

Immunomodulatory Effects of Oak Dust Exposure in a Murine Model of Allergic Asthma

Juha Määttä,^{*,1} Rita Haapakoski,^{*,1} Maili Lehto,^{*} Marina Leino,^{*} Sari Tillander,^{*} Kirsti Husgafvel-Pursiainen,[†] Henrik Wolff,^{†,‡} Kai Savolainen,[§] and Harri Alenius^{*,2}

^{*}Unit of Excellence in Immunotoxicology, [†]Team for Biological Mechanisms and Prevention of Work-related Diseases, [‡]New Technologies and Risk Analysis, Finnish Institute of Occupational Health, Topeliuksenkatu 41 a A, FIN-00250, Helsinki, Finland; and [§]Department of Pathology, Helsinki University Central Hospital, Helsinki, Finland

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Repeated airway exposure to wood dust has been reported to cause adverse respiratory effects such as asthma and chronic bronchitis. In our recent study, we found that exposure of mice to oak dust induced more vigorous lung inflammation compared to birch dust exposure. In the present study, we assessed the immunomodulatory effects of repeated intranasal exposure to oak dust both in nonallergic and in ovalbumin-sensitized, allergic mice. Allergen-induced influx of eosinophils and lymphocytes was seen in the lungs of allergic mice. Oak dust exposure elicited infiltration of neutrophils, lymphocytes, and macrophages in non-allergic mice. Interestingly, oak dust-induced lung neutrophilia as well as oak dust-induced production of the proinflammatory cytokine TNF- α and chemokine CCL3 were significantly suppressed in allergic mice. On the other hand, allergen-induced expression of IL-13 mRNA and protein was significantly reduced in oak dust-exposed allergic mice. Finally, allergen-induced airway hyperreactivity to inhaled metacholine was significantly suppressed in oak dust-exposed allergic mice. The present results suggest that repeated airway exposure to oak dust can regulate pulmonary inflammation and airway responses depending on the immunological status of the animal.

Key Words: wood dust; inflammation; asthma; lung; cytokine.

INTRODUCTION

It has been estimated that approximately 3.6 million workers are exposed to inhalable wood dust in the European Union (Kauppinen *et al.*, 2006). Several studies have indicated that occupational exposure to wood dust increases the risk for nonmalignant respiratory diseases such as allergic rhinitis, asthma, and organic dust toxic syndrome (Bohadana *et al.*, 2000; De Zotti and Gubian, 1996; Douwes *et al.*, 2001; Enarson and Chan-Yeung, 1990; Goldsmith and Shy, 1988). Respiratory symptoms have been linked to the dust of different tree species, some of which have been identified as allergenic

(Douwes *et al.*, 2001; Hessel *et al.*, 1995; Noertjojo *et al.*, 1996; Schlunssen *et al.*, 2002).

In studies on healthy volunteers, wood chip mulch exposure has been shown to cause an enhancement of proinflammatory cytokine levels and an elevation in neutrophil percentage in bronchoalveolar lavage (BAL) fluid (Wintermeyer *et al.*, 1997). Furthermore, an increase in BAL fluid total cell concentration and recruitment of eosinophils, T lymphocytes, and mast cells into the lungs following inhalation of pinewood dust have been demonstrated (Gripenback *et al.*, 2005). The work environment in the wood-handling industries may, however, contain many other hazardous inhalable substances such as formaldehyde, fungal β -glucans, and mould spores (Alwis *et al.*, 1999; Kauppinen and Niemela, 1985; van Kampen *et al.*, 2000). Furthermore, a mixed exposure to several species of wood dust as well as a wide distribution in the exposure levels between workers is very common, complicating the assessment procedure (Demers *et al.*, 1997; Kauppinen *et al.*, 2006).

