

ORIGINAL ARTICLE

T-cell polarization depends on concentration of the danger signal used to activate dendritic cells

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Although several studies have focused on allergic sensitization by dendritic cells, to date it is still open under which conditions these antigen-presenting cells are able to induce an allergic immune response. Our study reveals that BMDCs pulsed with LPS-free ovalbumine did not induce allergic disease. However, when BMDCs were activated with low-dose LPS during pulsing with allergen, these cells expressed an inflammatory set of cytokines and co-stimulatory molecules like CD86 and OX40L. Moreover, activated cells were able to prime mice for massive eosinophilic inflammation of the lung, airway hyper-reactivity, IgE production and production of Th2 cytokines by lymphocytes. Blocking experiments showed that expression of OX40L is not involved in induction of Th2 response. Interestingly, BMDCs that were activated with high dose of LPS lose their Th2-sensitizing capacity. Instead these cells induce a Th17 type immune response. We conclude that presentation of allergen by dendritic cells generated with GMCSF is not sufficient to lead to induction of allergic immune response. Further activation of BMDCs is required to prime mice for allergic immune response. In this study, we show that LPS is a suitable stimulus. However, when cells were activated with high dose LPS they tended to induce a Th17 response.

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Several animal studies have shown that airway sensitization could be achieved by priming mice with allergen-pulsed dendritic cells.^{1–3} Lambrecht *et al.*² have shown that these antigen-presenting cells are critically involved in both sensitization phase and in ongoing allergic immune response.^{2,4} However, in all of these studies ovalbumine preparations were used that were contaminated with LPS. There is some evidence from the literature that a TLR-mediated signal is required for effective induction of Th2 immunity.^{5,6} In particular, stimulation via TLR4 seems to be a suitable stimulus as shown for example by Eisenbarth *et al.*⁷ These authors showed that treatment with ovalbumine via the airways is not sufficient to sensitize mice. Only when low doses lipopolysaccharide were mixed to OVA, mice were sensitized towards the allergen.⁷ On the basis of this observation Nigo *et al.*⁸ have shown that allergy-promoting activity of low-dose LPS led to activation of mast cells via TLR4. Moreover, a recent publication of Hammad *et al.*⁹ showed that LPS activation of epithelial cells has a significant impact on allergic sensitization towards house dust mite allergens. From these experiments the question arises, whether direct activation of DCs with LPS is sufficient or an LPS-induced cytokine milieu due to activation of mast cells and/or airway epithelium is required to arm these cells for sensitization of mice. As there is some evidence that GMCSF is involved in induction of Th2 response *in vivo* the possibility must be considered that Th2 induction could be generally attributed to GMCSF-generated dendritic cells.^{10,11}

Despite several years of research, the question still remains, which

phenotype BMDCs must acquire to induce an allergic immune response. Now there is increasing evidence that OX40 ligand expression is critically involved in Th2 polarization. This ligand was shown to be involved in the induction of the allergic immune response in a mouse model of asthma and also in a primate model of asthma.^{12,13} Furthermore, thymic stromal lymphopoietin is supposed to be an important inductor molecule for OX40L.¹⁴ However, the question is unsolved whether direct expression of OX40L by dendritic cells is of importance for the induction phase of the Th2 response.

To study the importance of dendritic cell activation by a danger signal for their Th2-inducing properties we used an adoptive transfer model where dendritic cells were generated *ex vivo* and activated with increasing doses of LPS. Subsequently, allergen-pulsed cells were used to sensitize mice via the airways. As activation of cells with LPS occurs *ex vivo*, influence of other cell types like mast cells and epithelial cells is excluded in this model, giving us the opportunity to study the relevance of expression of cytokines and costimulatory molecules like CD86 and OX40L for induction of the allergic immune response.

RESULTS

Ovalbumine from commercial distributors contains different amounts of LPS

To quantify LPS contamination of commercially available OVA preparations, we subjected ovalbumine purchased from Sigma, Worthington or Profos to a cell-based assay for LPS activity. As

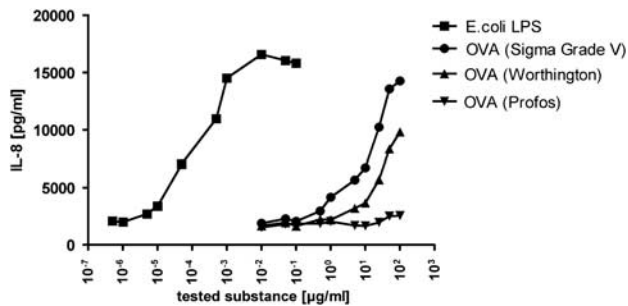


Figure 1 Analysis of LPS content in different OVA preparations by means of stimulation of HEK293 cells transfected with CD14/TLR4/MD2. IL-8 release was measured after stimulation with increasing concentrations of *E. coli* LPS to generate a standard curve. Unknown LPS concentration in OVA was calculated from this standard curve.

shown in Figure 1 increasing concentrations of *E. coli* LPS dose dependently induced production of IL-8 by CD14/MD2/TLR4-transfected HEK293 cells. This standard curve was used to determine TLR4-stimulating capacity in solubilized OVA preparations. Measured activity was expressed as *E. coli* LPS equivalent in ng ml^{-1} . With this method we could show that OVA from Sigma contained 10 ng LPS per 1 mg OVA, OVA from Worthington 2 ng LPS per 1 mg OVA and OVA from Profos <0.01 ng LPS per 1 mg OVA.

