

A novel synthetic lipopeptide is allergy-protective by the induction of LPS-tolerance

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Summary

Background Exposure to the environment of traditional farms can protect children from some allergic disease. Due to this exposure, TLR2 expression in these children is increased. TLR2 ligands derived from gram-positive bacteria are found in the dust of these farms.

Objectives We proved whether a synthetic lipopeptide binding to the TLR1/2 heterodimer is able to protect from allergic disease in two different murine models of allergy. We also investigated the immunological mechanisms underlying the protective properties of the lipopeptide.

Methods We synthesized a lipopeptide derived from a germination lipoprotein of *Bacillus cereus* (LPGerD). We evaluated the immunomodulatory activity of LPGerD in a murine model of systemic sensitization (OVA/Alum) and in a model in which mice were sensitized with OVA pulsed bone-marrow-derived dendritic cells (BMDCs) via the airways. Furthermore, the induction of LPS tolerance was studied.

Results Treatment of mice with LPGerD in a mouse model of asthma led to protection against sensitization and airway inflammation. Similarly, bone-marrow-derived dendritic cells (BMDCs) pre-treated with LPGerD were not able to prime mice for allergic immune response. We observed that pre-treatment with LPGerD led to the induction of a LPS-tolerant state in BMDCs. These cells secreted markedly lower amounts of pro-inflammatory cytokines upon LPS stimulation. Furthermore, we observed an up-regulation of IRAK-M mRNA in BMDCs pre-treated with LPGerD.

Conclusions and Clinical Relevance Our results suggest that induction of a LPS-tolerant state in antigen-presenting cells (APCs) may contribute to the protective effect of a farming environment. TLR2 agonists similar to those appearing in cowshed dust extracts, such as our synthetic LPGerD, lead to the ignorance of the LPS stimulus, which is important for the activation of APCs to mount a Th2 immune response. This substance might be a promising candidate for allergy-preventive treatments as LPGerD had only low pro-inflammatory characteristics.

Keywords asthma, farming environment, murine models

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Introduction

Contact with animal sheds during the first year of life can protect children from the development of some allergic diseases [1–4]. Dust samples were collected from cowsheds of traditional farms and extracts from this dust (cowshed dust extracts; CDE) mediated protection from the symptoms of allergic asthma in a murine model [5]. Two bacterial strains were found in the dust

in addition to a class of allergy-protective sugar molecules [6] which are able to exhibit allergy protection [7]. However, the exact immunological mechanisms by which these organisms mediate protection against allergic disease remain to be defined. Several studies have shown that TLR4 ligands, as well as gram-positive bacteria and TLR2 ligands, such as the lipopeptide Pam3CSK4, have allergy-protective properties [7–13]. TLR4 agonists are unsuitable for therapeutic approaches in allergic asthma because their beneficial effects are counterbalanced by severe inflammatory side-effects,

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such as the infiltration of neutrophils into lung tissue, persistent airway hyperreactivity and airway remodeling [7, 14], and an up-regulation of a Th1 immune response which inhibits the Th2 immune response [13]. However, we observed the down-regulation of both Th1 and Th2 immune responses by the application of CDE in our earlier studies [5]. This fact gave rise to our hypothesis that non-inflammatory immune-modulating substances are included in CDE which mediate protection. We focused on the role of TLR2 and its ligands in the mechanism of protection mediated by the farming environment because TLR2 and CD14 expression in blood samples of farm children showed that both receptors were expressed at a higher density than in blood samples of children without contact to animal sheds [15]. Consequently, in this study, we investigated CDE for the presence of TLR2 agonists. We synthesized a triacyl lipopeptide derived from the germination protein of *Bacillus cereus* (GerD). This lipopeptide GerD (LPGerD) not only exerted a strong reduction of allergic airway inflammation in two different animal models of allergy but it was also less inflammatory than LPS. We showed that the pre-treatment of bone-marrow-derived dendritic cells (BMDCs) with LPGerD led to a lower secretion of pro-inflammatory cytokines upon LPS stimulation. This phenomenon was accompanied by the up-regulation of IRAK-M mRNA in BMDCs, a molecule which has been shown to be essential for LPS tolerance [16]. We conclude that LPGerD exerts its allergy-protective ability by the induction of LPS tolerance in BMDCs because we know from our earlier study that LPS is critically needed to instruct antigen-primed BMDCs to mount a Th2 response [17]. These results may give insights into immunological mechanisms underlying the allergy-protective effect of the farming environment. To our knowledge, no studies are currently available which show that the induction of LPS tolerance exclusively in dendritic cells (DCs) is accompanied by an inability to induce a Th2 immune response.

Methods

Synthesis of a tri-acyl lipopeptide

The synthesis of the lipopeptide LPGerD was carried out by fully automated solid phase peptide synthesis and Fluorenylmethoxycarbonyl (Fmoc)/tert-Butyl chemistry on TCP resin. A sevenfold molar excess of single Fmoc-L amino acids was used for the coupling of amino acids. The peptide resin carrying the sequence AQ-EKEAKSELDYD was elongated with the unusual lipopeptide Pam3Cys-OH. The coupling was carried out in Dimethylformamide:CH₂Cl₂ (1 : 1) with DIC (Diisopropylcarbodiimid)/HOBt (1-Hydroxybenzotriazol) in threefold excess within 3 h and was monitored by Kaiser

assay. The lipopeptide was cleaved off the resin by treatment of the resin with trifluoroacetic acid/phenol/ethanedithiol/thioanisole (96 : 2 : 1 : 1) for 3 h. TFA was removed in vacuo and the lipopeptide was precipitated from cold diethylether. The precipitate was washed three times with cold diethylether and the lipopeptide LPGerD was dissolved in tert-butyl alcohol/water (4 : 1) and lyophilized. The structural characterization of the lipopeptide was carried out by electrospray mass spectrometry. In addition, it was analysed by HPLC-ESI-MS coupling. To prove that LPGerD is free of LPS as well as not an agonist of TLR4 by itself we have stimulated TLR4/CD14/MD2 transfected Hek293 cells (Cayla Invivogen Europe, Toulouse, France) with doses up to 1 µg/mL. These experiments showed that TLR4 is not activated by stimulation with LPGerD (data not shown).

Detection of TLR2 ligands in CDE

The measurement of TLR2 ligands in the CDE was performed using TLR2 transfected Hek293 cells (Cayla Invivogen Europe, Toulouse, France). A serial dilution of the synthetic diacyl lipopeptide FSL-1 was used (EMC microcollections GmbH, Tübingen, Germany) as a positive control. The Interleukin-8 production of cells was measured by ELISA after stimulation of the cells with either FSL-1 or CDE.