Since well-standardized experiments of wood dust exposure are rather difficult to execute in humans, knowledge of cellular mechanisms underlying wood dust-induced airway symptoms is still poorly understood. We reported recently that exposure to oak and birch dust could induce quantitative and qualitative differences in the outcome of pulmonary inflammation in healthy mice (Määttä *et al.*, 2006). In addition to elicitation of pulmonary inflammation in healthy subjects, wood dust exposure may also influence the development and severity of other pulmonary diseases, e.g., allergic asthma. In the present study, we investigated the effects of oak dust exposure on airway inflammation in both nonallergic and in ovalbumin (OVA)-sensitized, allergic mice.

MATERIALS AND METHODS

Oak dust and TiO₂. Preparation, physicochemical characterization, and the size distribution monitoring of oak (*Quercus alba*) dust used has been described in detail earlier (Määttä *et al.*, 2006). In the dust generated from oak (purchased from Puusepänilike Pöntinen KY, Lappeenranta, Finland), more than 99% of

¹ These authors contributed equally to this study.

² To whom correspondence should be addressed. Fax: +358-30-4742116. E-mail: harri.alenius@ttl.fi.

the oak dust particles had an aerodynamic diameter $\leq 5 \mu\text{m}$. The titanium dioxide (TiO_2) powder (Aldrich Chem. Co., Milwaukee, WI) was used in the experiment as a control substance. About 90% of the TiO_2 particles were smaller than $1 \mu\text{m}$ and 10% were between 1 and $5 \mu\text{m}$ in diameter (Määttä *et al.*, 2005). The size distribution measurement of TiO_2 particles was done with scanning electron microscopy JEOL JSM-6400 (JEOL Inc., Peabody, MA).

Mice and sensitization protocol. Female BALB/c mice, 6–8 weeks of age, were obtained from Taconic (Ry, Denmark) and housed under specific pathogen-free conditions. All the experiments were approved by the Social and Health Care Department at the State Provincial Office of Southern Finland.

Mice were sensitized ip on days 1 and 11 with $20 \mu\text{g}$ of OVA emulsified in alum (Sigma, St Louis, MO) in $100 \mu\text{l}$ of phosphate-buffered saline (PBS). The control groups were sham sensitized with alum in $100 \mu\text{l}$ of PBS. Part of the mice were treated with intranasal instillations of $50 \mu\text{g}$ of oak dust suspension in $50 \mu\text{l}$ of PBS under light anesthesia (Isoflurane; Abbott Laboratories Ltd, Queenborough, UK) two times a week for three and a half weeks (on days 2, 5, 8, 12, 15, 19, and 23 starting from the beginning of the experiment). The oak dust concentration used in the present study was selected on the basis of the results from our previous experiment (Määttä *et al.*, 2006). Control mice were given $50 \mu\text{l}$ of PBS or $50 \mu\text{g}$ of TiO_2 powder suspended in $50 \mu\text{l}$ of PBS intranasally at the same times. On days 22–24, all mice were challenged with 1% OVA solution via the airways for 20 min administered via an ultrasonic nebulizer (DeVilbiss, Glendale Heights, IL).

Measurement of airway responsiveness. Airway responsiveness was assessed on day 25 using a single chamber, whole-body plethysmograph system (Buxco, Troy, NY) as described earlier (Hamelmann *et al.*, 1997). Briefly, mice were exposed to increasing concentrations (1, 3, 10, and 30 mg/ml) of metacholine (MCh) (Sigma) delivered via an AeroSonic 5000 D ultrasonic nebulizer. Lung reactivity parameters were expressed as the percentage of baseline Penh (enhanced pause) values. After measurement of lung responsiveness, the mice were sacrificed and specimens collected for subsequent analysis.

Sample collections and lung preparations. The chest cavity was opened and the lungs were lavaged with $800 \mu\text{l}$ of PBS via the trachea. The BAL fluid sample was cytocentrifuged (Cytospin, Shandon Ltd, UK), and the cells were stained with the May Grünwald-Giemsa stain (Merck, Whitehouse Station, NJ) for inflammatory cell counting. The left lung was removed for RNA isolation, quick-frozen, and kept at -70°C . For histologic examination, the right lung was perfused with 10% formalin, embedded in paraffin, and cut into $5\text{-}\mu\text{m}$ thick sections. The slides were stained with hematoxylin and eosin (H&E) and Periodic Acid Schiff (PAS) solutions and examined under light microscopy.