LPS stimulation of allergen-pulsed DCs is obligatory to arm cells for induction of allergic immune response

To investigate the effects of the different ovalbumine preparations on the sensitizing capacity of dendritic cells, BMDCs were generated as described above and pulsed with ovalbumine preparations either containing LPS (Worthington) or not (Profos). Cells were used to prime mice via the airways. Ten days later mice were challenged on 3 consecutive days and subsequently evaluated for allergic airway inflammation.

Mice that were primed with BMDCs pulsed with ovalbumine free of LPS did not develop allergic airway inflammation. These mice showed low recruitment of leukocytes into the airways without infiltration into lung tissue (see Figures 2a, d and e). In contrast, mice that were primed with BMDCs pulsed with $100 \mu\text{g ml}^{-1}$ ovalbumine in the presence of LPS contamination 0.2 ng ml^{-1} exhibited significantly increased infiltration of airways in particular with eosinophils (see Figure 2a). Moreover, the lung tissue of these mice was strongly infiltrated by leukocytes (Figure 2b) and airways showed goblet cell metaplasia as revealed by Alcian Blue/PAS staining (Figure 2c). These experiments show that pulsing of DCs with ovalbumine free of TLR-activating substances is not sufficient to prime cells for induction of allergic airway inflammation in naive mice.

Distinct activation profile of DCs upon stimulation with raising LPS concentrations

To get deeper insights into the dose-dependent effects of lipopolysaccharide on dendritic cell biology we studied expression of costimulatory surface molecules and release of cytokines after pulsing of cells *in vitro*. As shown in Figure 3 we found increased fractions of OX40L- and CD86-positive cells due to stimulation of DCs with increasing amounts of LPS (see Figures 3a and b). However, no further increased frequency of CD86- and OX40L-expressing cells could be achieved with LPS concentrations above 1 ng ml^{-1} . Cytokine production showed a similar profile. Production of TNF- α and IL-12p70 increased with raising amounts of LPS reaching a maximum at

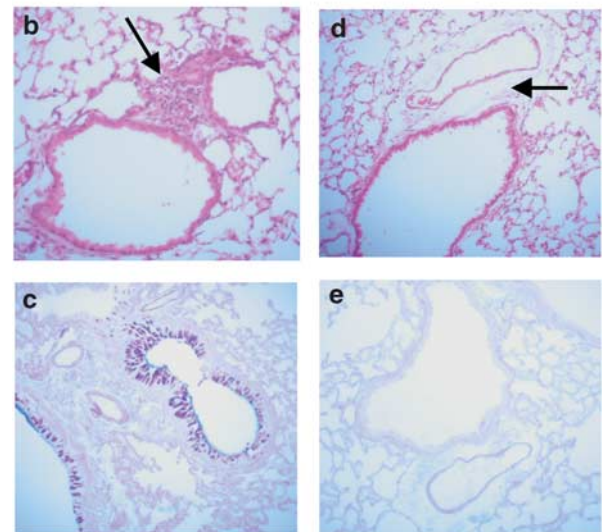
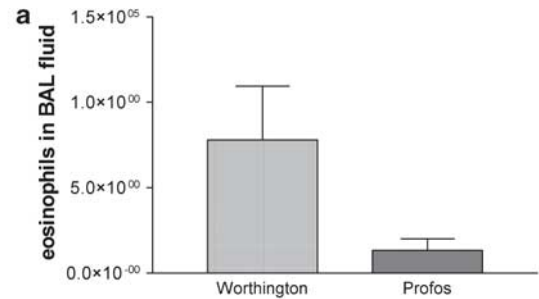


Figure 2 Number of eosinophils in BAL fluid of mice sensitized with BMDCs pulsed with either OVA purchased from Worthington or OVA from Profos (a). BAL was performed 3 days after challenge of mice on 3 consecutive days (mean $\pm$ s.e.m.). Arrow in photomicrographs indicates infiltration of leukocytes in lung tissue only in mice sensitized with BMDCs primed with LPS-contaminated OVA as revealed by routine H&E staining of lung slices (b: Worthington OVA; d: Profos OVA). Photomicrographs in C&E show Alcian Blue/PAS-stained tissue sections revealing goblet cell metaplasia in lungs of mice sensitized with BMDCs primed with LPS-contaminated OVA, (c) whereas goblet cell metaplasia is absent in lungs of mice primed with BMDCs pulsed with LPS-free OVA (e). The number of mice was $n=4$ per group. The experiment was repeated twice with similar results.

1 ng ml^{-1} LPS (see Figures 3c and d). No further amplification could be observed with higher LPS concentration, yet a not significant decrease of cytokine production could be measured. In contrast to TNF- α and IL-12, the production of IL-6 and TGF- β showed a different pattern (see Figures 3e and f). Concentration of these cytokines dose dependently increased with raising amounts of LPS without reaching saturation at 1 ng ml^{-1} LPS.

Besides, production of cytokines and expression of surface molecules we studied the T cell-stimulating capacity of BMDCs activated with LPS in an allogenic lymphocyte reaction. In accordance with their activation status the DCs led to increased activation of T-lymphocytes as measured by IFN- γ release in mixed lymphocyte cultures (data not shown).