Animals

Female BALB/c mice (Charles River, Sulzfeld, Germany) and TLR2 knockout mice on a C57/BL6 background (The Jackson Laboratory, Bar Harbor, MA, USA) aged 7–8 weeks were purchased and then adapted to the animal facility for 14 days prior to the experiments. The animals had access to food and water *ad libitum*. All experimental procedures were approved by the animal ethics committee at Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Germany (approval number: 50.8735.1 Nr. 107/3).

Murine OVA/Alum model of allergy

Mice were sensitized by two intraperitoneal injections of 20 µg ovalbumin (OVA gradeV; Sigma, St Louis, MO, USA) emulsified in 2.2 mg aluminium hydroxide (ImjectAlum; Pierce, Rockford, IL, USA) in a total volume of 200 µL on days 1 and 14 (see Fig. 1a). On days 28 and 38, the mice were challenged via the airways with 1% OVA-aerosol for 30 min using a PARI-Boy aerosol generator. For the treatments with the lipopeptide LPGerD, it was solubilized in dimethylsulfoxide (DMSO) in a concentration of 10 mg/mL. Just before administration to the mice the lipopeptide was further diluted with isotonic NaCl solution. During the sensitization period

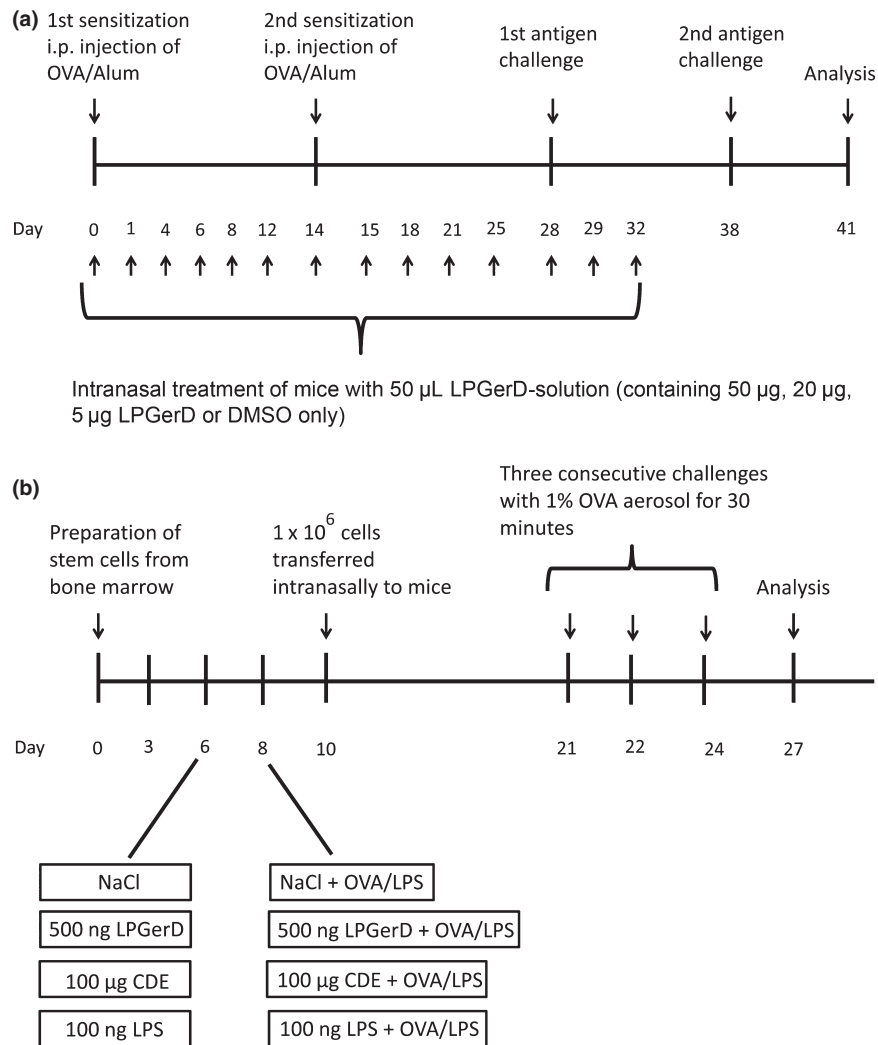


Fig. 1. Schematic depiction of the two mouse models used in this study. (a) Timeline of sensitization and challenge in a mouse model using systemic sensitization with OVA adsorbed to Alum. (b) Timeline of a mouse model using sensitization with OVA pulsed dendritic cells via the airways. Cells were cultured *in vitro* during the first ten days. On day ten, cells were administered intranasally to evaluate their sensitizing capacity.

and around the first antigen challenge, the mice were treated intranasally for a total of 14 times with 50 μ L of either NaCl or different concentrations of lipopeptide (50 μ g, 20 μ g or 5 μ g lipopeptide per application). To reveal possible pharmacological effects of the DMSO application a control group of mice was treated with 10% DMSO solution during sensitization. Three days after the last challenge, the mice were killed and the different parameters of allergic sensitization and airway inflammation were investigated.

Murine DC transfer model of OVA-allergy

The femora and tibia of naive mice were prepared and then flushed with phosphate-buffered saline (PBS) to harvest stem cells from the bone-marrow. Subsequently, erythrocytes were lysed by hypotonic shock. Bone-marrow cells were cultured with 20 ng/mL

rmuGMCSF (Immunotools, Friesoythe, Germany) for six days with one exchange of supplemented RPMI 1640 medium (RPMI 1640 with 5% fetal calf serum [FCS], 2 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin; all from Biochrom, Berlin, Germany) on day three (see Fig. 1b). On day six, low-adherent dendritic cells were collected and cultured with either PBS, 500 ng of lipopeptide, 100 μ g of CDE or 100 ng of LPS. On day eight, the medium was exchanged and the cells were loaded with 100 μ g/mL purified ovalbumin (OVA, Hyglos, Bernried am Starnberger See, Germany) in combination with 1 ng LPS for two days. In addition, the medium was supplemented with the same stimuli the cells received on day six, that is, either PBS, 500 ng of lipopeptide, 100 μ g CDE or 100 ng of LPS. On day ten, the cells were harvested and 1×10^6 cells were intranasally transferred to the anaesthetized mice. The mice were challenged with a 1% OVA-aerosol using a

PARI-Boy aerosol generator (PARI, Starnberg, Germany) on days 21, 22 and 24. Three days after the last challenge, the mice were killed and sera, as well as spleens, lungs and bronchoalveolar lavages were collected for further analysis.

