Thorax Online First, published on October 21, 2005 as 10.1136/thx.2005.049403

Inhalation of stable-dust extract prevents allergen-induced airway inflammation and hyperresponsiveness

Marcus Peters[1*], Marion Kauth[1*], Jürgen Schwarze[3,4], Cordula Körner-Rettberg[4], Josef Riedler[5], Dennis Nowak[6], Charlotte Braun-Fahrländer[7], Erika von Mutius[8], Albrecht Bufe[1], Otto Holst[2] and the ALEX Study Group

[1]Department of Experimental Pneumology, Ruhr-University Bochum, Bochum, Germany
[2]Division of Structural Biochemistry, Forschungszentrum Borstel, Leibniz-Center for Medicine and Biosciences, Borstel, Germany
[3]Department of Respiratory Medicine, NHLI, Imperial College, London, UK
[4]Klinik für Kinder- und Jugendmedizin, St Josef-Hospital, Bochum, Germany
[5]Kinderspital Schwarzach, Schwarzach, Austria
[6]Institute of Occupational and Environmental Medicine, University of Munich, Munich, Germany
[7]Institute of Social and Preventive Medicine, Basel, Switzerland
[8]Dr. von Hauner Children's Hospital, Munich, Germany
*MP and MK contributed equally to the manuscript

Corresponding author: Dr. M. Peters, Department of Experimental Pneumology, Ruhr-University Bochum, Bürkle-de-la-Camp-Platz 1, 44789 Bochum, Germany
Tel.: +492343024690; Fax: +492343074682
e-mail: marcus.peters@rub.de

Keywords: animal models, allergic asthma, hygiene hypothesis

Abstract

Background: Recent epidemiological studies have shown that growing up on a traditional farm protects from the development of allergic disorders like hay fever and allergic asthma. Here, we present experimental evidence that substances protecting from development of allergic diseases can be extracted from dust collected in stables of animal farms. Methods: Stable-dust was collected from 30 randomly selected farms all located in rural regions of the Alps (Austria, Germany and Switzerland). Dust was homogenized with glass beads and extracted with physiological sodium chloride solution. This extract was used to modulate immune response in a well established mouse model of allergic asthma. Results: Inhalative treatment of mice with stable-dust extract during sensitization to ovalbumin inhibits the development of airway hyperresponsiveness and airway eosinophilia upon challenge, as well as the production of IL-5 by splenocytes and of antigen-specific IgG1 and IgE. In addition, dust extract also suppresses the generation of human dendritic cells *in vitro*. The biological activity of dust extract was not exclusively mediated by lipopolysaccharide. Conclusions: Stable-dust from animal farms contains strong immune modulating substances. These substances can interfere with the development of both cellular and humoral immunity against allergens, thus suppressing allergen sensitization, airway inflammation and airway hyperresponsiveness in a murine model of allergic asthma.

Introduction

One explanation for the increase of allergic diseases during the last 30 years originates from the “hygiene hypothesis” which proposes that increases in the prevalence of allergic disorders are linked to changes in the microbial burden children are exposed to in early life.[1][2][3][4] Recently, epidemiological studies have shown that growing up on a traditional animal farm can protect from sensitization to allergens and the development of asthma and hay fever. The exposure of infants to the environment of stables during the first year of life seems to be crucial for this protection. In the search for pathogen derived factors, which inhibit the development of allergen sensitization, many studies have concentrated on lipopolysaccharides (LPS), which can induce allergy-protective Th1 type immune responses in animal experiments.[5][6][7] Interestingly, LPS levels in dust from mattresses of children were inversely related to the frequency of atopic sensitization and atopic asthma and overall endotoxin levels were significantly higher in dust from farming than from non-farming households.[2] A recent study showed that in addition to endotoxin, other microbial components such as peptidoglycans were associated with a reduction in non-atopic asthma symptoms.[4]

In animal models of allergen induced asthma, LPS treatment led to Th1 dominated immune responses.[7][8] Given systemically before sensitization, LPS reduced both, the production of allergen specific IgE and numbers of eosinophils in the bronchial mucosa demonstrating that the Th2 response normally associated with allergic sensitization in mice was inhibited. In contrast to these animal models, LPS activation of peripheral blood mononuclear cells in children, who themselves had been exposed to high amounts of endotoxin levels in early childhood, seemed to lead to immune tolerance to LPS.[2] This finding indicates that down regulation rather than activation of the immune response to bacterial pathogens is associated with protection from allergy. These conflicting results suggest that LPS is probably primarily a marker for microbial burden rather than the principle molecule responsible for the protective effect of the farm environment. It is possible that allergy-protection induced by the exposure to a farming environment is linked to the activity of T-regulatory cells (Treg) releasing TGF-β and IL-10 and suppressing both, Th1 and Th2 immune responses.[9][10]

So far, experimental confirmation for the epidemiological observation that exposure to stable-dust reduces the risk for allergic diseases is still missing. Therefore in the present study, dust was collected from stables of traditional farms and extracted with physiological sodium chloride solution. We tested whether inhalation of this extract could modify allergic responses in a mouse model of acute allergic asthma. Furthermore, the influence of the dust extract on generation of dendritic cells from human monocytes was determined *in vitro*.

Methods

Collection of stable-dust

A total of 30 farming households were selected, all located in rural regions of the Alps where the previous field studies have been conducted too.[1][2] Sediment dust out of cattle and goat stables was collected by scraping off all surfaces in a height of 0.05 to 0.15 m (racks, tables, tools) with a metal spatula. From every single farm 100 to 200 g dust was collected and passed through a conventional kitchen sieve. Finally, all dust samples were combined.

Extraction of stable-dust

For homogenization of the dust 15 g of glass beads (0.4 – 0.6 mm in diameter) were added to 10 g dust, and *aqua bidest* was added to a final volume of 70 ml. The sample was then homogenized in a Braun MSK homogenizer (B. Braun, Melsungen, Germany). Then, five such samples (= 50 g of dust) were combined and extracted with 0.9% aqueous sodium chloride (NaCl) solution (total volume: 5 l) for six hours at 22°C with continuous stirring. After centrifugation the dust extract was dialyzed against water and finally lyophilized.

Animals

Female BALB/c mice (Charles River, Sulzfeld, Germany) aged 7-8 weeks were used and acclimated to the animal facility for 14 days prior to experiments. Animals had access to food and water ad libitum. All experimental procedures were approved by the animal ethics committee at Bezirksregierung Arnsberg.

Sensitization and airway challenge

Mice were sensitized by intraperitoneal injection of 20 µg ovalbumin (OVA; GradeV; Sigma, St. Louis, MO) emulsified in 2.2 mg aluminum hydroxide (ImjectAlum; Pierce, Rockford, IL) in a total volume of 200 µl on days 1 and 14. On days 28 and 38 mice were challenged via the airways with 1% OVA aerosol for 20 min (fig 1) using a PARI-Boy aerosol generator. Controls were injected with aluminum hydroxide alone and challenged with PBS aerosol (non-sensitized).

