA/LPS groups) co-adsorbed to alum and challenged twice with LPS-free OVA. Control group consisted of non-manipulated animals. The experiments were performed 24 h after the last OVA challenge. Airway hyper-reactivity to increasing doses of methacholine (a). Mucus index (b) was determined by periodic acid-Schiff staining as indicated in M&M. Representative lung sections showing normal lung histology in control group (upper left) and massive cell infiltration around bronchi and vessels (c). Note: mucus plug in OVA group (upper right). Peribronchovascular inflammation in OVA/LPS 0.1 was very discrete (bottom left) and in OVA/LPS 10 group was almost absent (bottom right). Results are expressed as mean $\pm$ SEM for groups of four to five mice. *Significant difference ($P < 0.05$) when compared with control group. #Significant difference ($P < 0.05$) when compared with OVA group.

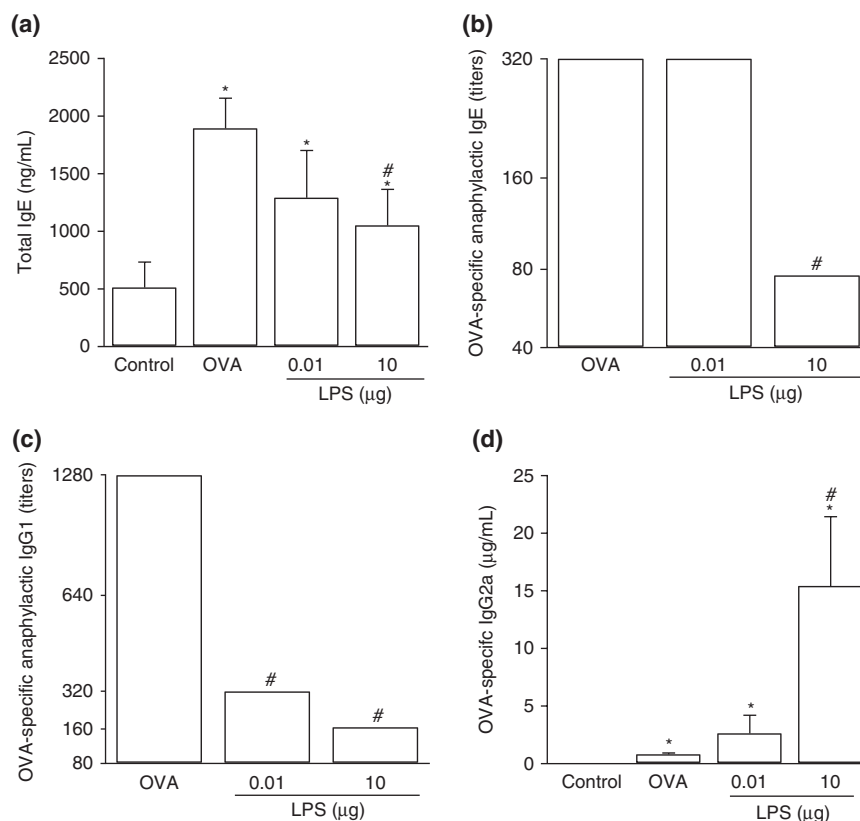


Fig. 3. Ovalbumin (OVA)-induced antibody responses in animals sensitized with OVA or OVA plus different concentrations of lipopolysaccharide (LPS). Total IgE (a) and IgG2a antibodies (d) were determined by sandwich ELISA. Results are expressed as mean $\pm$ SEM for groups of five mice and are representative of two experiments. IgE (b) and IgG1 (c) anaphylactic antibodies were determined by passive cutaneous anaphylactic reaction (PCA) and titres were expressed as the reciprocal of the highest dilution that gave a lesion of > 5 mm in diameter. Results are from five animals per group. *Significant difference ($P < 0.05$) when compared with control group. #Significant difference ($P < 0.05$) when compared with OVA group.

found that the MyD88 pathway is essential for the inhibition of allergic inflammation because in MyD88-deficient mice, LPS did not down-modulate the eosinophilic in-

flammation when compared with OVA group (Fig. 4c). We conclude that TLR4 and MyD88 signaling are essential for down-regulation of allergic inflammation by LPS.