Treatment of BMDCs for allergy protection

The BMDCs were treated with different stimuli on days six and eight of cell culture. The cells were pre-treated with either 500 ng/mL LPGerD, 100 ng/mL LPS, 100 µg/mL CDE or were sham treated with PBS on day six of the *in vitro* cell culture. On day eight, the different groups were treated again with these stimuli. In addition, all the groups of differently treated BMDCs were armed for induction of an OVA-specific Th2 immune response by application of 100 µg/mL ovalbumin in combination with 1 ng/mL LPS to the cell culture. On day ten of the cell culture, 1×10^6 cells were intranasally transferred into the mice.

Investigation of the pro-inflammatory immune response in vitro

The BMDCs received stimuli with LPGerD or LPS in different concentrations on day eight of the *in vitro* cell culture. The cell culture supernatants were collected 24 h after the stimulation and stored at -20°C until ELISA.

Quantification of IRAK-M mRNA by qPCR

The isolation of RNA from the BMDCs was performed using a RNeasy kit (Qiagen, Hildesheim, Germany), as described by the manufacturer. cDNA was synthesized with a Quantitect RT kit 8 (Qiagen), as described by the manufacturer. The quantification of mRNA was performed using the following primers: forward primer: CATCTGTGGTACATGCCAGAA and reverse primer: CCAGAGAGAAGAGCTTTCAG; 10 µL of SYBR Fast Mix (Peqlab) were combined with 0.4 µL ROX High Mix (Peqlab) and 1 µL of 7.5 µM forward and reverse primer. To this solution was added 7.6 µL of distilled water, and then 5 µL of cDNA preparation were added to this premix and qPCR was performed as follows: (1) 1 s 50°C ; (2) 10 min 95°C ; (3) 15 s 95°C ; (4) 1 min 60°C ; steps three and four were repeated a total of 39 times. The housekeeping gene GUS was measured to calculate ΔCT .

ELISA kits used to measure concentration of cytokines and total IgE

The cytokine concentration in the supernatants of the cell culture and the IgE concentration in the serum and

BALF of mice were evaluated by ELISA. The following ELISA kits were used: huIL-8, muIL-1b, muIL-5, muIL-10 and muIgE, all from BD Biosciences (Heidelberg, Germany); muTNF- α , muIL-6 and muIL-13, all from eBiosciences (Frankfurt, Germany).

Measurement of OVA-specific IgE in BAL

BAL was collected by flushing the airways with 2 mL PBS. To determine the concentration of OVA-specific IgE sample-wells of a Nunc maxisorb ELISA plate (Nunc, Wiesbaden, Germany) were coated with 5 µg/mL OVA overnight and then blocked with 1% bovine serum albumin. After incubation with diluted BALF, bound OVA-specific IgE antibodies were detected with a biotinylated rat anti-mouse IgE antibody (clone R35-72, BD Biosciences). The latter antibody was detected by horseradish peroxidase conjugated extravidin (Sigma). Levels of OVA-specific IgE were related to a combined standard serum generated in our laboratory and expressed as arbitrary units per mL.

Measurement of airway responsiveness

Twenty-four hours after the last challenge airway resistance was measured in either conscious, unrestrained mice using whole-body plethysmography (Buxco Electronics) by measuring the enhanced pause [5] or in anaesthetized orotracheally intubated, spontaneously breathing animals by measuring the airway resistance [6]. Hyperreactivity of airways was determined by provocation with methacholine aerosol for 1 min followed by a recording period of 3–5 min at increasing concentrations (0, 6, 12, 25 and 50 mg/mL).

Statistical analysis

All the data from the *in vivo* experiments were analysed by the Kruskal–Wallis test and the Dunn's multiple comparison test using the Graph Pad Prism software, Version 5 (La Jolla, CA, USA). Values of $P < 0.05$ were considered statistically significant ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$). Results are presented as medians or means including SDs as indicated.

Results

Identification of TLR2 agonists in CDE

Gram-positive bacteria included in cowshed dust are able to mediate an allergy-protective effect [18]. We focused on the identification of TLR2 agonists, such as lipoproteins, derived from the bacterial cell wall in CDE. We investigated the potency of CDE to induce the release of IL-8 in a cell-based assay using TLR2-trans-

fects HEK293 cells. We observed that CDE induced the secretion of IL-8, as shown in Fig. 2, whereas HEK293 cells that were transfected with an empty control vector did not release IL-8 upon stimulation with CDE (data not shown). We concentrated on the identification of lipoproteins from *B. cereus* because this gram-positive microorganism typically occurs in the dust of traditional farms [19, 20]. We identified a lipoprotein from the endospores of *B. cereus* which was active in our TLR2 assay, namely the GerD protein. We fused 13 N-terminal amino acids of this protein (AQ-EKEAKSELDYD) to S-[2,3-Bis(palmitoyloxy)-(2RS)-propyl]-N-palmitoyl-[R]-cysteine (Pam3Cys-OH) to synthesize a lipopeptide with structural similarity to the naturally occurring GerD protein.

Lipopeptide LPGerD protects from allergic airway inflammation and sensitization in a murine model of asthma

We tested the allergy-protective properties of LPGerD in a murine model of systemic allergic sensitization (OVA/Alum model). We applied LPGerD intranasally a total of 14 times during the sensitization phase in a scheme described earlier [5]. We used different concentrations of the lipopeptide ranging from 5 µg/application to 50 µg/application. The application of 50 µg or 20 µg of LPGerD led to a significant reduction of infiltrating leucocytes and eosinophils into the lungs (Figs 3a and b). These cell infiltrations were also reduced by 5 µg of LPGerD, but this effect was not significant. The splenocytes secreted significantly less IL-10 and IL-5 upon antigen restimulation if the mice were treated with LPGerD (Figs 3c and 3d). This reduction was not signifi-

cant if 50 µg of lipopeptide were applied to mice. In addition, IFN-γ secretion of splenocytes upon OVA restimulation was low and did not differ between the different groups, indicating that no Th1 immune response was induced (data not shown). We did not observe an effect on the IgE titres in serum from the treatment of the mice with LPGerD (data not shown). However, we observed a significant reduction of the local concentration of OVA-specific IgE (Fig. 3e) and total IgE in the lung (Fig. 3f) when the lowest dose of lipopeptide (5 µg) was administered. Similarly, the strongest treatment effect on airway reactivity was observed with the 5 µg dose as determined by measurement of Penh by whole-body plethysmography (Fig. 3g). With this dose, Penh values were significant lower as compared to the sham-treated mice. ($P < 0.01$ for the methacholine concentrations 25 and 50 mg/mL). In contrast, higher doses of lipopeptide led to a non-significant reduction of airway reactivity. To confirm the reduction of airway reactivity by treatment with the 5 µg dose, we have measured airway reactivity of mice by invasive lung function measurement (Fig. 3h). This measurement confirmed the significant reduction of airway reactivity by treatment with LPGerD ($P < 0.01$ for the methacholine concentrations 25 and 50 mg/mL). Taken together, these data show a strong allergy-protective effect of LPGerD on airway inflammation and sensitization in the murine OVA/Alum model, while treatment with the 5 µg dose tends to be more effective than treatment with higher doses.