Endocytotic activity and apoptosis of DC stimulated with increasing dose of LPS

To define whether the endocytotic capacity of DCs is influenced by increasing dosage of LPS we analyzed the uptake of Fluorescein-labeled dextran by LPS-stimulated DC. The stimulation of DCs with

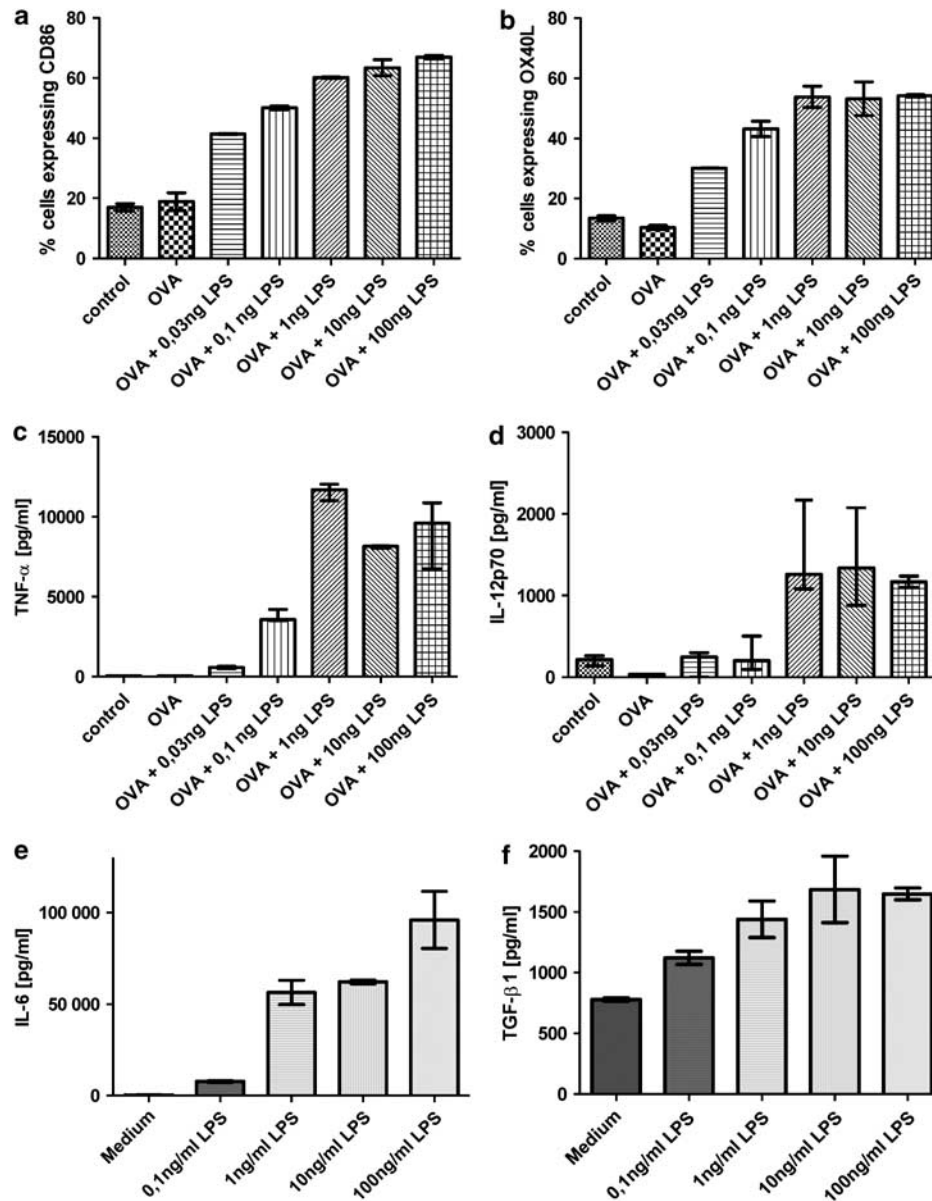


Figure 3 BMDCs were pulsed with LPS-free OVA and stimulated with increasing dose of LPS from day 8–10. Subsequently cytokine concentration was measured in cell culture medium and cells were stained for surface marker expression. Percent-positive BMDCs for CD86 (a) and OX40L (b) are shown. Cytokine concentration in cell culture medium (c=TNF- α , d=IL-12p70, e=IL-6, f=TGF- β 1) was measured by ELISA in three independent treated cell culture wells. Median concentration is given in pg ml⁻¹. The experiment was repeated twice with similar results.

LPS resulted in a reduced endocytotic activity (data not shown). However, we found no difference between cells stimulated with more than 0.1 ng ml⁻¹ LPS.

Furthermore, it was important to know whether DCs stimulated with high-dose LPS went into apoptosis. To analyze this, DCs were stained with Annexin V and propidiumiodide after activation by LPS. Results show that stimulation of cells with increasing doses of LPS did not lead to a significant increase of apoptosis: $4.29 \pm 0.54\%$ apoptotic BMDCs in cell cultures without LPS versus $3.70 \pm 0.17\%$ in BMDCs stimulated with 100 ng ml⁻¹ LPS.