Real-time PCR. Total RNA from lungs was extracted using TRIzol reagent (Gibco/Invitrogen, Carlsbad, CA) as described earlier (Lehto *et al.*, 2003). PCR primers and probes for murine IFN- γ , IL-1 β , IL-13, TNF- α , CCL3/MIP-1 α , CXCL2/3/MIP-2, and CXCL5/LIX were purchased from Applied Biosystems (Foster City, CA).

Protein levels in BAL fluid. IL-13, TNF- α , and CCL3 protein levels in BAL fluid were determined using commercial ELISA kits (R&D Systems, Minneapolis, MN). The lower limits of detection were 31.3 pg/ml for IL-13, 4 pg/ml for TNF- α , and 4.7 pg/ml for CCL3.

Measurement of serum antibodies. For the analysis of OVA-specific IgG2a, ELISA plates were coated with OVA (BD Biosciences, San Diego, CA). Serum samples (1:60 and 1:180 dilutions) were added to the plate and incubated overnight. Biotinylated anti-mouse IgG2a (BD Biosciences) was added and streptavidin horseradish peroxidase (BD Biosciences) followed by substrate (ABTS Microwell Peroxidase Substrate System, KPL, Gaithersburg, MD) was used to detect the bound antibody levels. Absorption was read at 405 nm with ELISA plate absorbance reader (Multiskan MS, Labsystems, Helsinki, Finland).

For the analysis of OVA-specific IgE, the protocol was slightly modified. Plates were coated with anti-mouse IgE antibody (BD Biosciences). Serum

samples (1:20 dilution) were added to the plate and incubated overnight. Biotinylated OVA was added and streptavidin horseradish peroxidase followed by substrate was used to detect the bound antibody levels. Optical density was measured at 405 nm as described above.

Statistical analysis. Statistical tests were performed using GraphPad Prism Software (GraphPad Software, Inc., San Diego, CA). Single-group comparisons were conducted by the nonparametric Mann-Whitney *U*-test. The results are expressed as mean $\pm$ SEM. *p* values < 0.05 were considered to be statistically significant.

RESULTS

Oak Dust-Induced Pulmonary Neutrophilia Is Reduced in Allergic Mice

Oak dust exposure provoked a significant influx of lymphocytes into the lungs of both allergic and nonallergic mice (Fig. 1A). However, there was greater infiltration of lymphocytes in BAL in oak dust-exposed allergic mice when compared to the oak dust-exposed nonallergic mice (Fig. 1A). This increase in the number of lymphocyte-like cells was observed also in H&E-stained lung sections of oak dust-exposed mice (data not shown). The number of eosinophils was significantly increased in BAL of allergic mice, whereas eosinophils were virtually absent in the BAL of nonallergic mice (Fig. 1B). Oak dust exposure had no detectable effect on eosinophil infiltration in either group of mice. In contrast, as shown in Figure 1C, oak dust exposure induced a marked increase ($p < 0.001$) in the number of neutrophils both in OVA allergic and nonallergic mice. However, oak dust-induced infiltration of neutrophils was significantly reduced in BAL of allergic mice compared to nonallergic mice ($p < 0.01$). Furthermore, oak dust exposure induced a comparable, significant increase in the number of macrophages in the BAL of allergic and nonallergic mice ($p < 0.001$) (Fig. 1D). Administration of TiO_2 dust did not evoke any significant changes in the numbers of inflammatory cells when compared to allergic mice given PBS (data not shown). A slight enhancement of neutrophils (< 5 cells per high-power fields) was detected in nonallergic mice when compared to mice treated with PBS (no neutrophils observed).

Oak Dust Exposure Downregulates Allergen-Induced Airway Hyperreactivity

Mice sensitized and challenged to OVA exhibited a significant increase in airway hyperreactivity (AHR) to inhaled MCh as compared to nonallergic mice ($p < 0.05$) (Fig. 2A). Interestingly, oak dust exposure in allergic mice significantly reduced allergen-induced airway reactivity to inhaled MCh when compared to mice treated with OVA only ($p < 0.05$) (Fig. 2A).