Lipopeptide induces a lower inflammatory immune response than LPS

Although we administered LPGerD to the mice in high doses (up to 50 µg per application), we did not observe a septic phenotype or any signs of illness. We hypothesized that lipopeptide acts in a less inflammatory way than LPS. To prove this, we generated BMDCs *in vitro* and treated them with LPS or lipopeptide in increasing concentrations. After two days of cell culture, we analysed the secretion of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 (Figs 4a, b and c) in the cell-culture supernatants. By comparing the two different TLR ligands, it is obvious that LPS induces a higher production amount of the pro-inflammatory cytokines than the TLR2 ligand LPGerD at all concentrations tested. Moreover, one must keep in mind that the molar ligand to receptor ratio is higher for each concentration tested for LPGerD due to its lower molecular weight in comparison to LPS. Taken together, these data indicate that lipopeptide acts in a less inflammatory way than LPS. These results may also explain why the mice tolerated LPGerD in high doses without showing a septic phenotype.

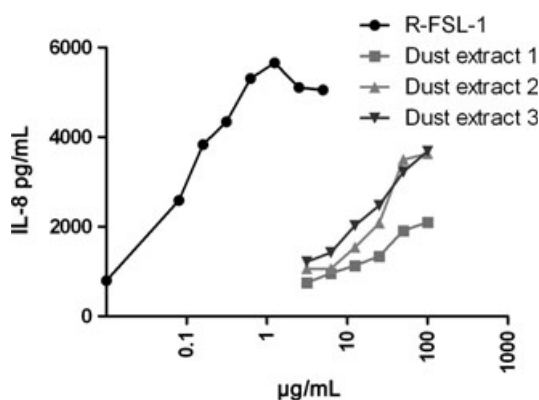


Fig. 2. Determination of the activity of TLR2 agonists in cowshed dust extracts (CDE) in a cell-based assay using TLR2-transfected HEK-293 cells. R-FSL1 (EMC microcollections) was used as a standard and IL-8 secretion was measured 24 h after stimulation of the cells. Dust extract samples were applied to the cells with the indicated concentrations.

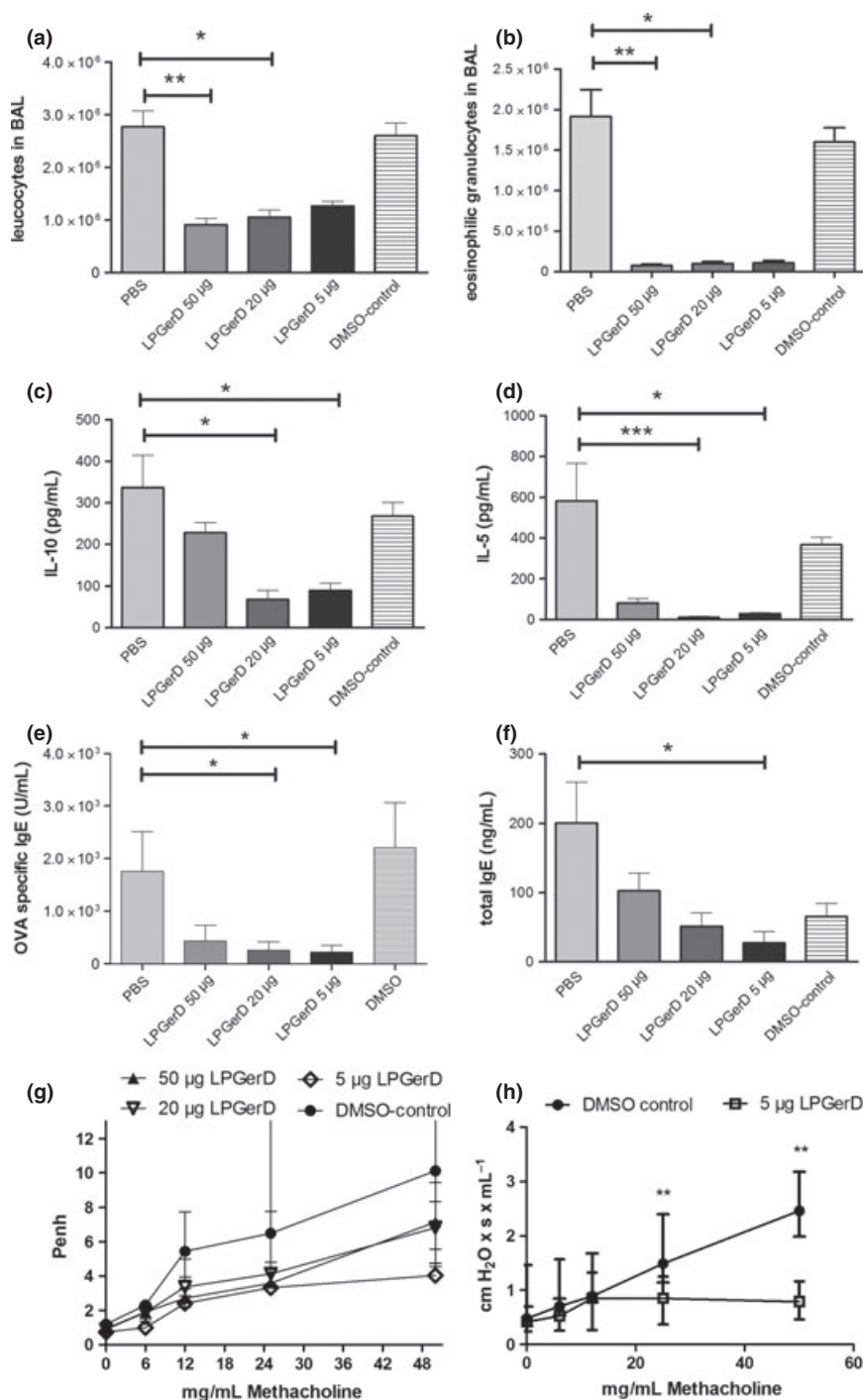


Fig. 3. Mice were sensitized by two intraperitoneal injections of OVA/Alum, each injection two weeks apart. During the sensitization phase, mice were treated a total of 14 times with different concentrations of lipopeptide, that is, 50 μ g, 20 μ g or 5 μ g, and DMSO as a negative control. After the challenge, different parameters of allergic airway inflammation were analysed: (a) Infiltration of leucocytes into BALs of mice; (b) Differential cell counts of eosinophilic granulocytes infiltrating into BALs; (c, d) IL-10/IL-5 as measured by ELISA in cell culture supernatants of *in vitro* restimulated splenocytes from differently treated mice; (e) OVA-specific IgE titres in BAL of differently treated animals; (f) Total IgE titres as measured by ELISA in BALs of differently treated mice. (g) Lung function measurement was done by whole-body plethysmography; Penh values were determined by challenge with increasing concentrations of methacholine. (h) Lung function measurement by invasive determination of airway resistance; dynamic resistance was determined by challenging with increasing concentrations of methacholine. $N = 5-8$ mice in each group.