Treatment of animals with stable-dust extract

Mice were treated with stable-dust extract during the sensitization phase (fig 1). Extract was administered as aerosol generated from a 10 mg/ml solution. For treatment mice were placed in a plexiglass chamber (11 l volume) for 20 min. The chamber was connected to a PARI-Boy Turbo jet stream aerosol generator (PARI, Starnberg, Germany). Mice were allowed to inhale the aerosol for 20 min. Mice were treated 14 times beginning on the day of the first OVA injection and the last treatment six days before the second OVA-aerosol challenge (fig 1). Control groups were sensitized as described above and treated 14 times with either phosphate buffered saline (PBS) or 0.8 µg/ml *E.coli* lipopolysaccharide (LPS) during the sensitization. The LPS concentration was chosen to match the concentration in the dust extract detected by Limulus amebocyte lysate assay (LAL). The *E.coli* LPS used for our study was purified at Forschungszentrum Borstel. In stimulation assays with human MNC it is highly biological active. It induces maximal TNF-α release at a concentration of 2 ng/ml and maximal IL-10 release at 0.4 ng/ml, respectively.

Airway responsiveness (AR)

24 h after the last aerosol challenge, airway hyperresponsiveness (AHR) to methacholine aerosol (0, 6, 12, 25 and 50 mg/ml, Sigma) was evaluated in conscious, unrestrained mice using whole-body plethysmography (Buxco Electronics) by measuring the enhanced pause (Penh).[11][12][13] Previous studies have shown that the AHR to methacholine reaches its maximum 24 h after OVA challenge.[12] Furthermore, it was demonstrated that in this experimental setting AHR assessed by Penh correlates closely with increases in airway resistance in ventilated animals.[12]

In vitro cytokine production of mouse splenocytes

Spleens were harvested three days after the second aerosol challenge. Single cell suspensions were prepared by mechanical disruption and erythrocytes were lysed. Spleen cells were then cultured at a concentration of $5x10^6$/ml in complete tissue culture medium (CTCM) (RPMI 1640 with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, all from Biochrom, Berlin, Germany). To stimulate specific cytokine production OVA was added to a final concentration of 50 µg/ml. After 48 h of culture, supernatants were taken and stored at –80°C until analysis. Levels of IL-5 and IL-10 were assessed by using optEIA kits (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions.

Bronchoalveolar Lavage (BAL)

Three days after OVA challenge lungs were lavaged via a tracheal tube with 2x1 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.

Measurement of OVA-specific IgG1, IgG2a and IgE in serum and BAL

Blood was collected by tail vein puncture two days after the second aerosol challenge. BAL was collected as described above. Levels of OVA-specific IgG1, IgG2a and IgE in Serum or BAL fluid were determined by ELISA. Briefly, 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 samples, bound OVA-specific antibodies were detected with isotype specific antibodies: rat anti mouse IgG2a (clone R19-15), rat anti mouse IgG1 (clone X56) both conjugated to alkaline phosphatase or biotinylated rat anti mouse IgE (clone R35-72) (all from BD Biosciences). The latter antibody was detected by horseradish peroxidase conjugated extravidin (Sigma). In control experiments isotype specific rat anti mouse IgE antibody does not cross-react with IgG1 or IgG2a isotype. Serum levels of OVA-specific IgG1 and IgE were related to combined standard sera generated in our laboratory and expressed as arbitrary units per ml. Levels of OVA-specific IgG2a in 1/10 diluted serum were expressed as optical density measured at a wavelength of 405 nm (OD_{405}).

Generation of human dendritic cells *in vitro*

Human monocyte-derived dendritic cells (MoDC) were generated essentially as described.[14] Briefly, mononuclear cells were isolated from peripheral blood of

healthy volunteers by Ficoll-Paque density gradient centrifugation. Monocytes were purified by centrifugal elutriation or adherence to plastic for 2 h at 37°C each yielding similar results. Purified monocytes were cultured at 37°C, 5% CO_2 for seven days at 1 x 10^6/ml in CTCM supplemented with 500 U/ml recombinant human GM-CSF, 500 U/ml recombinant human IL-4 (both from PeproTech, London, UK) and with or without 25 µg/ml dust extract or 2 ng/ml LPS from *E. coli* (Sigma) as a matched control to the LPS content of dust extract. Every other day 50% of the medium was replaced by fresh, pre-warmed medium containing the same supplements. To induce maturation, 250 ng/ml LPS (Sigma) were added on day 6 and cells were incubated for another 24 h.

Flow cytometric analysis

Flow cytometric analysis of monocyte- and DC-specific surface antigens was performed on a FACSCalibur flow cytometer (BD Biosciences). The following fluorescein-isothiocyanate- (FITC-) or phycoerythrin- (PE) conjugated mouse anti-human monoclonal antibodies (mAbs) were used for direct labeling of the cells: antiCD1a-PE (clone BL6), antiCD14-FITC (RMO52), both from Beckman-Coulter, Krefeld, Germany. Isotype-matched mAbs of irrelevant specificity were used as controls.

Allogenic mixed lymphocyte reaction (MLR)

The T cell stimulatory capacity of MoDC generated in the presence or absence of dust extract was determined by measuring induction of IFN-γ in an allogenic mixed lymphocyte reactions. 1 x 10^4 MoDC were incubated with 1 x 10^5 allogenic lymphocytes in a 96-well flat bottom cell culture plate (Nunc, Wiesbaden, Germany) for 96 h in CTCM. Concentrations of IFN-γ were assessed in cell free supernatants by ELISA (IFN- γ OptEIA Set, BD Biosciences).

Statistical Analysis

Statistical analysis was done to test whether treatment with dust extract leads to a significant reduction of several parameters associated with allergic disease. For this purpose data from the mouse model were analyzed by the two tailed Mann-Whitney test. *In vitro* studies with human leukocytes were analyzed by two tailed Wilcoxon matched pairs test. Graph Pad Prism Software (Version 3.03) was used for analysis. Values of $p<0.05$ were considered statistically significant. All results are presented as median with the 25th to 75th percentile in parenthesis.

Results

Treatment of mice with stable-dust extract during sensitization inhibits the development of airway hyperresponsiveness

Following sensitization and challenge with OVA, mice develop marked airway hyperresponsiveness (AHR) compared to non-sensitized controls (fig 2). To examine the influence of stable-dust on allergen induced AHR, mice were exposed to aerosol of dust extract during sensitization and first challenge. Mice that were treated with dust extract were nearly as unresponsive to methacholine provocation as non-sensitized mice. In contrast, treatment of mice with a solution containing 0.8 µg/ml *E. coli* LPS as a matched control to the LPS content of dust extract did not reduce AHR. Treatment of non-sensitized mice with dust extract did not induce changes in airway reactivity (data not shown).