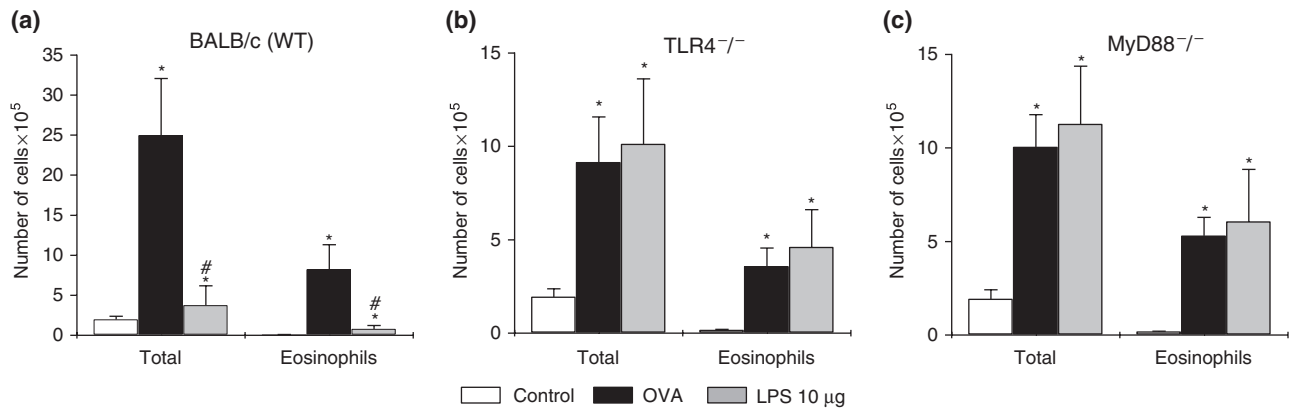


Fig. 4. Inhibition of airway allergic inflammation by lipopolysaccharide (LPS) requires toll-like receptor 4 (TLR4) and MyD88. Groups of wild type (WT), TLR4^{-/-} or MyD88^{-/-} mice were sensitized twice (s.c.) with ovalbumin (OVA) or with OVA containing 10 µg of LPS adsorbed on alum and challenged twice with OVA by intranasal route. Control group consisted of non-manipulated animals. The experiments were performed 24 h after the last OVA challenge and total cell number or the number of eosinophils were determined in bronchoalveolar lavage (BAL) fluid of WT (a), TLR4^{-/-} (b) or MyD88^{-/-} (c) mice. Results are expressed as mean ± SEM for groups of four to five mice. *Significant difference ($P < 0.05$) when compared with control group. #Significant difference ($P < 0.05$) when compared with OVA group.