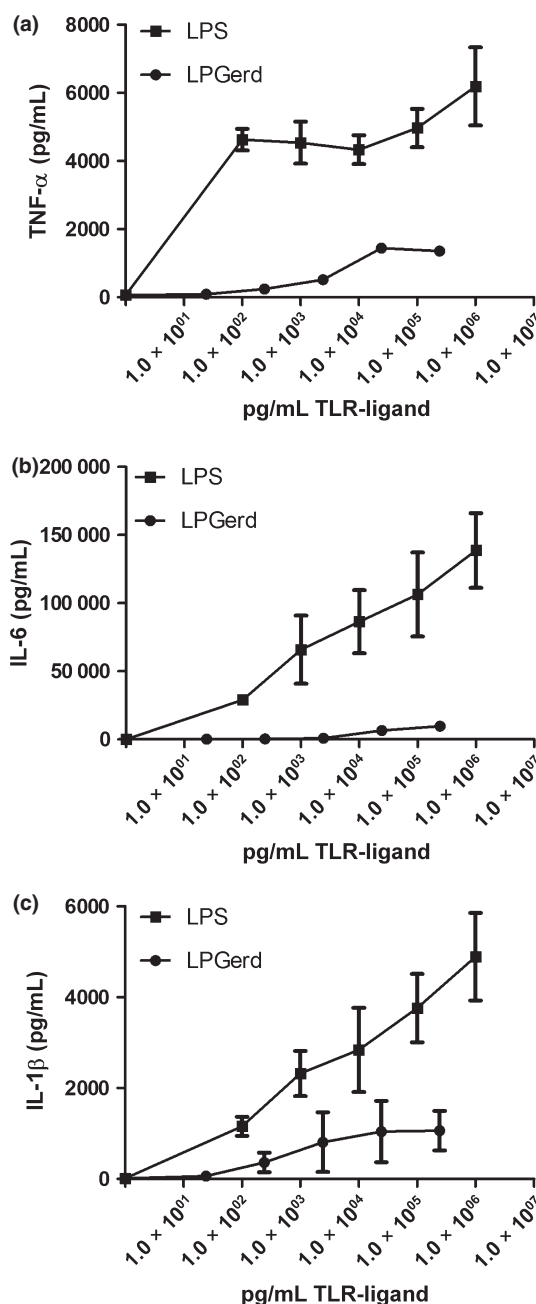


Fig. 4. Comparison of LPS and LPGerD induced secretion of TNF- α (a), IL-6 (b) or IL-1 β (c), respectively. The cells were stimulated on day eight with the indicated amounts of LPGerD or LPS. Supernatants were collected 24 h later and cytokines were determined by ELISA. This experiment was repeated three times with similar results.

Lipopeptide protects from allergic asthma in a transfer model of DCs

It is well known that DCs are one of the key players in the development of allergic airway inflammation and sensitization. We took advantage of a well-established transfer model of DCs [21] and investigated the effect of LPGerD, LPS and CDE on the sensitization capacity

of DCs. The treatment of BMDCs with LPGerD, LPS or CDE led to a significant reduction of infiltrating leucocytes (Fig. 5a), as well as eosinophils and lymphocytes into the lungs (Fig. 5b). We observed that treatment of BMDCs with LPGerD or CDE led to a significant reduction of systemic and local titres of total IgE (Figs 5c and d). A reduction of OVA-specific IgE was also detectable in serum. The following titres were measured by ELISA PBS: 57 ± 15 kU/mL; LPGerD: 1 ± 0.6 kU/mL; CDE: 0.6 ± 0.4 kU/mL; LPS: 1.9 ± 1.1 kU/mL ($P < 0.05$ for PBS vs. LPGerD and PBS vs. CDE). OVA-specific IgE in BALF was below the detection range of the ELISA. Interestingly, there was no significant reduction of IgE titres observable when BMDCs were treated with LPS. Moreover, we determined the levels of the Th2 cytokines IL-5 and IL-13 in the BALF of mice sensitized with BMDCs (Figs 5e and f). We observed a significant reduction of IL-5 by the treatment of BMDCs with LPGerD or LPS and of IL-13 by treatment with LPGerD, LPS or CDE. In contrast, splenocytes which had been restimulated with ovalbumin showed no significant reduction of the release of these Th2 cytokines, suggesting that protection against the exaggerated Th2 response is achieved in a local manner (data not shown).

Induction of a LPS-tolerant state in BMDCs treated with LPGerD

It is known that TLRs play a critical role in instructing BMDCs to mount a Th2 immune response [22, 23]. We have previously shown that LPS as a TLR4 agonist is of critical importance upon antigen loading to enable BMDCs to induce a Th2 immune response towards ovalbumin in our mouse model [6]. However, it has also been described that a repetitive exposure of DCs to LPS and TLR2 agonists leads to a LPS-tolerant state which is characterized by a reduced secretion of the pro-inflammatory cytokine TNF- α upon LPS stimulation [24–27]. We determined the amounts of TNF- α , IL-1 β and IL-6 in cell culture supernatants 24 h after BMDCs were stimulated with LPS (Figs 6a–c). In some approaches, the cells were pre-treated with LPS or with LPGerD two days before giving a final activation stimulus with 1 ng LPS on day eight. BMDCs receiving 1 ng LPS without pre-treatment secreted high amounts of TNF- α , IL-1 β and IL-6 24 h after the stimulation. In contrast, cells that had been pre-treated with 1 ng LPS secreted markedly lower amounts of these cytokines upon the LPS stimulus. Even more importantly, cells that were pre-treated with 500 ng of LPGerD secreted markedly lower amounts of all three cytokines upon LPS stimulation. This effect was completely abolished when BMDCs from TLR2 knockout mice were used, highlighting the specificity of the response. These