Suppression of airway eosinophilia following treatment with stable-dust extract

In addition to AHR, allergic asthma is characterized by airway inflammation, with predominant infiltrates of eosinophils.

As shown in figure 3, the BAL of non-sensitized mice contains only moderate numbers of macrophages, no lymphocytes and no eosinophils. After sensitization and challenge with OVA primarily eosinophils but also lymphocytes and macrophages are recruited to the airways resulting in 9.6 x 10^5 (7.6 to 11.5 x 10^5) eosinophils and 1 x 10^5 (0.8 to 1.1 x 10^5) lymphocytes in BAL. Treatment of mice with LPS did not have any significant influence on cell recruitment to the airways induced by sensitization and challenge. In contrast, treatment with stable-dust extract led to a remarkable suppression of recruitment of eosinophils and lymphocytes to the airways. The BAL of dust extract treated mice contained only 0.4 x 10^5 (0.2 to 0.7 x 10^5) eosinophils and 0.3 x 10^5 (0.2 to 0.7 x 10^5) lymphocytes.

Influence of treatment with dust extract on production of Th2 cytokines

Airway eosinophilia is dependent on the secretion of Th2 type cytokines, especially IL-5, from Th2 lymphocytes as a consequence of allergic sensitization.[15] In accordance, we found an induction of Th2 cytokines after sensitization, with increased *in vitro* production of IL-5 and IL-10 in splenocyte cultures re-stimulated with OVA (tab 1). However, *in vitro* production of IL-5 by splenocytes from sensitized and dust-treated mice was reduced by about 63% compared to sensitized mice sham-treated with PBS. In contrast, treatment with LPS did not result in a significant reduction in sensitization induced IL-5 production. On the contrary, LPS inhalation tended to increase IL-5 concentrations *in vitro*.

Production of IL-10 did not seem to be affected by inhalation of dust extract.

Table 1 Effect of inhaled dust extract on IL-5 and IL-10 production of murine splenocytes

Treatment	IL-5 (pg/ml)	IL-10 (pg/ml)
PBS	834 (625.5 to 1002)	1459 (1164 to 1782)
LPS	1080 (806.5 to 1420)	1816 (1482 to 2030)
dust extract	309 (248 to 402.3), p<0.0001	1275 (842.8 to 1742), p=0.4398
non sensitized	5.2 (0 to 16.5)	91.5 (78 to 124)

In vitro production of IL-5 and IL-10 was measured by ELISA after re-stimulation of splenocytes with OVA for two days. n ≥ 8 per group, results were presented as

median with the 25th to 75th percentile in parenthesis. Dust extract treated mice were compared with PBS treated mice by the two tailed Mann-Whitney test.

Influence of stable-dust extract on development of humoral immune responses to allergens

Serum levels of OVA-specific IgG1 and IgE antibodies increased substantially after sensitization (table 2). Following treatment with dust extract during sensitization, the production of both, OVA-specific IgG1 and IgE was reduced compared to mice sham-treated with PBS during sensitization. Inhalation of LPS during sensitization did not have any significant influence on the production of either antibody isotype. Interestingly, OVA-specific IgG2a antibodies, which are associated with Th1 responses and which were only marginally but significantly elevated in the serum after sensitization and challenge, did not increase in concentration following treatment with dust extract.

Table 2 Effect of inhaled dust extract on serum antibody production in mice

Treatment	IgE x 10^5 (U/ml)	IgG1 x 10^5 (U/ml)	IgG2a OD_{405}
PBS	2.7 (1.7 to 5.6)	11.1 (8.1 to 19.6)	0.15 (0.13 to 0.27)
LPS	2.2 (1.3 to 5.6)	13.3 (8.4 to 26.7)	0.14 (0.13 to 0.3)
dust extract	1.3 (0.9 to 2), p=0.017	5.5 (2 to 8.1), p=0.001	0.17 (0.13 to 0.2)
non sensitized	0	0	0.09

Serum levels of OVA specific IgE, IgG1, IgG2a were measured by ELISA and expressed as arbitrary units. $n \geq 8$ per group, results were presented as median with the 25th to 75th percentile in parenthesis. Dust extract treated mice were compared with PBS treated mice by the two tailed Mann-Whitney test.

To determine local antibody responses in the airways during allergic inflammation, OVA-specific IgE was also measured in BAL fluid.[16] IgE titers of sensitized and sham-treated mice were significantly elevated compared to non-sensitized mice which did not have any detectable OVA-specific antibodies in BAL fluid. Inhalation of dust extract significantly reduced IgE-titers in BAL fluid (fig 4). In contrast, treatment with LPS did not induce significant changes of IgE titers in BAL fluid.

Reduced T cell stimulatory capacity of human dendritic cells generated in the presence of stable-dust extract

Allergen induced airway inflammation and AHR are thought to depend on T cell responses. T cells in turn require activation by specialized antigen presenting dendritic cells. To determine whether dendritic cell differentiation can be inhibited by substances present in the dust extract, we tested the influence of the extract on *in vitro* generation of monocyte derived dendritic cells (MoDC).

Monocytes isolated from human blood were cultured in the presence of GM-CSF and IL-4 for seven days, resulting in dendritic cell differentiation as demonstrated by expression of CD1a and down regulation of CD14 (fig 5). The presence of 2 ng/ml *E. coli* LPS (concentration matched to LPS concentration in the dust extract) during culture did not seem to have an influence on dendritic cell differentiation with respect to up regulation of CD1a and down regulation of CD14. In contrast, the presence of dust extract inhibited the GM-CSF and IL-4 (G/I) induced differentiation of monocytes, reducing up regulation of CD1a while partially maintaining CD14 expression. These incompletely differentiated cells had a reduced T cell stimulatory capacity in allogenic mixed lymphocyte reactions (MLR) as measured by IFN-γ

release. In the presence of cells differentiated with dust extract, IFN-γ production was significantly reduced by 37.1% compared to IFN-γ concentrations in MLR with MoDC generated in the absence of dust extract (G/I=7.0 ng/ml (5.5 to 32.9 ng/ml); G/I+dust extract=4.4 ng/ml (1.2 to 15.1 ng/ml); n=6; p=0.03). MoDC from matched LPS control cultures did not change IFN-γ production in MLR significantly (G/I+LPS=6.6 ng/ml (5.4 to 19.1 ng/ml), not significant (p=0.16) versus G/I).