Sensitization of mice with allergen-pulsed DCs depends on dosage of LPS

To study the effect of LPS on sensitization more detailed, we pulsed BMDCs with ovalbumine (LPS free) in the presence of increasing

amounts of LPS. The addition of 0.03 ng ml⁻¹ LPS during pulsing of BMDCs already led to robust sensitization of mice. These mice showed hyper-responsiveness of airways (see Figures 4a and b), infiltration of eosinophils into airway lumen (see Figure 4c), antibody (see Figures 5a–e) and Th2 cytokine production (see Figures 4d and e). Induction of airway hyper-responsiveness reached significance ($P < 0.05$) at 12 mg ml⁻¹ methacholine for treatment groups OVA compared with OVA + 0.03–1 ng ml⁻¹ LPS (see Figure 4b). Eosinophilic airway infiltration reached its maximum when BMDCs used for sensitization were pre-activated with 1 ng ml⁻¹ of LPS. In contrast, concentrations of LPS greater than 1 ng ml⁻¹ during priming of DCs led to reduced airway infiltration with eosinophils and reduced AHR in pulsed mice. Analysis of antibody and cytokine production supported these data. IFN γ release (data not shown) and IgG2a (see Figures 5c and d) production both characteristic for a Th1 response

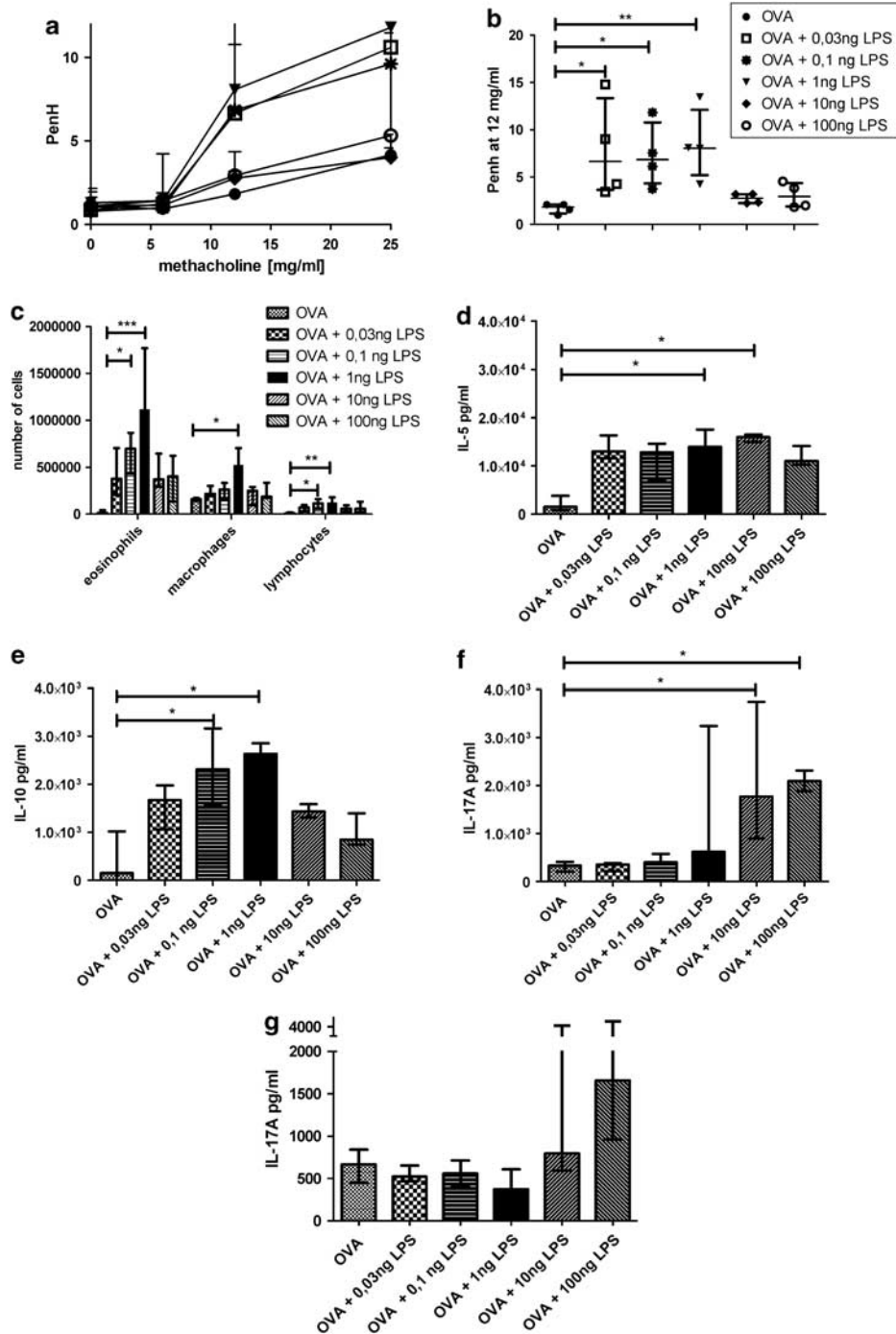


Figure 4 Mice were sensitized with OVA-pulsed DCs stimulated with increasing concentrations of LPS. Subsequently, sensitized animals were challenged with OVA via the airways for a total of six times. Airway hyper-reactivity of mice was measured by whole body plethysmography after challenge with increasing concentrations of methacholine (**a** and **b**). Median with interquartile range of Penh values is shown for increasing doses of methacholine (**a**); at 12 mg ml⁻¹ methacholine Penh of different groups of mice were compared by one-way ANOVA test (**b**). Percentage of leukocyte subsets of the BAL fluid was assessed on cytopins after staining for routine microscopy (**c**). Cytokine production was measured in tissue culture supernatants of lung cells (**d-f**) and in supernatant of spleen cells (**g**). Median with interquartile range is shown. **P* < 0.05. All groups were *n* = 4. The experiment is representative for two independent experiments.