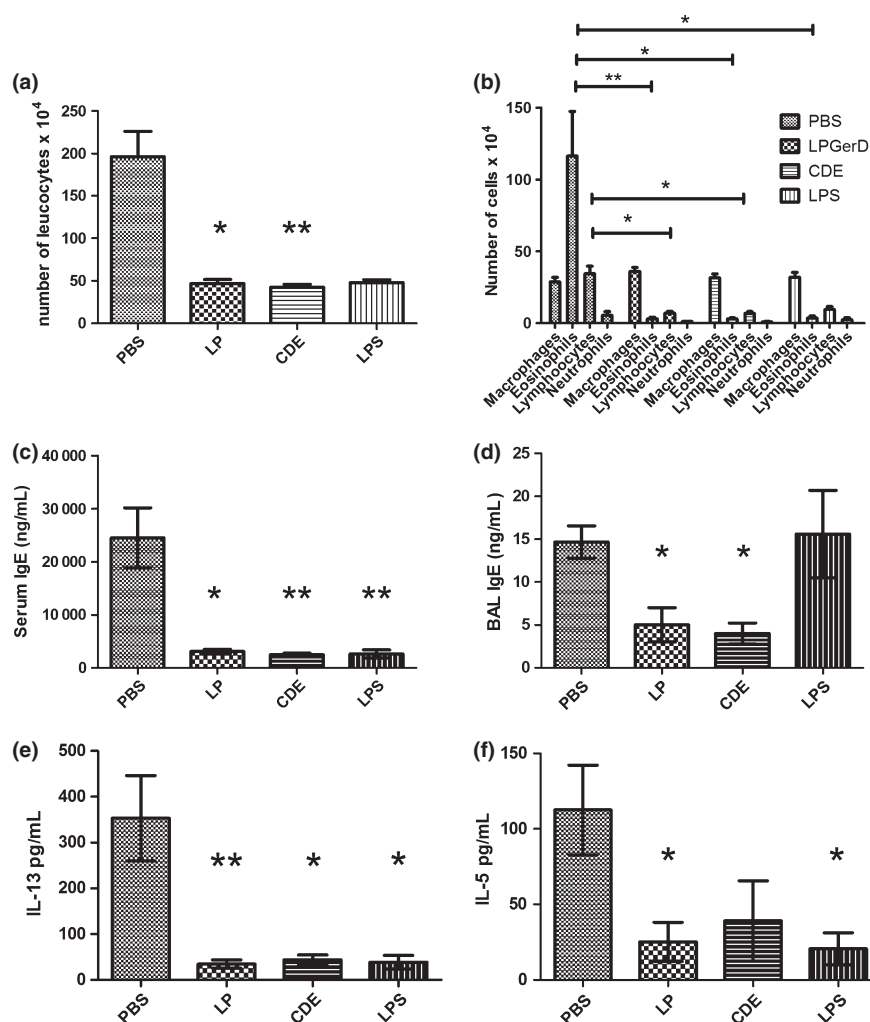


Fig. 5. Investigation of the allergy-protective properties in the DC-OVA transfer model. BMDCs were either treated on day six and eight of the *in vitro* cell culture with either 500 ng LPGerD (LP), 100 ng LPS (LPS) or 100 µg CDE (CDE), or they were left untreated (PBS). All cells were loaded with 100 µg OVA in combination with 1 ng LPS on day eight, before the cells were transferred intranasally to mice on day ten. (a) Total cell counts of leucocytes in BALs; (b) Differentiated cell counts of BALs; (c) Serum total-IgE titres; (d) total-IgE titres in BALs; (e) Concentration of IL-13 measured in supernatant of BAL fluid; (f) Concentration of IL-5 measured in supernatant of BAL fluid. $N = 6$ per group. * $P < 0.05$. The experiment was performed twice in two different mouse strains with similar results.

results indicate that LPGerD induces a LPS-tolerant state in BMDCs by binding to TLR-2, thereby disabling them to react to the LPS stimulus.

The induction of a LPS-tolerant state in BMDCs is accompanied by the up-regulation of IL-10 production

We have shown in previous studies that IL-10 is secreted by DCs upon treatment with CDE and that this cytokine is critically involved in the mediation of the allergy-protective properties of CDE [28]. Thus, we investigated if LPGerD treatment of BMDCs also leads to the secretion of IL-10. Interestingly, we observed that no IL-10 could be detected in the cell culture supernatants of BMDCs 24 h after a single stimulation with 500 ng of LPGerD, and a low dose of LPS (1 ng) did

not lead to the production of IL-10. Although we did not observe IL-10 production after a single treatment of the cells with LPGerD, we did observe IL-10 production when cells were treated for a second time with LPGerD (Fig. 7). IL-10 secretion increased when the cells were pre-treated with LPGerD and stimulated two days later with a combination of 1 ng LPS and lipopeptide (LP 500 ng/LPS 1 ng + LP 500 ng). This effect was not caused by LPS, because the stimulation of the cells with LPS alone did not induce IL-10 secretion (PBS/LPS 1 ng). However, the stimulation with 1 ng LPS induced IL-10 secretion if the cells received a pre-treatment with 500 ng of lipopeptide (LP 500 ng/LPS 1 ng). These results indicate that the pre-treatment with LPGerD instructs BMDCs to secrete IL-10 upon stimulation with LPGerD or LPS two days later.