Discussion

Here, we present evidence that dust collected from stables of traditional farms contains biologically active factors which after extraction with physiological sodium chloride solution and inhalative administration can protect mice from allergic sensitization, eosinophilic airway inflammation and resulting AHR. This is the first experimental approach to confirm the epidemiological observation that exposure to the stable environment of traditional farms reduces the risk of allergic diseases.[1][2][3][4]

In our study, a method was used to extract substances from stable-dust that is probably comparable to the natural situation on the bronchial mucosa where salt containing mucosal fluid likely extracts similar substances from inhaled stable-dust particles. The effects of dust extract inhalation were particularly strong regarding AHR and airway inflammation. In comparison, the protective activity of inhaled dust extract on systemic sensitization was less pronounced. Average antibody titers (IgG1 and IgE) in serum of mice treated with dust extract were only slightly reduced. This indicates that inhaled dust extract mainly acts locally in the airways, inhibiting consequences of airway allergen challenges and only to a lesser extent the systemic responses to intraperitoneal sensitization with OVA and aluminum hydroxide. This interpretation is further supported by the observation that in contrast to serum levels, local concentrations of OVA specific IgE in BAL fluid were markedly reduced after dust extract inhalation. These findings indicate that the anti-allergic effects of dust extract would be even more pronounced in a model of sensitization exclusively via the airways. Such a model may reflect allergen sensitization in infants more closely than intraperitoneal hyperimmunization used in this study.

Several authors observed similar reductions in airway symptoms after treatment with solutions of microbes or microbial compounds; Broide et al. showed that bacterial CpG-oligodeoxynucleotides led to suppression of allergen induced asthma in mice, Hopfenspirger et al. found that treatment with mycobacterial antigens could attenuate allergen induced airway eosinophilia and deterioration of lung function even in mice with established allergic airway inflammation.[7][8] [11] [17][18][19] Furthermore, there is a body of information regarding effects of LPS in mouse models of asthma, demonstrating that systemic treatment with LPS in relative high doses results at least in partial reduction of allergic symptoms.[7][8] In all of these experiments LPS seemed to induce a shift towards Th1 responses. This is in contrast to our findings of slight enhancement of Th2 responses following LPS treatment and a lack of Th1 response after inhalation of stable-dust extract. This difference may be due to the comparatively low concentration of LPS in stable-dust extract and the matched LPS concentration used in our control experiments. It has been shown previously, that low concentrations of LPS are not only unable to inhibit allergic sensitization and Th2 responses, but that they can even enhance these.[8] Although endotoxin concentration in dust extract is too low to inhibit sensitization of mice, we can not exclude that it acts synergistically with other immune modulatory substances of farm dust as Roy et al. have shown for IL-10 and IL-12 release of human PBMC after stimulation with barn dust DNA *in vitro*.[20]
Considering the lack of a Th1 shift it is likely that the immune modulation induced by stable-dust extract was based on the induction of immunological tolerance or suppression. This hypothesis is supported by our results showing that human MoDC generated in the presence of dust extract showed a much weaker T cell stimulatory capacity than MoDC generated without dust extract. This lack of stimulatory capacity

was associated with persistent CD14 and reduced CD1a expression indicating incomplete differentiation from monocytes into dendritic cells in the presence of dust extract. Thus, the stable-dust extract may well exert influence on allergic immune responses by modulating differentiation and function of antigen presenting cells (APC). It is tempting to speculate, that modulation of APC differentiation by components of stable-dust may lead to unresponsiveness of allergen specific T cells. Although we do not present direct evidence for this hypothesis, our data indicate down regulation of the T cell response rather than activation.
Finally, the finding that stable-dust extract used in our study contained immune modulatory substances which were able to inhibit the development of allergic sensitization, eosinophilic airway inflammation and changes in lung function could lead the way to new prophylactic treatments for the prevention of allergic diseases. Especially, the observation that inhalation of stable-dust extract induced suppression or unresponsiveness rather than inducing Th1 responses against the allergen, makes stable-dust derived compounds interesting candidates for therapeutic approaches in humans, since the lack of Th1 induction may reduce the risk of undesirable side effects like auto-immunity.[21]

In summary, we have established an experimental schedule which will allow the identification of relevant biologically active compounds in the inhaled stable-dust extract. Utilizing this experimental schedule our study demonstrates that it is possible to extract highly active substances from stable-dust which can suppress sensitization in a mouse model of allergic asthma. This encouraging observation will make it a priority to define the active factors in stable-dust and to delineate their precise mode of action.

Acknowledgements: This study was supported by grants from the Deutsche Forschungsgemeinschaft (DFG: BU-762/5-1, HO-1259/4-1).
We are indebted to our collaborators of the ALEX Study Group: David Carr and Rudolf Schierl, Munich, Germany; Waltraud Eder, Tucson, USA; Udo Herz and Harald Renz, Marburg, Germany; Roger P. Lauener, Zurich, Switzerland; Marco Waser, Basel, Switzerland. We like to thank Britta Steeger, Petra Fritz, Petra Behrens and Erika Flacke for excellent technical assistance.

All authors declare that they do not have any competing financial or other interest in relation to this work.

References

1 Riedler J, Braun-Fahrländer C, Eder W, *et al.* Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *The Lancet* 2001;**358**:1129-33.

2 Braun-Fahrländer C, Riedler J, Herz U, *et al.* Environmental exposure to endotoxin and its relation to asthma in school-age children. *New Engl J Med* 2002;**347**:869-77.

3 Von Ehrenstein OS, von Mutius E, Illi S, *et al.* Reduced risk of hay fever and asthma among children of farmers. *Clin Exp Allergy* 2000;**30**:187-93.

4 van Strien RT, Engel R, Holst O, *et al.* Microbial exposure of rural school children, as assessed by levels of *N*-acetyl-muramic acid in mattress dust, and its association with respiratory health. *J Allergy Clin Immunol* 2004;**113**:860-7.

5 Gerhold K, Blümchen K, Franke A, *et al.* Exposure to endotoxin and allergen in early life and its effect on allergen sensitization in mice. *J Allergy Clin Immunol* 2003;**112**:389-96

6 Gerhold K, Blümchen K, Bock A, *et al.* Endotoxins and allergy: lessons from the murine model. *Pathobiology* 2002-2003;**70**:255-9.

7 Gerhold K, Blümchen K, Bock A, *et al.* Endotoxins prevent murine IgE production, T(H)2 immune responses, and development of airway eosinophilia but not airway hyperreactivity. *J Allergy Clin Immunol* 2002;**110**:110-6.

8 Eisenbarth SC, Piggott DA, Huleatt JW, *et al.* Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med* 2002;**196**:1645-51.

9 Romagnani S. The increased prevalence of allergy and the hygiene hypothesis: missing immune deviation, reduced immune suppression, or both? *Immunology* 2004;**112**:352-63.

10 Zuany-Amorim C, Sawicka E, Manlius C, *et al.* Suppression of airway eosinophilia by killed Mycobacterium vaccae-induced allergen-specific regulatory T cells. *Nat Med* 2002;**8**:625-9.