Mucus-producing cells (counted as the number of PAS+ cells in the airway epithelia) were significantly increased in OVA-treated allergic mice when compared to the situation in nonallergic mice ($p < 0.001$) (Fig. 2B). Overall, PAS+ cell numbers were abundant in allergic mice and, as compared to

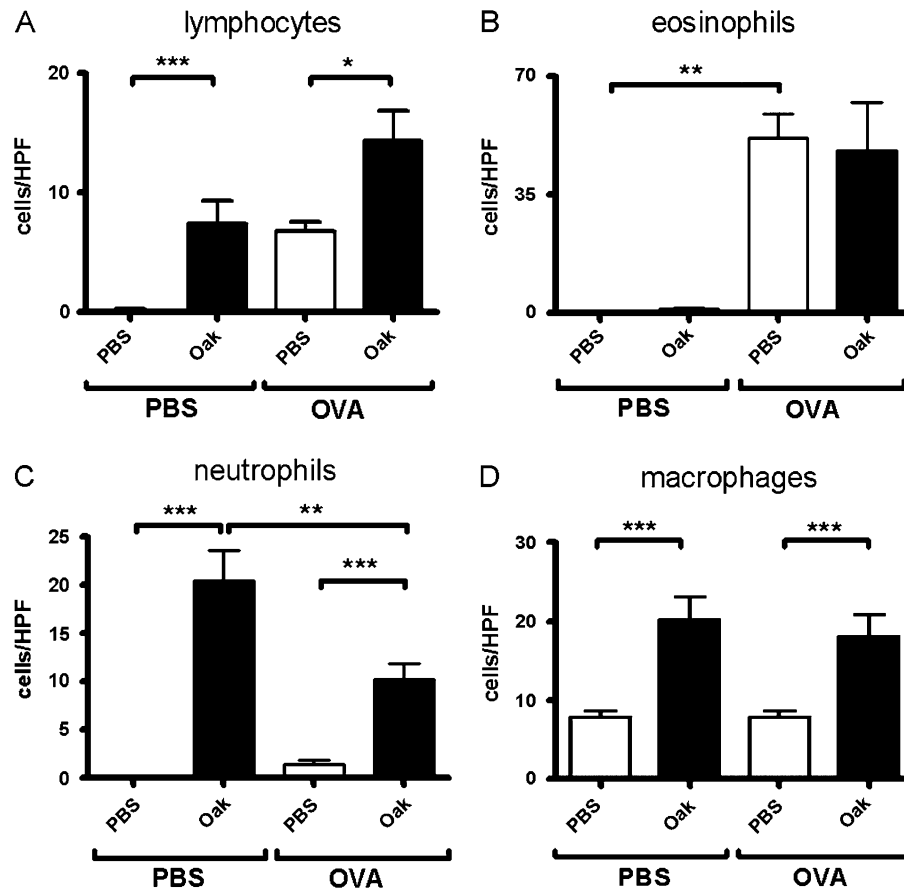


FIG. 1. Effect of oak dust administration on the amount of (A) lymphocytes, (B) eosinophils, (C) neutrophils, and (D) macrophages in BAL fluid in allergic (OVA) and in nonallergic (PBS) mice. Cells are counted as the average of positive cells in four different randomly selected lung areas/mice ($n = 9$ mice per group). Original magnification $\times 40$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

nonallergic mice, this effect was unaltered after oak dust exposure (Fig. 2B). Mucus-producing cells were absent in nonallergic control mice and only a few PAS+ cells were found in oak dust-exposed nonallergic mice (Fig. 2B).