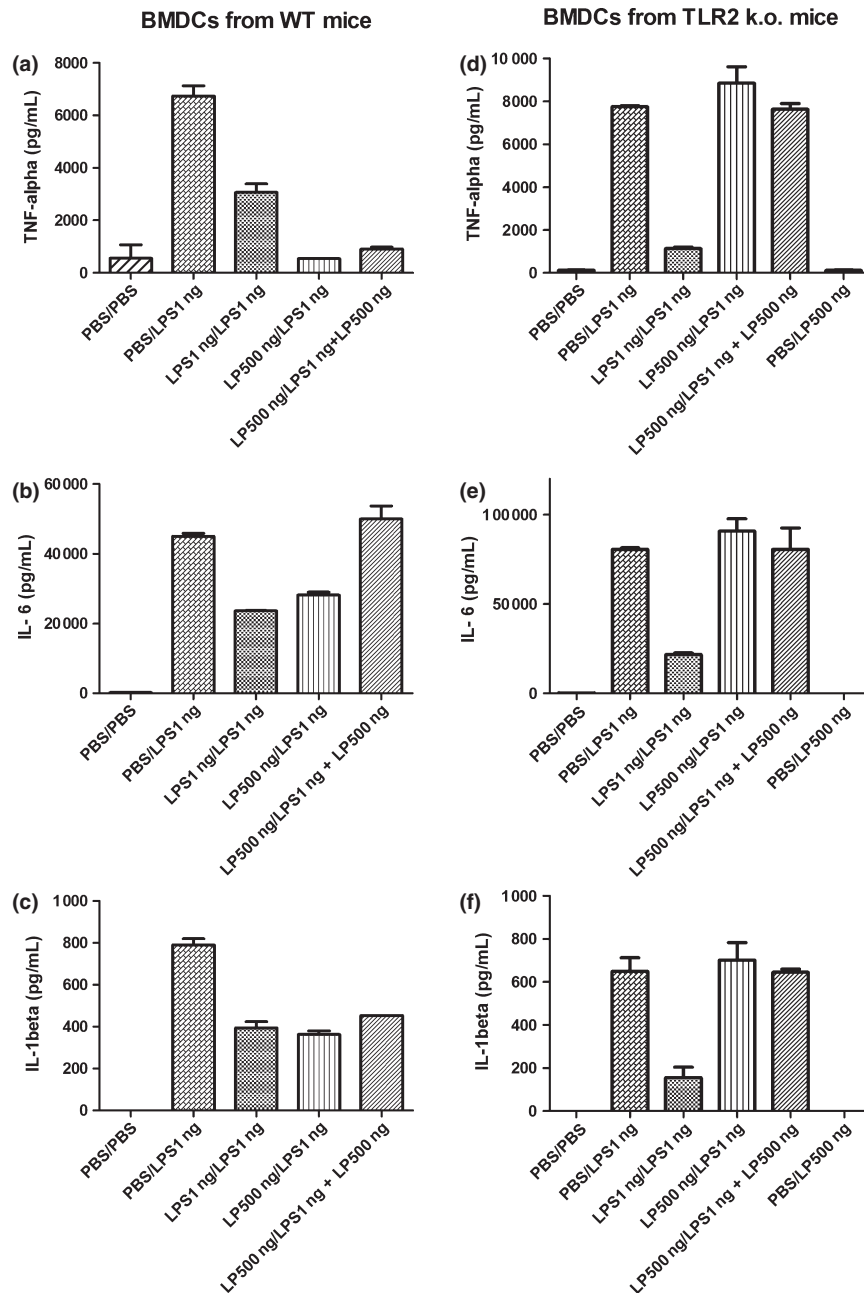


Fig. 6. Investigation of LPS tolerance induced by LPGerD in DCs. Determination of the cytokines TNF- α , IL-6 and IL-1 β in cell culture supernatants of *in vitro* stimulated BMDCs 24 h after the last stimulation of cells from either wild-type mice (a–c) or TLR2 knockout mice (d–f). The first stimulation was performed on day six, the medium was changed on day seven and a further stimulus was added to the cell culture on day eight. Substances appearing before the slash refer to stimulation on day six; stimuli behind the slash refer to stimulation on day eight; for example, LPS1 ng/LP500 ng means stimulation with 1 ng/mL LPS on day six and stimulation with 500 ng/mL LPGerD on day eight. Numbers appearing behind the stimulus (e.g. LPGerD500) represent the amount of the stimulus used (e.g. 500 ng). This experiment was repeated three times with similar results.

Lipopeptide-induced LPS tolerance is accompanied by up-regulation of IRAK-M mRNA

We next examined whether IRAK-M mRNA is up-regulated in DCs due to the stimulation with LPGerD, because IRAK-M is known to be critically involved in

the induction of LPS tolerance [16]. We observed that IRAK-M mRNA was up-regulated in BMDCs in a time-dependent manner after stimulation with either LPS or LPGerD. A first maximum of IRAK-M mRNA expression was reached 24 h after stimulation (Fig. 8) and rested until 48 hours after the stimulus. The cells were treated

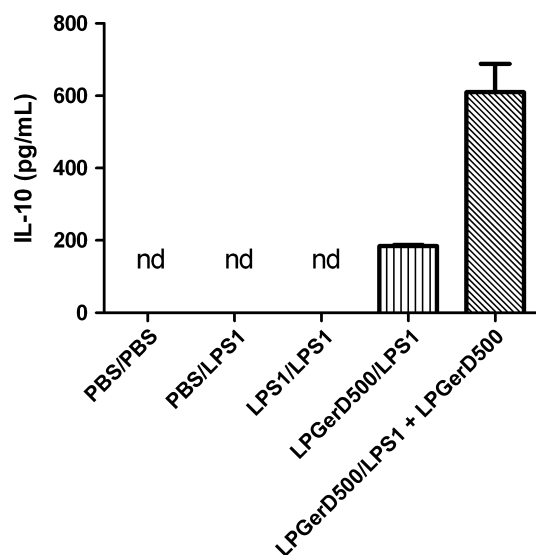


Fig. 7. Determination of production of IL-10 by *in vitro* stimulated BMDCs. Cells were treated twice on the days six and eight with different amounts of either LPGerD or LPS respectively (e.g. LPS100, meaning 100 ng of LPS). IL-10 in cell culture supernatants was determined by ELISA 24 h after the last stimulation. This experiment was repeated twice with similar results. nd, not detectable.

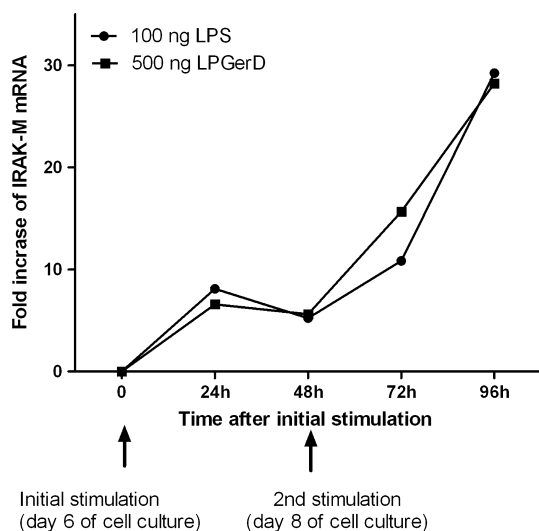


Fig. 8. Bone-marrow-derived dendritic cells were stimulated with either 100 ng LPS or 500 ng LPGerD on day 6 and day 8 of cell culture. A sample of cells was lysed every 24 h. Subsequently, quantification of IRAK-M mRNA by quantitative PCR was performed. $\Delta\Delta CT$ values of stimulated cells were related to unstimulated cells to calculate the fold increase in mRNA expression. This experiment was repeated twice with similar results.

for a second time 48 h after the first stimulation with 100 ng LPS or 500 ng of LPGerD, and IRAK-M mRNA was determined 24 and 48 h afterwards. LPS and LPGerD led to a further increase of the expression of

IRAK-M mRNA. The increase of IRAK-M mRNA was the same for both LPS and LPGerD.