11 Liu YH, Kao MC, Lai YL, *et al.* Efficacy of local nasal immunotherapy for Dp2-induced airway inflammation in mice: Using Dp2 peptide and fungal immunomodulatory peptide. *J Allergy Clin Immunol* 2003;**112**:301-10.

12 Tomkinson A, Cieslewicz G, Duez C, *et al.* Temporal association between airway hyperresponsiveness and airway eosinophilia in ovalbumin-sensitized mice. *Am J Respir Crit Care Med* 2001;**163**:721-30.

13 Hamelmann E, Schwarze J, Takeda K, *et al.* Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. Am *J Respir Crit Care Med* 1997;**156**:766-75.

14 Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* 1994;**179**:1109-18.

15 Hamelmann E, Cieslewicz G, Schwarze J, *et al.* Anti-interleukin 5 but not anti-IgE prevents airway inflammation and airway hyperresponsiveness. *Am J Respir Crit Care Med* 1999;**160**:934-41.

16 Chvatchko Y, Kosco-Vilbois MH, Herren S, *et al.* Germinal center formation and local immunoglobulin E (IgE) production in the lung after an airway antigenic challenge. *J Exp Med* 1996;**184**:2353-60.

17 Broide D, Schwarze J, Tighe H, *et al.* Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J Immunol* 1998;**161**:7054-62.

18 Hopfenspirger MT, Agrawal DK. Airway hyperresponsiveness, late allergic response, and eosinophilia are reversed with mycobacterial antigens in ovalbumin-presensitized mice. *J Immunol* 2002;**168**:2516-22.

19 Saito K, Yajima T, Nishimura H, *et al.* Soluble branched beta-(1,4)glucans from Acetobacter species show strong activities to induce interleukin-12 in vitro and inhibit T-helper 2 cellular response with immunoglobulin E production in vivo. *J Biol Chem* 2003;**278**:38571-8.

20 Roy SR, Schiltz AM, Marotta A, *et al.* Bacterial DNA in house and farm barn dust. *J Allergy Clin Immunol* 2003;**112**:571-8.

21 Stephens R, Eisenbarth SC, Chaplin DD. T helper type 1 cells in asthma: friend or foe? *Curr Opin Allergy Clin Immunol* 2002;**2**:31-7

Figure 1 Design of *in vivo* experiments.
OVA i.p., denotes systemic immunization with OVA adsorbed in aluminum hydroxide. OVA-aerosol, indicates challenges with 1% OVA aerosol via the airways. AR, denotes measurements of airway responsiveness. Analysis, indicates harvest of BAL cells, serum and splenocytes for analysis.

Figure 2 Airway responsiveness following exposure to stable-dust extract during sensitization.
Airway responsiveness to methacholine was measured 24 h after the second challenge with OVA in mice treated by inhalation of dust extract (n=16) or sham-treated with PBS (n=16) during sensitization. Another control group was treated during sensitization by inhalation of LPS as a matched control to LPS content of dust extract (n=8). Median Penh values from groups of mice were related to the methacholine concentrations used for provocation. Whiskers represent the 25th to the 75th percentile of median values. Significant differences: ***P<0.001 dust extract treated mice compared to PBS treated mice.

Figure 3 Effect of exposure to stable-dust extract on allergen induced airway inflammation.
Total number of eosinophils, lymphocytes and macrophages in BAL fluid recovered from mice treated by inhalation of dust extract (n=16) or sham-treated with PBS (n=16) during sensitization were counted and differentiated to evaluate recruitment of leucocytes to the airways. Another control group was treated during sensitization by inhalation of LPS as a matched control to LPS content of dust extract (n=8). Boxes represent the 25th to the 75th percentile with a line at the median. Whiskers show the highest and lowest values.

Figure 4 Effect of dust extract inhalation on allergen specific IgE levels in the airways.
BAL fluid was collected following sensitization to OVA and two OVA aerosol challenges. Concentrations of OVA specific IgE antibodies were measured by ELISA. Individual values expressed as arbitrary units and medians are represented in the graph. No OVA-specific IgE was detected in non-sensitized mice (data not shown).

Figure 5 Surface expression of CD14 and CD1a on MoDC.
Peripheral blood monocytes from six donors were cultured for seven days with GM-CSF and IL-4 (G/I), GM-CSF/IL-4 and LPS (G/I + LPS) or GM-CSF/IL-4 and dust extract (G/I + dust extract). Resulting cells were evaluated by FACS for expression of CD14 (A) and CD1a (B). Boxes represent the 25th to the 75th percentile with a line at the median. Whiskers show the highest and lowest values.