Effects of Oak Dust Exposure on Cytokine Levels in the Lungs

The expression of proinflammatory cytokine TNF- α mRNA was clearly enhanced after administration of oak dust in both allergic ($p < 0.01$) and nonallergic mice ($p < 0.001$) (Fig. 3A). Interestingly, the oak dust-induced secretion of TNF- α protein was significantly decreased in allergic mice when compared to nonallergic mice treated with oak dust ($p < 0.05$) (Fig. 3A). The expression of IL-1 β mRNA was significantly induced only in the lung tissue of nonallergic mice treated with oak dust ($p < 0.05$).

Both Th2 (IL-13) and Th1 (IFN- γ) type cytokines were enhanced in the lungs of allergic mice at mRNA level compared to nonallergic mice (Fig. 3B). However, oak dust exposure inhibited significantly the expression of IL-13 at the protein level ($p < 0.01$) in allergic mice when compared to nonallergic mice (Fig. 3B). The expression of a major Th1-type

cytokine, IFN- γ , exhibited also a decline in allergic mice after oak dust exposure although the difference did not reach statistical significance (Fig. 3B).

Effect of Oak Dust Exposure on Chemokine Expression in the Lungs

Exposure of nonallergic mice to oak dust significantly induced the expression of all of the chemokines tested (Figs. 4A and 4B). A significant induction of CCL3 mRNA and protein was found in oak dust-exposed healthy mice ($p < 0.001$) (Fig. 4A). On the contrary, oak dust-induced secretion of CCL3 protein was markedly inhibited in allergic mice (Fig. 4A). In addition, allergen-induced expression of CXCL5 and CXCL2/3 chemokines appeared to be reduced by oak dust in allergic mice although the inhibition did not reach statistical significance (Fig. 4B).

Effect of Oak Dust Exposure on Serum IgE and IgG2a Levels

IgE levels were significantly induced only in allergic mice ($p < 0.001$) (Fig. 5A). OVA-specific IgG2a levels were similarly induced in allergic mice exposed to oak dust ($p < 0.01$) and

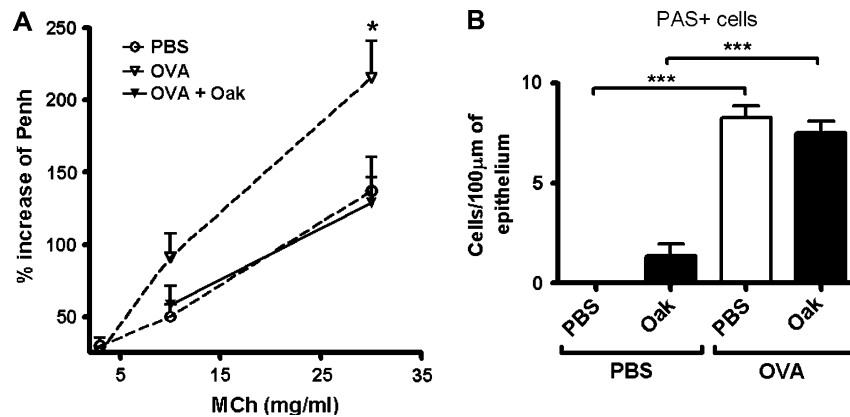


FIG. 2. (A) Reduction of airway responsiveness to MCh in allergic mice after oak dust exposure. Results are shown as percent increase of average enhanced pause (Penh) values in relation to increasing doses (1–30 mg/ml) of aerosolized MCh. (B) Effect of oak dust administration on the amount of mucus-producing cells around the bronchioles. Results are indicated as the average of PAS+ cells/100 μ m of bronchus counted from three bronchioles per mouse ($n = 8$ mice per group). Original magnification $\times 40$. * $p < 0.05$, *** $p < 0.001$.

in mice nonexposed to oak dust ($p < 0.001$) when compared to nonallergic control groups (Fig. 5B).

DISCUSSION

We have recently demonstrated that repeated exposure to oak dust induced an influx of inflammatory cells and upregulation of various cytokines and chemokines in the lungs of nonallergic mice (Määttä *et al.*, 2006). The purpose of the

present study was to examine the immunomodulatory effects of oak dust exposure in OVA-sensitized, allergic mice. Our results indicate that repeated exposure to oak dust can modulate airway inflammation in quite distinct ways depending on the immune status of the animal. Interestingly, the expression of several cytokines and chemokines as well as AHR to inhaled MCh was significantly suppressed in oak dust-exposed allergic mice compared to nonexposed allergic mice.