also reached maximum in mice sensitized with BMDCs activated with 1 ng ml⁻¹ LPS. Interestingly, priming of mice with DC pulsed with ovalbumine in the presence of 10 or 100 ng ml⁻¹ LPS led to reduced production of the Th2 cytokine IL-10 and reduced IgG1 (see

Figure 5e) and IgE (see Figures 5a and b) levels. However, lymphocytes from the lung of these mice showed a dose-dependent increase of IL-17A production (see Figure 4f) suggesting that this cytokine is induced especially by BMDCs activated with high LPS concentrations.

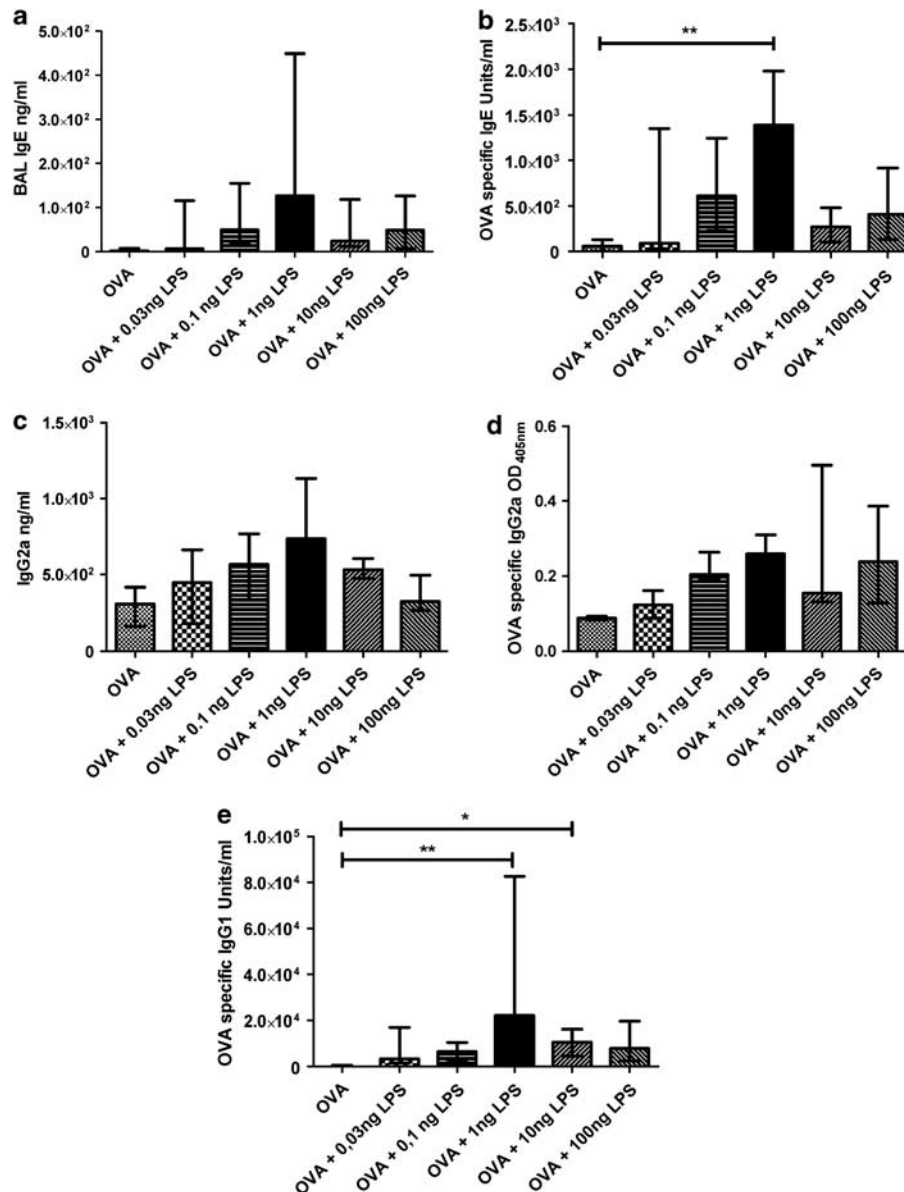


Figure 5 Mice were sensitized with OVA-pulsed DCs stimulated with increasing concentrations of LPS. Subsequently, sensitized animals were challenged with OVA via the airways for a total of six times. Total (**a** and **c**) and specific (**b**, **d** and **e**) immune globulins were measured in BAL fluid. Median with interquartile range is shown. * $P < 0.05$. All groups were $n = 4$. Experiment is representative for two independent experiments.

These data are supported by IL-17A production of spleen cells from mice sensitized with BMDCs activated with 100 ng ml⁻¹ LPS (see Figure 4g).

Blocking of OX40L with specific antibodies resulted in reduced Th1 response

As OX40L expression on DCs was found to be associated with a Th2 response we aimed at inhibiting this costimulation by OX40L-blocking antibodies to understand the role of LPS activation in this experimental setting. For comparison we also blocked CD86, a molecule known for its costimulatory activity on T-cell responses. Cells were stimulated with LPS and an antibody against OX40L or CD86 was added simultaneously. FACS analysis with antibodies binding to a different epitope of the surface molecule revealed that OX40L and CD86 were completely blocked under these conditions (data not shown). Subsequently, these cells were used for priming of mice.