Discussion

It is known that exposure to the environment of traditional farms during early childhood has a protective impact on the development of allergic disorders [1–4]. It has been shown using animal models that CDE includes substances which are able to protect mice from allergic sensitization and airway inflammation [5]. Among these substances are arabinogalactans, large polysaccharides that consist predominantly of arabinose and galactose [6]. These polysaccharides are able to act in a immunomodulatory way in the sense of allergy protection. However, there are other substances beside arabinogalactans included in CDE which have not yet been identified. Earlier studies in humans revealed that genetic variations in TLR2 are of great importance for the susceptibility to allergic asthma [29]. Importantly, allergy-protective, gram-positive bacteria had been identified in CDE, [18] and TLR2 agonists, such as Pam3CSK4, had been identified to be allergy-protective in other studies [29–32]. These facts led to the hypothesis that TLR2 ligands might be another group of allergy-protective substances included in CDE. In this study, we synthesized a triacyl-lipopeptide derived from the germination D protein of *B. cereus* (LPGerD) because these bacteria and their spores occur in cowsheds [19, 20]. For the first time, we proved LPGerD for its allergy-protective properties in two murine models of allergy. In a first approach, we applied LPGerD in different doses intranasally to mice during the sensitization phase. We showed that 5 μ g of LPGerD per application were sufficient to alleviate allergic airway inflammation and sensitization. In fact, it seems that the 5 μ g dose is more effective than the higher doses, particularly in the reduction of IgE and airway hyperreactivity. One reason for this might be the hydrophobic nature of LPGerD. It is possible that due to the hydrophilic environment of the airways, LPGerD forms aggregates and that the tendency to aggregate increases the higher the concentration is. This would reduce the amount of free LPGerD available to stimulate the TLR2 receptor.

Although it has been shown that TLR4 or TLR2 activation leads to the induction of an allergy-protective Th1 immune response [8, 30], we were not able to detect any differences in IFN- γ secretion of splenocytes upon OVA restimulation between the different groups (data not shown). We also could not detect any significant differences in lung neutrophilia between the differently treated groups, indicating that no severe airway inflammation was induced by the application of LPGerD. Importantly, the mice tolerated lipopeptide in high doses up to 50 μ g per application without showing

a septic phenotype. This is a very important finding bearing in mind that TLR4 agonists are known for their pro-inflammatory potency [33–36] and are, therefore, not suitable for preventive treatment of allergic diseases. We verified the low inflammatory potency of LPGerD *in vitro* where LPGerD induced a lower inflammatory response than LPS. These findings suggest that the synthetic LPGerD used here could be a candidate molecule for allergy-preventive vaccine development, provided it also proves not to be pro-inflammatory in humans. We investigated the immunological mechanisms underlying the protective effect of LPGerD. Therefore, we narrowed the effect of LPGerD down to the level of DCs because these cells are critically involved in the induction of the mucosal allergic immune response [21]. We showed in this study that BMDCs which were pre-treated *in vitro* with LPGerD, CDE or LPS before they were loaded with OVA/LPS were not able to induce an allergic phenotype *in vivo*. Thus, we hypothesized that pre-treatment of BMDCs with LPGerD during *in vitro* culture of BMDCs leads to the ignorance of the danger signal LPS during antigen loading two days later. In fact, we observed that BMDCs secreted markedly lower amounts of TNF- α , IL-1 β and IL-6 in response to the LPS stimulus if they were pre-treated with LPGerD. It is important to note that a reduced TNF- α production is a common readout for endotoxin tolerance, meaning that cells which received an endotoxin stimulus as pre-treatment secrete less TNF- α if they receive this stimulus for a second time [37–41]. We observed induction of LPS tolerance by a TLR2 agonist in this study. This phenomenon is referred to as endotoxin cross-tolerance [42] and has previously been described for other TLR2 agonists, such as MALP-2 [43, 44]. However, it has not yet been shown that induction of endotoxin cross-tolerance in antigen-presenting cells is directly associated with a reduced ability of these cells to mount a Th2 immune response. Our study is consistent with the findings of others who showed that TLR2 signalling induces the secretion of IL-10 upon a later stimulation with a TLR4 agonist [45]. This is interesting because IL-10 is known to be an important cytokine for the induction of Tregs [46] which, in turn, have been shown to be critically involved in the down-regulation of allergic Th2 immune responses [47]. Furthermore, it is known from a recent study that IL-10 acts on BMDCs and instructs them to induce an antigen-specific regulatory T cell response *in vivo* [48]. One observation from our experiments that supports the induction of a local regulatory immune response is the distinct behaviour of T lymphocytes from the lung compared to cells from the spleen upon contact to the allergen. Specifically, in the BMDC transfer model we found a local suppression of Th2 cytokine release, whereas cytokine production of spleen

cells seems to be unaltered due to pre-treatment of BMDCs with LPGerD, LPS or CDE. However, whether these differences can be explained by a local induction of regulatory T cells in the lung has to be investigated in further studies.

LPS-tolerance has been reported to be accompanied by both down-regulation of TLR4-MD2 complex on the cell surface and the suppression of intracellular signalling by molecules, such as IRAK-M [16]. Stimulation of TLR2 does not lead to the down-regulation of TLR4 [25], so that we conclude that TLR4 down-regulation is not responsible for the LPGerD-induced LPS tolerance in our experiments. However, we observed the upregulation of IRAK-M mRNA by the treatment of BMDCs with LPGerD. Knowing the importance of the danger signal LPS for the ability of BMDCs to mount a maladaptive Th2 immune response towards OVA *in vivo*, we conclude that the induction of an LPS-tolerant state in BMDCs is an explanation for the LPGerD-induced allergy protection. This is the first report showing that induction of endotoxin tolerance in antigen-presenting cells by treatment with the synthetic lipopeptide LPGerD is directly associated with an inability of these cells to induce a Th2 immune response. As the synthetic lipopeptide used in this study did not induce a septic phenotype in mice, we further suggest that LPGerD is a promising new candidate for allergy prophylaxis. Our study gives insights into the immunological mechanisms underlying the protective effect of the farming environment. It is tempting to speculate that the exposure of children to animal sheds, and thus to bacterial compounds such as lipoproteins, may induce a TLR2-mediated LPS tolerance. Interestingly, previous epidemiological studies described LPS tolerance of PBMCs in children who grew up in a traditional farming environment, leading to the conclusion that our results in the murine model are also relevant for the human situation [2]. However, it is unlikely that the induction of LPS tolerance is the only driving mechanism of protection against allergic diseases. Further studies will elucidate what the significance of LPS tolerance is in the context of other allergy-protective mechanisms, such as the tolerization of DCs by IL-10 [28], the possible induction of T regulatory cells [49] and epigenetic factors transferred by mothers to their children during pregnancy [50].

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Conflict of interests

The authors declare no conflict of interests.

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