In the present study, we found elevated numbers of lymphocytes, neutrophils, and macrophages in the lungs of nonallergic

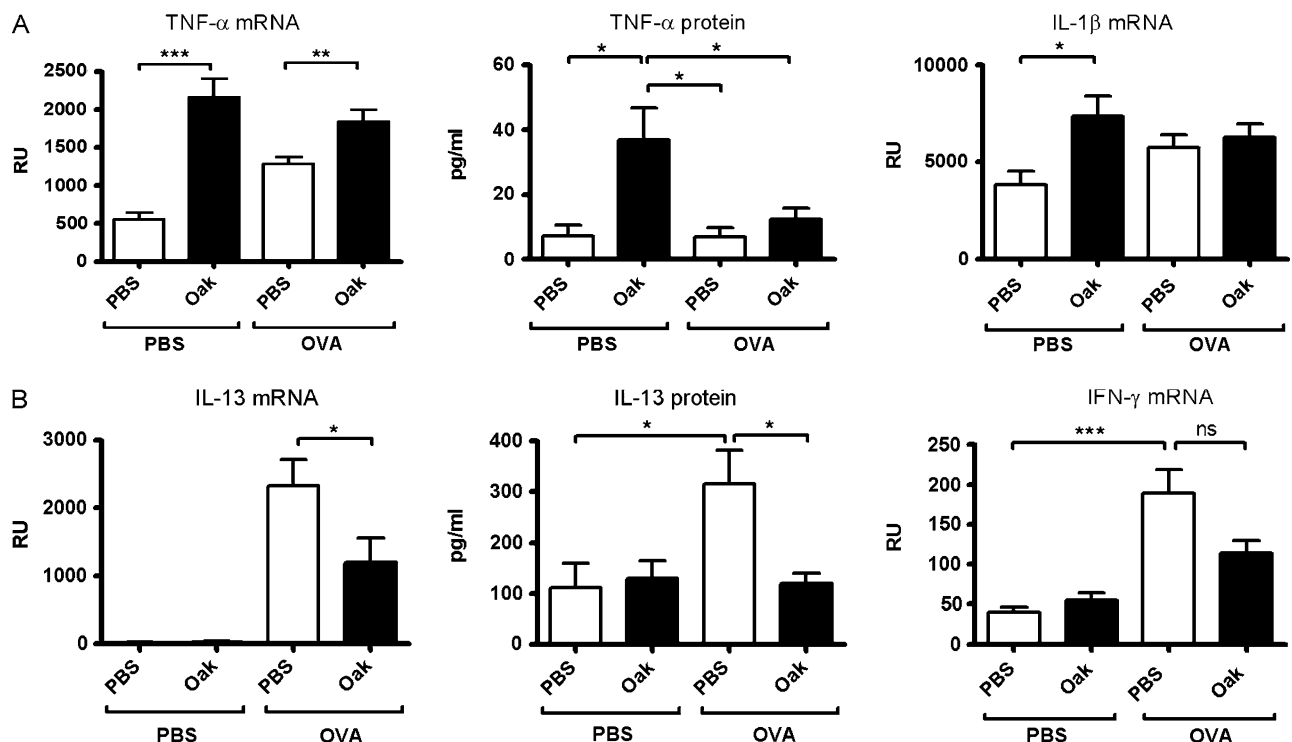


FIG. 3. (A) Induction of proinflammatory cytokines in the lung tissue after oak dust exposure in nonallergic mice. (B) Inhibitory effect of oak dust on Th2- and Th1-type cytokines in the lung tissue and in BAL fluid in allergic mice exposed to oak dust. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns, not significant.