Surprisingly, this experiment revealed that blocking of OX40L led to reduction of Th1 response as measured by IFN γ production of lymphocytes (see Figure 6b) and IgG2a titer (see Figures 6c and d) rather than reducing Th2-type response, whereas airway infiltration with eosinophilic granulocytes was not affected (see Figure 6a). In contrast, blocking of CD86 resulted in a non-significant reduction of airway inflammation with reduced eosinophilic infiltration. Here, we conclude that in our experiments the expression of OX40L may rather lead to the induction of Th1 immunity and has only limited importance for the induction of a Th2 response in the BMDC transfer model used here.

DISCUSSION

In this study, we show for the first time that dendritic cells must be stimulated with a low dose of LPS to induce an allergic immune response in naive mice. This is an important observation as since it

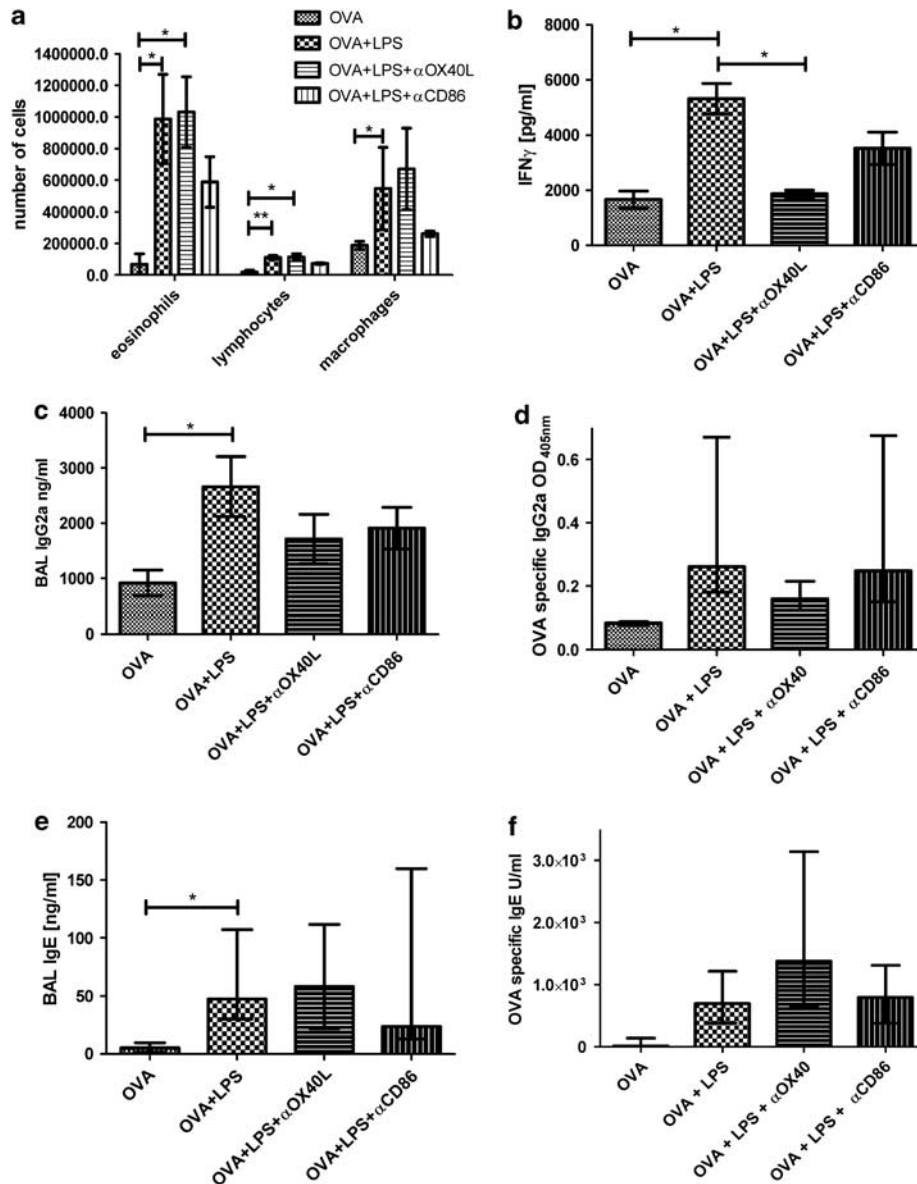


Figure 6 BMDCs were stimulated with 1 ng ml^{-1} LPS and pulsed with OVA (OVA+LPS, $n=5$) in the presence of CD86 (OVA+LPS+ α CD86, $n=4$) or OX40L (OVA+LPS+ α OX40L, $n=4$) neutralizing antibody. Control cells were stimulated with LPS-free OVA alone (OVA, $n=4$). Cells were then used to sensitize mice via the airway. Subsequently, mice were challenged for a total of six times with OVA aerosol. Cellular composition of BAL fluid (**a**), IgE (**e**) and IgG2a (**c** and **d**) in serum and BAL fluid and IFN- γ production (**b**) of splenic lymphocytes is shown. Median with interquartile range is shown. * $P < 0.05$.

was yet unknown whether GMCSE, which is used for generation of dendritic cells, is polarizing these cells to a Th2-inducing phenotype.^{10,11} Moreover, we found that dendritic cells activated with a high dose of LPS induce a Th17 response.