figure 1

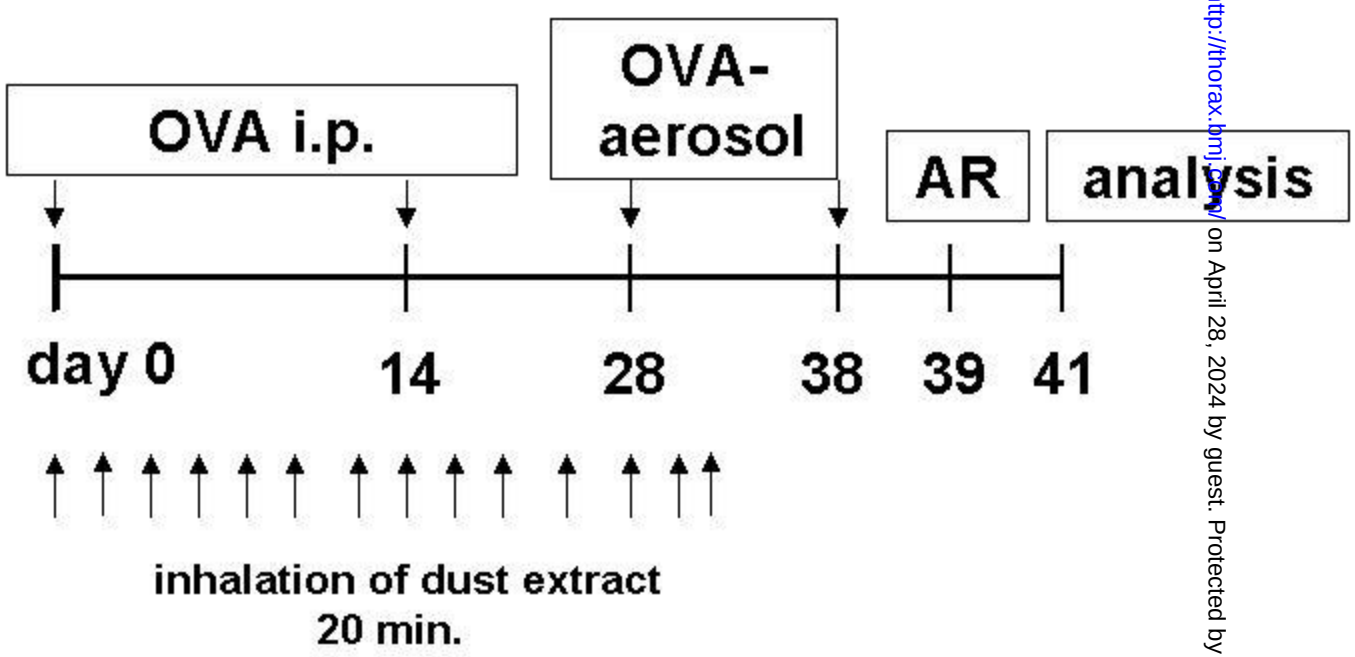


Figure 2

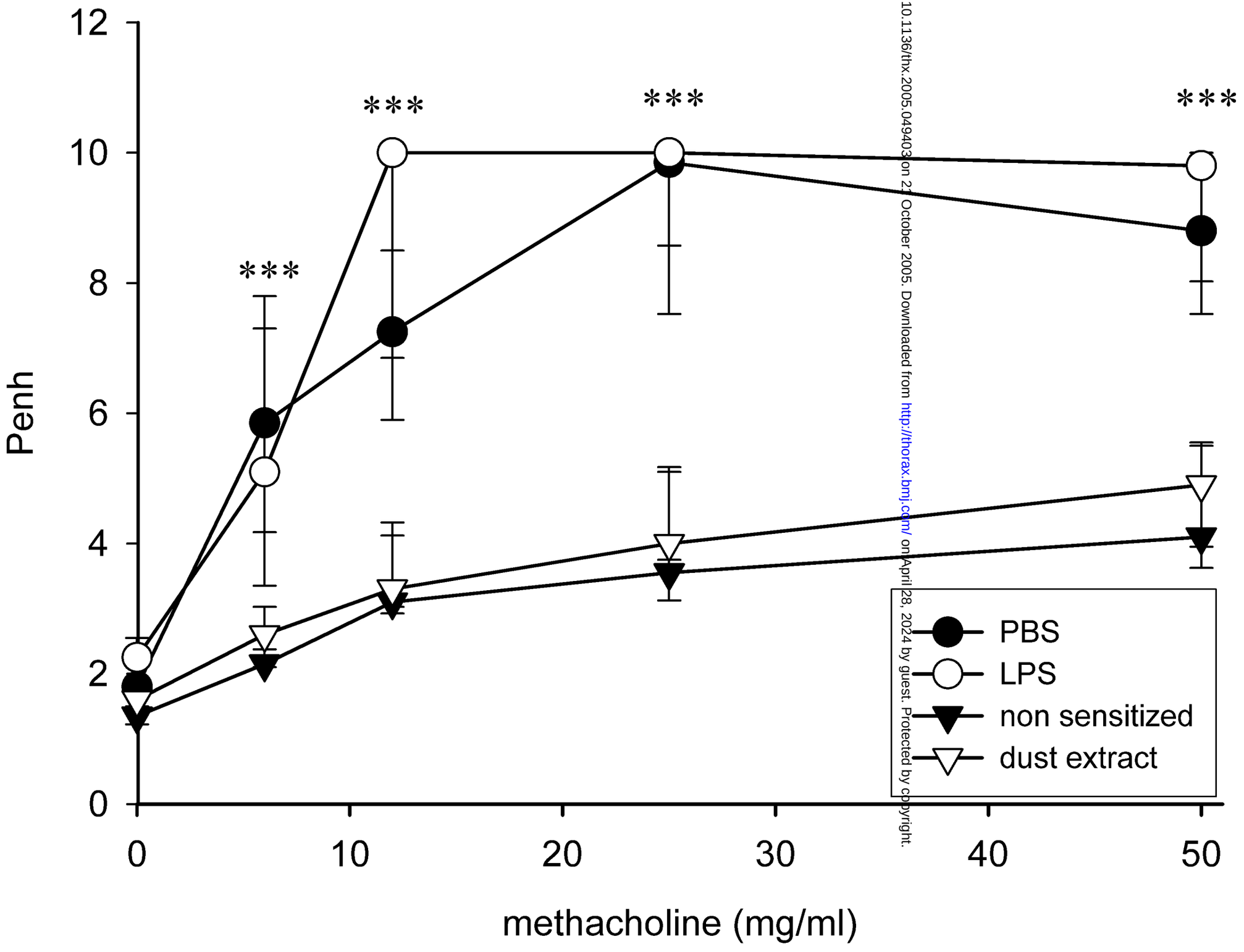


Figure 3

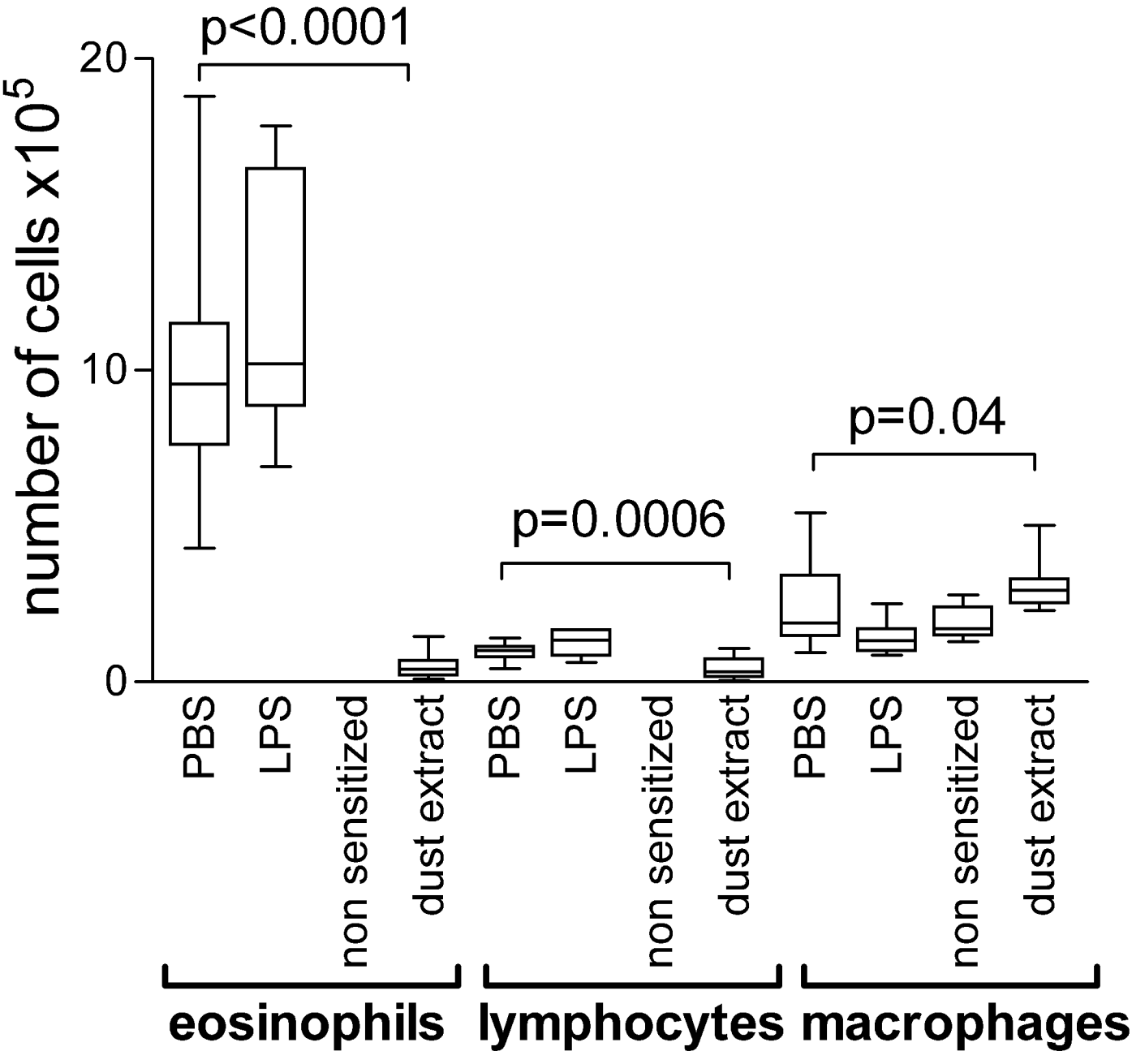

p<0.0001
p=0.0006
p=0.04
number of cells x10^5
20
10
0
PBS
LPS