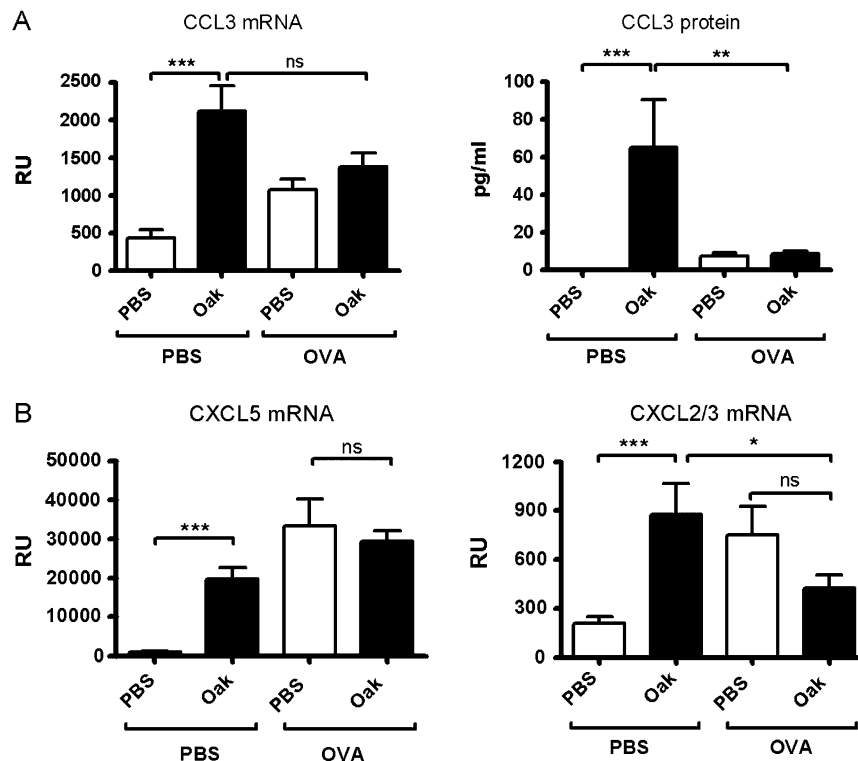


FIG. 4. (A) Effect of oak dust administration on CCL3 mRNA in the lung tissue and CCL3 protein level in BAL fluid. (B) mRNA expression levels of CXCL5 and CXCL2/3 in the lung tissue after oak dust exposure. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns, not significant.

mice exposed to oak dust compared to nonexposed control mice. Moreover, expression of proinflammatory cytokines and chemokines (TNF- α , IL-1 β , CCL3) in the lung tissue and BAL was elevated in control mice exposed to oak dust. The present results confirm our recent findings (Määttä *et al.*, 2006) and indicate that oak dust exposure in nonallergic control mice promotes the development of pulmonary inflammation, in which macrophages, neutrophils, and nonpolarized lymphocytes as well as proinflammatory cytokines and chemokines play a central role. It has been reported earlier that an elevation in the number of neutrophils in BAL fluid as well as an increase in IL-6 and TNF- α cytokine levels are detected in healthy, nonasthmatic subjects after exposure to wood chip dust (Wintermeyer *et al.*, 1997). Moreover, pine dust has been shown to induce the expression of TNF- α in rat alveolar macrophages (Long *et al.*, 2004). Recently, induction of IL-1 β expression in the lung tissue was found to cause pulmonary inflammation as characterized by neutrophil and macrophage infiltrates (Lappalainen *et al.*, 2005). These findings are in accordance with our present observations showing an influx of neutrophils as well as an increase in the expression of proinflammatory cytokines in the lungs of nonallergic mice after oak dust administration.