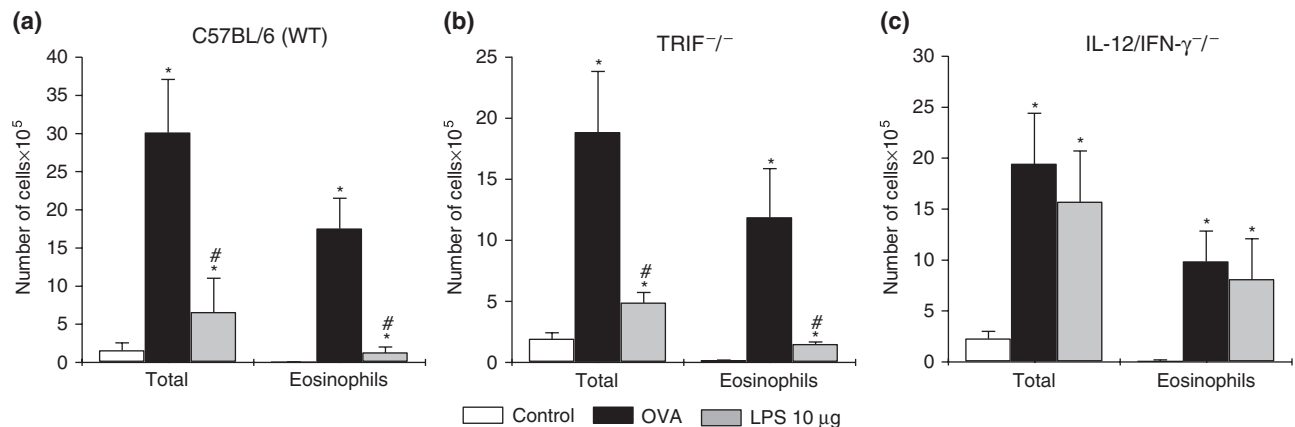


Fig. 5. Inhibition of airway allergic inflammation by lipopolysaccharides (LPS) is independent of TRIF pathway but dependent of IL-12/IFN-γ axis. Groups of wild type (WT), TRIF^{-/-} or IL-12/IFN-γ^{-/-} were sensitized twice (s.c.) with ovalbumin (OVA) or with OVA containing 10 µg of LPS adsorbed on alum and challenged twice with OVA by i.n. route. Control group consisted of non-manipulated animals. The experiments were performed 24 h after the last OVA challenge and the total cell number or the number of eosinophils were determined in BAL fluid of WT (a), TRIF^{-/-} (b) or IL-12/IFN-γ^{-/-} (c) mice. Results are expressed as mean ± SEM for groups of four to five mice. *Significant difference ($P < 0.05$) when compared with the control group. #Significant difference ($P < 0.05$) when compared with OVA group.

Lipopolysaccharide inhibits Type 2 T helper cells-driven airway allergic responses in TRIF-deficient but not in interleukin-12/interferon-γ-deficient mice

Another TLR4 signaling pathway is via toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF) [36]. This pathway generates IFN-β and nitric oxide, both products that could potentially suppress asthma [12, 37, 38]. Thus, we investigated whether the TRIF pathway could also mediate the LPS effects using TRIF-deficient mice. We found that the TRIF pathway appears to be dispensable for the suppression because in TRIF-deficient animals, LPS prevented the development of allergic lung inflammation similar to WT mice (Figs 5b and a). We conclude that LPS

inhibits allergic responses signaling through MyD88 but not TRIF. As MyD88 pathway is essential for the production of IL-12, a key cytokine for IFN-γ production [39], and because MyD88 is also involved in IFN-γ signaling [40], and these cytokines are known to down-regulate allergic asthma [41] we studied the effect of LPS in IL-12/IFN-γ double KO mice. We found that LPS failed to inhibit allergic lung inflammation in double KO mice (Fig. 5c). In these animals, LPS also did not inhibit AHR, type 2 cytokines and anaphylactic antibodies (data not shown). The above experiments indicate that MyD88 and IL-12/IFN-γ molecules are critically involved in LPS-mediated down-modulation of allergic sensitization.

Comparison of systemic bioactivities induced by lipopolysaccharides with ER-803022, a synthetic lipid A analogue with toll-like receptor 4 agonist activity

Several molecules such as monophosphoryl lipid A, a purified LPS-derived from *Salmonella minnesota* or synthetic lipid A analogues have been developed in an attempt to maintain LPS-like bioactivities but decrease endotoxicity [42, 43]. Thus, we were interested in determining whether a synthetic lipid A analogue (ER-803022) that possesses adjuvant effects via TLR4 signaling [23] could down-modulate Th2 responses. Before testing the effect of ER on allergic sensitization, we first determined its bioactivities that are associated with systemic inflammation. Thus, we compared the systemic effects of LPS with ER after intravenous injection by measuring serum levels of TNF- α and NO, two products involved in LPS toxicity [1]. As expected, i.v. LPS injection resulted in the production of high levels of TNF and NO when compared with the control group (Figs 5a and b). Surprisingly, the injection of ER did not induce the production of TNF or NO and the values were similar to control group (Figs 6a and b). Moreover, animals injected with LPS showed discrete signs of toxicity such as diarrhoea, curly hair and lethargy while animals injected with ER did not show these symptoms. Although we used the same doses of TLR4 agonists in our study, it should be stressed that molar concentration ER is roughly 250-fold higher than LPS. Collectively, these results show ER is clearly less toxic than LPS and does not induce an apparent systemic inflammation at the time-point examined.