non sensitized
dust extract
PBS
LPS
non sensitized
dust extract
PBS
LPS
non sensitized
dust extract
eosinophils
lymphocytes
macrophages

Figure 4

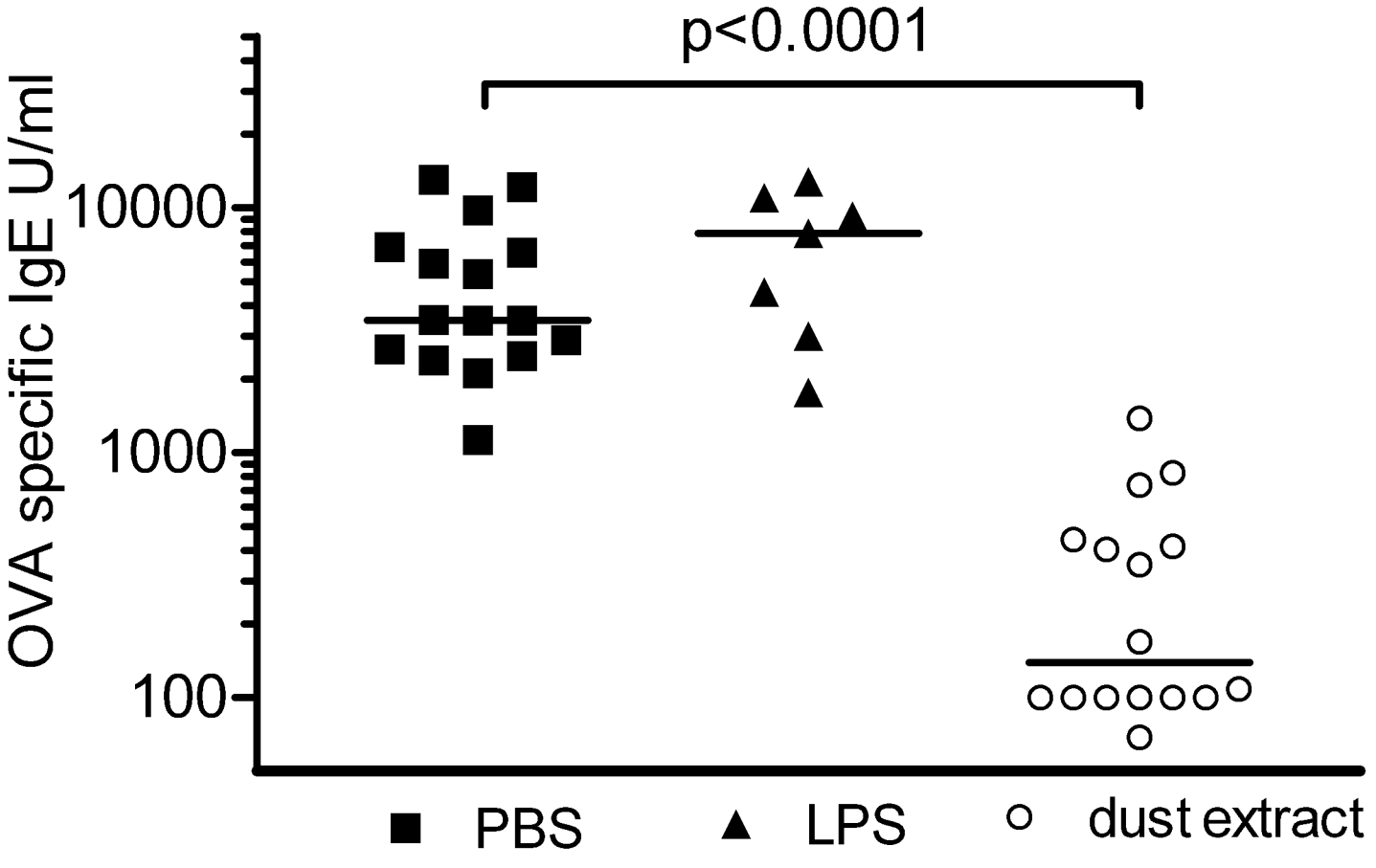
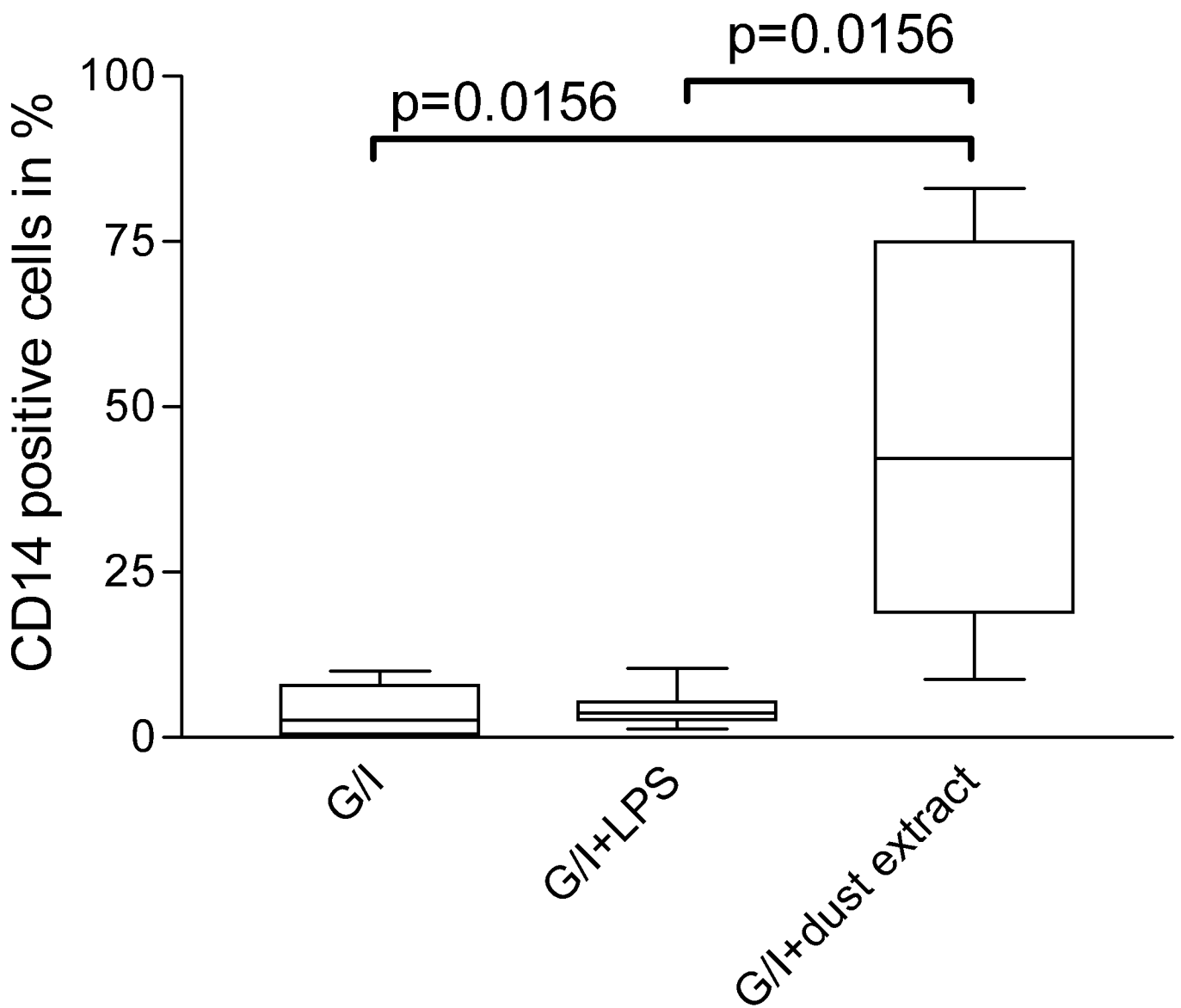