In our experiments BMDC cultivated with increasing doses of LPS up to 1 ng ml^{-1} were activated to produce an inflammatory set of cytokines. Besides, production of cytokines activated BMDCs exhibited increased expression of co-stimulatory molecules on the cell surface. In line with the literature we found upregulation of CD86 due to activation with LPS. In addition, even more important, we found upregulation of OX40L on LPS-activated cells. Increasing interest has focused on the function of OX40L and its contribution to allergic disorders.^{12–15} Hoshino *et al.*¹² have shown that neutralization of OX40L led to reduced allergic sensitization indicating the important contribution of this molecule in induction of allergic

immune response in the mouse model of asthma used by these authors. However, it remained undefined whether the expression of OX40L on dendritic cells due to TLR activation is relevant for induction of allergic airway inflammation and sensitization. Surprisingly, however, we found that blocking of OX40L did not lead to inhibition of Th2 response but rather blocked the induction of a Th1 response as measured by IFN γ production of spleen cells and IgG2a production in serum and BAL fluid. One explanation for this discrepancy could be that experiments from Hoshino *et al.*¹² were performed in a mouse model of asthma where mice were systemically sensitized with OVA adsorbed to Alum by i.p. injection in contrast to our model using pulsed DCs applied i.n. into mice. It cannot be excluded that OX40L is having a role in Alum adjuvant activity for induction of Th2 response. In contrast, in our study the contribution of OX40L expressed on BMDCs could be studied more directly.

Interestingly, in a recent publication by Dannull *et al.*¹⁶ our observation is supported, namely that the expression of OX40L by DC is responsible for induction of a Th1 rather than a Th2 response. In contrast to OX40L, blocking of CD86 seems to reduce at least partially eosinophilic airway infiltration, confirming the importance of this molecule for costimulation and induction of allergic immune response.^{17,18}

Another surprising result in our transfer model is the fact that IL-12 production by dendritic cells did not preclude induction of a Th2 response. This is in contrast to reports which showed that the presence of IL-12 inhibits Th2 polarization.¹⁴ However, this may be due to the low concentration of IL-12 produced in our experiments. In contrast, Ito *et al.*¹⁴ have shown in an *in vitro* experimental setting using human DCs and T cells that in the presence of IL-12 concentrations as high as 10 ng ml⁻¹ Th2 development is suppressed.

Another interesting observation in our study is that high concentrations of LPS (10 and 100 ng ml⁻¹) led to a Th17 response as measured by IL-17 production of splenic lymphocytes. This may explain the results of Kuipers *et al.*¹ who found that high-dose LPS treatment of BMDCs led to a suppression of Th2 response independent of IL-12p70 production, an observation which the authors themselves did not understand. In accordance, we found in our study that BMDCs activated with 100 ng ml⁻¹ LPS did not induce an allergic immune response but led to a Th17 type of immune response with high production of IL-17A by lung and spleen cells. This is likely due to induction of high concentrations of IL-6 and TGF- β 1 by BMDCs activated with high dose LPS, because these two cytokines are important differentiation factors which when used for polarization of T-helper cells lead to generation of Th17 cells.^{19,20} Interestingly, we find that in contrast to IL-12 and TNF- α , which reached their maximum already at 1 ng ml⁻¹ LPS, production of IL-6 and TGF- β 1 continuously increased to reach highest levels at 100 ng ml⁻¹ LPS.

In conclusion, a low dose of a danger signal seems to be necessary for activation of a Th2 response by dendritic cells, whereas a higher dose of LPS led to the induction of Th17 immune response and reduced Th2 immunity. One could speculate that there is a threshold concentration for danger signals where a Th2-inducing phenotype of BMDCs is switched to a Th17-inducing phenotype potentially resulting in a more aggressive immune response.

Finally, it is important to note that this improved model of airway sensitization characterized by applying primed dendritic cells to naive mice is a relevant option to study the induction of allergic immune response because animals show airway hyper-reactivity, high IgE titres and eosinophilic airway infiltration comparable to the conventional OVA/Alum model. However, by omitting adjuvants for sensitization, the investigation of induction of T-helper cell responses is more focused on the phenotypic appearance of the dendritic cell, namely the expression of costimulatory molecules and production of cytokines than in the OVA/Alum model.

METHODS

Animals

Female BALB/c mice (Charles River, Sulzfeld, Germany) aged 5–6 weeks were used and acclimated to the animal facility for 14 days before experiments. 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.

Determination of LPS concentration in OVA preparations

Ultra pure *E. coli* LPS was a generous gift from Dr Ulrich Zähringer (Forschungszentrum Borstel, Germany). CD14/MD2/TLR4-transfected HEK293

cells and control cells were purchased from Invivogen (Cayla, France). Cells (2×10^5) were cultured in a 96 Depot cell culture plate in DMEM medium containing 10% fetal calf serum. A standard curve was generated by stimulating cells with *E. coli* LPS reaching from 1 pg ml⁻¹ to 100 ng ml⁻¹ for 24 h and subsequently measuring huIL-8 production in tissue culture supernatants with an optEIA kit (BD Biosciences, Heidelberg, Germany). This standard curve was used to determine TLR4 stimulating capacity in ovalbumine preparations and expressed as *E. coli* LPS equivalent in ng ml⁻¹. Ovalbumine from the following companies was analysed: Grade V OVA from Sigma-Aldrich (Cat. No. A5503, St Louis, MO, USA), chromatographically purified OVA from Worthington Biochemical Corporation (Cat. No. LS003056, Lakewood, CO, USA) and EndoGrade OVA from Hyglos formerly named Profos (Hyglos GmbH, Regensburg, Germany).