Co-adsorption of ER to alum suppresses the development of airway allergic responses

As ER-induced bioactivities including toxicity were less robust than LPS, we tested whether ER administration

during allergen sensitization at dose of 10 μ g could affect asthma development. For comparison, we used a group of mice that received 10 μ g of LPS. We first determined AHR, airway inflammation and Th2 cytokine in BAL fluid of animals that were sensitized with OVA or OVA co-adsorbed with LPS or ER to alum. Animals sensitized with OVA containing LPS or ER did not develop AHR as indicated by Penh values that were similar to control group and significantly different from OVA group (Fig. 7a). Allergic airway inflammation was also suppressed in animals sensitized in the presence of ER or LPS as revealed by total and eosinophils cell counts (Fig. 7b). Quantification of BAL IL-5 and IL-13 confirmed that LPS or ER were equally effective in reducing OVA-induced Th2 cytokine secretion (Fig. 7c). Next, we measured the serum levels of OVA-specific IgE, IgG2a and IgG1 antibodies. Both LPS and ER suppressed IgE production and again the suppressive activity was similar for both compounds (Fig. 7d). Regarding IgG2a antibody production, animals from OVA/LPS and OVA/ER groups showed increased level of this isotype when compared with OVA group (Fig. 7e). However, in the OVA/LPS group the levels of OVA-specific IgG2a were significantly higher than in the OVA/ER group (Fig. 7e). Finally, all groups except control animals produced similar levels of OVA-specific IgG1 antibodies (Fig. 7f). In conclusion, these experiments indicate that ER is as efficient as LPS in inhibiting allergic lung responses and OVA-specific IgE antibodies.

Discussion

The present work provides data indicating that co-adsorption of natural or synthetic TLR4 agonist with allergen to alum impairs the development of Th2-driven allergic airway disease. We found that LPS inhibited the

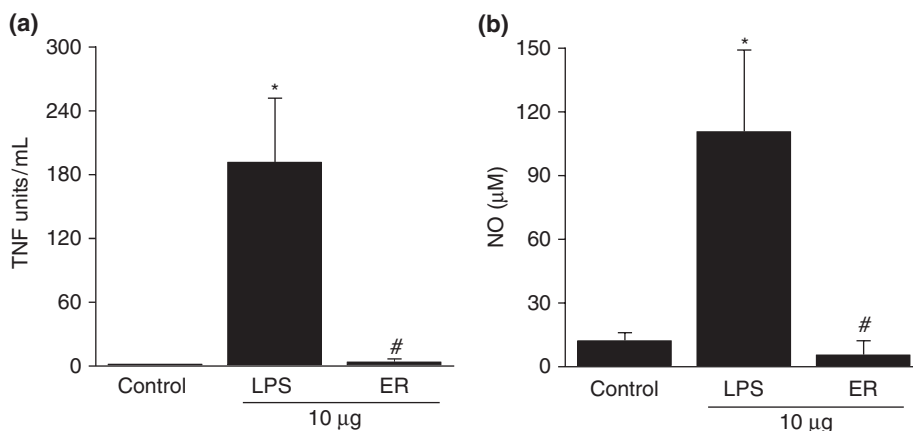


Fig. 6. Systemic effects of natural (lipopolysaccharides, LPS) or synthetic (ER803022) toll-like receptor 4 (TLR4) agonists. Groups of mice received 10 μ g of LPS or ER by intravenous route and 90 min after serum TNF- α levels were measured (a). Twenty-four hours after intravenous LPS or ER injections, the serum nitrate was converted to nitrite to determine nitric oxide production (b). Results are expressed as mean $\pm$ SEM for groups of five mice and are representative of two experiments. *Significant difference ($P < 0.05$) when compared with LPS group. #Significant difference ($P < 0.05$) when compared with control group.