p<0.0001
OVA specific IgE U/ml
10000
1000
100
PBS
LPS
dust extract

Figure 5A



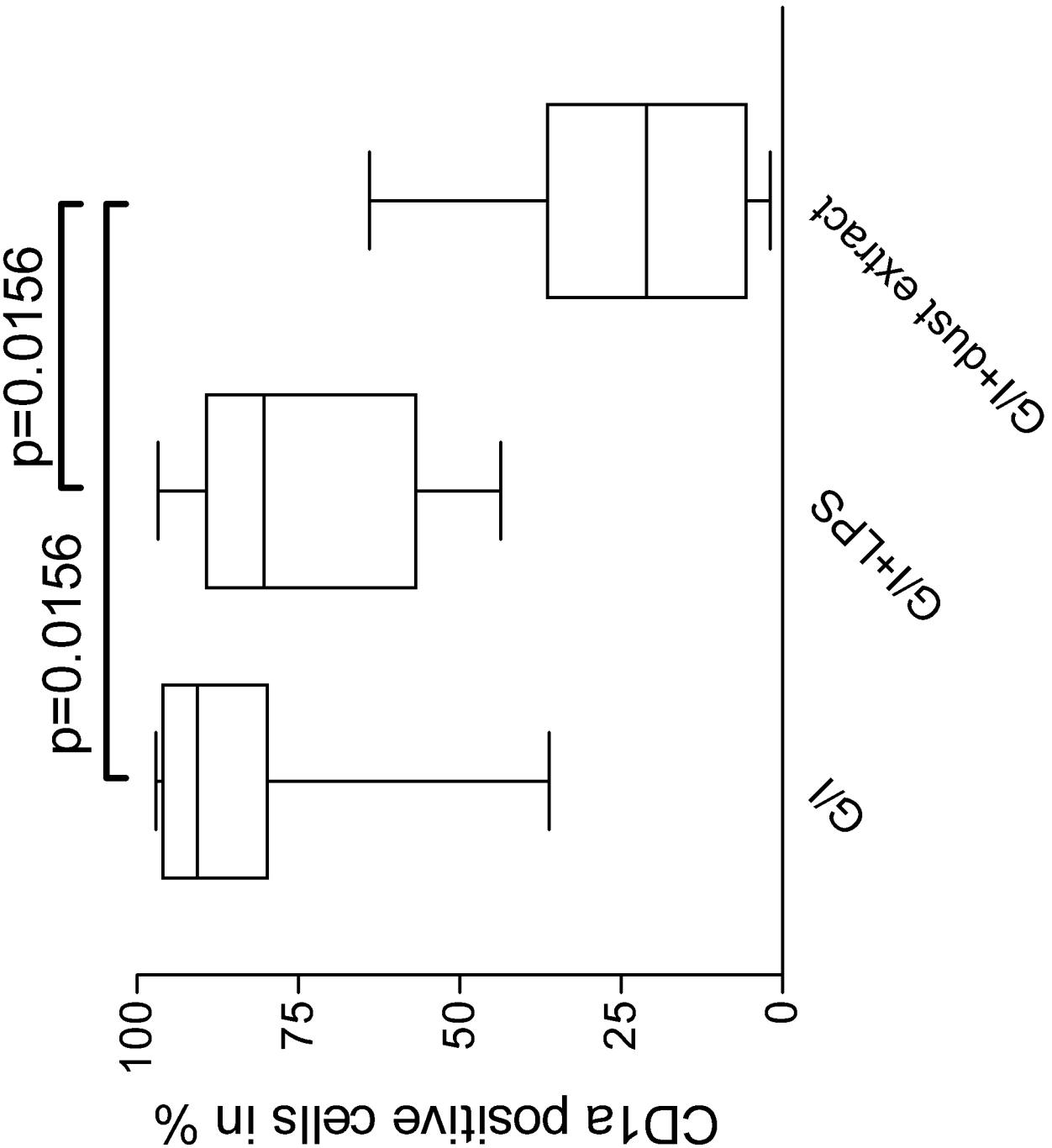


Figure 5B