Generation of BMDCs *in vitro*

BMDCs were generated by cultivation of bone marrow cells in the presence of GM-CSF as described elsewhere.²¹ On day eight cells were pulsed with 100 μ g ml⁻¹ chromatographically purified ovalbumine (Worthington Biochemical Corporation, New York, USA) or 100 μ g ml⁻¹ ovalbumine free of LPS (Profos AG now named Hyglos GmbH). In some experiments ovalbumine free of LPS was supplemented with *E. coli* lipopolysaccharide at different concentrations. In some experiments 5 μ g ml⁻¹ of CD86 (clone PO3.1, eBioscience, San Diego, CA, USA) or 5 μ g ml⁻¹ of OX40L (clone 182601, R&D Systems, Minneapolis, MN, USA) neutralizing antibody was added to the culture medium on day eight.

Sensitization of mice with OVA-pulsed BMDCs and airway challenge

For sensitization of mice with dendritic cells via the airways animals were anesthetized with a mixture of Ketamin and Rompun. 10⁶ OVA-pulsed BMDCs were administered intranasally in 50 μ l PBS. For induction of airway inflammation mice were challenged on days 11, 12 and 13 with 1% OVA aerosol for 30 min using a PARI-Boy aerosol generator (PARI, Starnberg, Germany). Where indicated, mice were challenged more frequently for induction of antibody response and hyper-reactivity on days 12, 19, 26, 33, 34 und 35. Controls were treated with OVA aerosol alone.

Airway responsiveness (AR)

Forty-eight hours after the third aerosol challenge, airway hyper-responsiveness (AHR) to methacholine aerosol (0, 6, 12, 25 mg ml⁻¹, Sigma) was evaluated in conscious, unrestrained mice using whole-body plethysmography (Buxco Electronics, Wilmington, NC, USA) by measuring the enhanced pause (Penh).²²

In vitro cytokine production of mouse lymphocytes

Spleens and lungs were harvested 3 days after the last aerosol challenge. Single-cell suspensions were prepared from spleens by mechanical disruption or from lungs by digestion with collagenase IV (Sigma). Erythrocytes were lysed by hypotonic shock. Lymphocytes were then cultured at a concentration of 10⁷ ml⁻¹ in complete tissue culture medium (RPMI 1640 with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin, all from Biochrom, Berlin, Germany). After 48 h of culture, supernatants were taken and stored at -80 °C until analysis.

Measurement of cytokines in cell culture supernatants

Levels of IL-5, IL-10, IFN- γ were assessed by using optEIA kits (BD Biosciences, Heidelberg, Germany), IL-4, IL-6, IL-12p70, IL-13, IL-17A and TNF- α were measured with a commercial ELISA set (eBioscience), TGF- β 1 was measured with a DuoSet (R&D Systems) according to the manufacturer's instructions. This ELISA kit detects both TGF- β 1 produced by murine cells and bovine TGF- β derived from FCS in cell culture medium. As 800 pg ml⁻¹ TGF- β are detectable in unconditioned RPMI containing 5% FCS this background was subtracted from all values measured in cell culture medium conditioned by BMDCs.

Bronchoalveolar lavage (BAL)

Three days after the last OVA challenge lungs were lavaged via a tracheal cannula with 2×1 ml PBS, and leukocytes in the lavage fluid were counted. After centrifugation, BAL fluid was frozen for further analysis. Cytospin slides of BAL cells were stained with a fast staining procedure (HAEME-Schnellfärbung, Labor+Technik Eberhard Lehmann, Berlin, Germany), according to the manufacturer's instructions. The percentages of eosinophils, lymphocytes and macrophages in BAL samples were determined by light microscopy. At least 300 cells per sample were differentiated by a blinded investigator.

Flow cytometric analysis

Analysis of DC-specific surface antigens was performed by flow cytometry (Partec, Münster, Germany). The following fluorescein isothiocyanate- (FITC-) or phycoerythrin- (PE) conjugated rat anti-mouse monoclonal antibodies (mAbs) were used for direct labeling of the cells: antiCD11c-PE (clone N418) from BD Bioscience, anti OX40L PE (clone E23-45), antiCD86-FITC (clone GL1), antiMHCII-FITC (clone M5/114.15.2) from eBioscience. Isotype-matched mAbs of irrelevant specificity were used as controls. For analysis of apoptosis cells were stained with Annexin V- FITC (Immunotools, Friesoythe, Germany) and Propidiumiodide (Sigma-Aldrich). Phagocytotic activity of DCs was evaluated by cultivation of cells for 48 h with fluorescein-labeled Dextran (Sigma-Aldrich).

Statistical analysis

All data were analyzed by a non parametric ANOVA test. When the Kruskal-Wallis test reported a significant difference between the compared groups, sub-analysis was performed between two groups by Dunnett's *post hoc* test. Graph Pad Prism Software (Version 5) was used for analysis. Values of $P < 0.05$ were considered statistically significant.

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