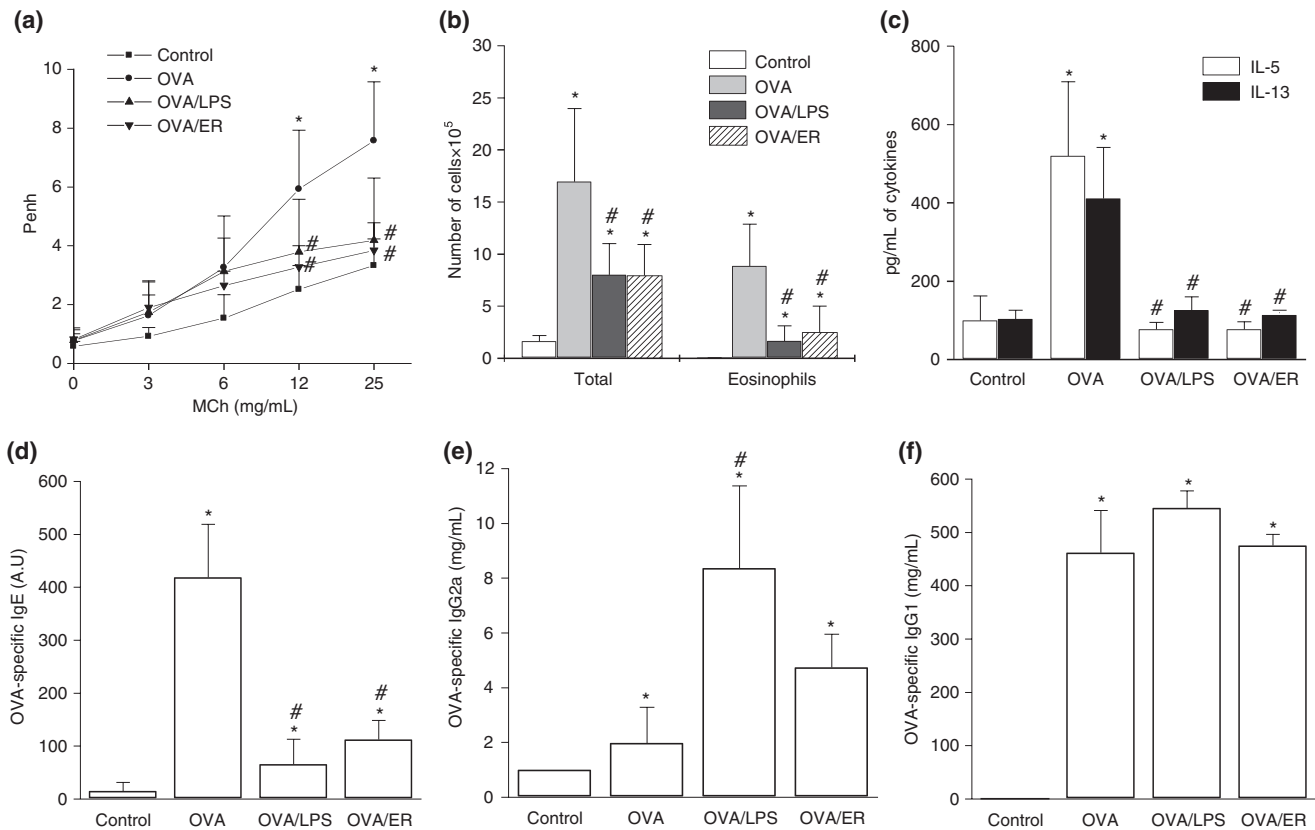


Fig. 7. Allergen sensitization with lipopolysaccharides (LPS) or ER suppresses the development of Th2 immunity. Groups of BALB/c mice were sensitized twice (s.c.) with ovalbumin (OVA) (OVA group) or with 10 μ g of LPS or ER (LPS or ER groups) adsorbed on alum and challenged twice with LPS-free OVA control group consisted of non-manipulated animals. The experiments were performed 24 h after the last OVA challenge. Airway hyper-reactivity to increasing doses of methacholine (a). Total cell numbers and the number of eosinophils were determined in bronchoalveolar lavage (BAL) fluid (b). Cytokines in BAL fluid (c). OVA specific antibodies were measured by sandwich ELISA IgE (d), IgG2a (e) and IgG1 (f). Results are expressed as mean $\pm$ SEM for groups of five mice. #Significant difference ($P < 0.05$) when compared with OVA group. *Significant difference ($P < 0.05$) when compared with the control group.

development of airway eosinophilic inflammation, AHR, type 2 cytokine production and mucus hyper-secretion in a dose-dependent manner.

The minimal dose of LPS that affected lung inflammation was 10 ng and by increasing the doses up to 10 μ g, the cellular and humoral Th2-mediated responses became increasingly suppressed. Based on these results, we concluded that LPS always exerted a negative regulation on Th2 immunity. These results confirm and extend previous reports showing that LPS given during sensitization prevents allergen-induced airway inflammation [20] and that AHR is markedly suppressed when commercial OVA that is contaminated with LPS is used [19]. It has been reported that B6 TLR4-sufficient mice challenged for long periods of time with OVA containing low doses of LPS develop an attenuated allergic response when compared with B6 TLR4-deficient mice [15]. In contrast, Dabbagh et al. [18] showed that TLR4 expression is required for optimal Th2 responses. It was also reported that the expression of TLR4 and MyD88 molecules was necessary for allergic sensitization achieved by repeated intranasal administrations of OVA containing low doses of LPS [13]. However, in a

subsequent work, the authors showed that TLR4 signaling appears to be dispensable for allergic sensitization when other route is used or when sensitization is done with allergen adsorbed to alum [17].

It is reasonable to assume that TLR4 signaling is dispensable for allergic sensitization because alum is a potent Th2 adjuvant that does not contain microbial products. Indeed, we found that TLR4-deficient animals sensitized with LPS-free OVA plus alum developed allergic lung inflammation. We found that co-adsorption of natural or synthetic TLR4 agonist with allergen to alum exerted a negative regulation on allergic lung disease. Mechanistically, we found that LPS down-regulated allergic lung disease signaling through TLR4 and MyD88, but not the TRIF pathway. Moreover, the inhibitory effect of LPS was not observed in animals deficient in IL-12/IFN- γ molecules. Thus, one possible explanation for the protective mechanism of LPS on allergic lung disease might be the development of TLR4-stimulated IL-12-dependent Th1 immune response because IFN- γ inhibits IgE synthesis, airway eosinophilia and augments IgG2a production [44, 45]. Indeed, we found that IgE production and airway

eosinophilia were consistently inhibited in mice sensitized with OVA in the presence of LPS and the serum levels of IgG2a were increased. However, we do not favour the notion that LPS shifted the immunity to a fully polarized Th1 response because we could not detect an increased IFN- γ production in the lungs of animals sensitized in the presence of LPS. Moreover, we could not find any evidence for Th1-mediated airway disease as the OVA/LPS groups did not develop AHR or neutrophilic inflammation that are common features of type 1 asthma phenotype [21, 22]. In addition, histological analyses showed that mucus formation and lung cellular infiltrates of animals sensitized with OVA/LPS were drastically decreased when compared with that of OVA group (Figs 2b and c).

We speculate that a polarized T cell activation towards Th2 or Th1 pattern does not occur due to two possible mechanisms: (1) TLR4 signaling antagonizes the Th2 enhancing properties of alum [46, 47] and alum, in turn, might impair the Th1-promoting activity of TLR4 signaling [13, 15, 19] and (2) induction of regulatory T cell by LPS [48] could suppress the development of Th2 or Th1 cells. We favour the first possibility because s.c. sensitization with OVA plus LPS but in the absence of alum-induced airway neutrophilia that is characteristic of Th1-mediated airway inflammation [33].

It remains to be explained why TLR4 signaling promoted an increase in the production of IgG2a antibodies without concomitant increase in inflammatory Th1 cells. One possibility is that sensitization with LPS generated IFN- γ -producing cells in draining LN or spleen where they helped B cell to produce IgG2a antibodies and inhibited alum's ability to effectively initiate a Th2 response but these cells did not acquire the phenotype of fully polarized inflammatory Th1 cells [33] and therefore lacked the ability to migrate to lung.

Using similar methodology, Jankovic et al. [49] reported that adsorption to alum promotes the activity of IL-12 as an adjuvant for type 1 cytokine response to HIV-1 gp120. It should be noted that in this study, 1 μ g of IL-12 was used. Although we did not determine how much IL-12 was induced *in vivo* in our protocol, it is likely, based on *in vitro* experiments with LPS and macrophages, that the amount of IL-12 generated in our study might be much lower than that required for Th1 polarization.

An important observation that emerged from our study is that OVA given with a mixture of two adjuvants with opposing effects on immune responses dampened the development of airway inflammatory responses characteristic of Th2 or Th1 cells, but spared antibody production. This type of immune modulation might be applied for the development of vaccines against microbial products or against allergy. In this line, it was shown that subcutaneous administration of a vaccine of ragweed-TLR 9 agonist appeared to offer long-term clinical efficacy in the treatment of patients with allergic rhinitis [50]. Having

this in mind, in the present study we used the s.c. route because it is less invasive than the i.p. route and as such more suitable for immune intervention in humans.

Alum is currently used as adjuvant in human vaccination and monophosphoryl lipid A, a new generation of soluble adjuvants is currently being investigated [51]; however, the association of both adjuvants have not been addressed. Here, we show that the association of ER, a synthetic lipid A analogue that did not induce a systemic inflammatory response, with alum given s.c. was as effective as LPS in the inhibiting allergic responses. When viewed in combination, our work indicates that TLR agonists plus allergens co-adsorbed to alum might represent a novel strategy for down-modulating allergic responses.

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