AHR, pulmonary eosinophilia, and expression of Th2 cytokine IL-13 in the BAL fluid were detected exclusively in OVA-allergic mice. Interestingly, oak dust exposure significantly downregulated Th2-type inflammation in allergic mice

by preventing the expression of IL-13 and reducing AHR to MCh. On the other hand, oak dust significantly suppressed the expression of the proinflammatory cytokine, TNF- α , as well as CCL3 chemokine levels in the lungs of allergic mice compared to nonallergic mice. Oak dust was observed to dramatically suppress pulmonary neutrophilia in the airways of allergic mice. Similarly, the level of CXCL2/3 mRNA in the lung tissue of allergic mice was reduced by oak dust exposure. Since CXCL2/3 is believed to act as a potent chemoattractant for neutrophils in mice (Van Den Steen *et al.*, 2003; Wuyts *et al.*, 1996), the observed reduction in its levels may be connected to the downregulation of neutrophil infiltration detected in the lungs of allergic mice after oak dust exposure. These results highlight how the immunological status of the mice critically affects the inflammatory outcome after exposure to oak dust. It can be speculated that anti-inflammatory Th2 response elicited by allergen sensitization may suppress the oak dust-induced proinflammatory response. At the same time, however, the proinflammatory response elicited by oak dust exposure may suppress the strength of the Th2 response.

It should be noted, however, that simultaneous exposure to particulate matter, e.g., fungal spores, during the allergen sensitization may also cause a marked enhancement of the inflammatory response instead of suppression. We reported recently, with the same experimental approach used as in the present study, a synergistic effect between the allergen sensitization and mold exposure (*Stachybotrys chartarum*), resulting

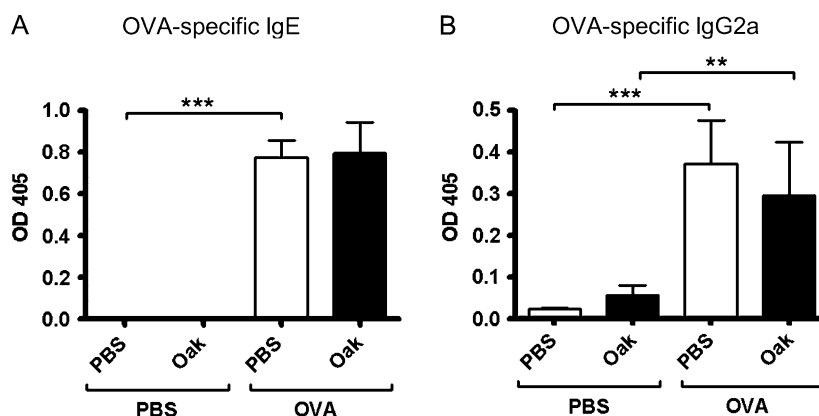


FIG. 5. (A) Ovalbumin-specific IgE and (B) IgG2a antibodies in serum measured by ELISA. Results are represented as absorbance units (405 nm). ** $p < 0.01$, *** $p < 0.001$.

in enhanced and qualitatively different granulomatous pulmonary inflammation as reflected by an increase in the levels of proinflammatory cytokines and chemokines (Leino *et al.*, 2006). In contrast to the results of the present study, secretions of TNF- α and CCL3 protein were drastically increased in the BAL fluid of allergic mice exposed to mold. It is likely that mold spores contain several pathogen-associated molecular patterns which can activate innate immunity, resulting in a marked enhancement of the inflammatory response in allergic mice. Our results suggest that exposure to oak dust without significant microbial contaminants may suppress rather than enhance allergic airway inflammation in allergic mice. It is also possible that the chemical components present in oak wood such as tannins and flavonoids (IARC, 1995) may participate in this process.

Oak dust exposure together with allergen sensitization, mimicking Th2-dominating immunity in atopic patients, did not seem to enhance allergic inflammation or AHR. In contrast, AHR to inhaled MCh was decreased in allergic mice exposed to oak. This may be due to the decreased levels of IL-13, which has been shown to play an important role in the development of AHR (Hershey, 2003). The mechanisms of IL-13-induced AHR is thought to be mediated partly by the combined actions of IL-13 on airway epithelial cells and smooth muscle cells, leading to mucus overproduction and airway obstruction. It is suggested that although IL-13 is able to redirect the recruitment of eosinophils into the airways, they are not required for induction of AHR. (Hershey, 2003; Wills-Karp and Chiaramonte, 2003). According to a recent study by Schlunssen *et al.* (2004), atopic woodworkers were more susceptible than nonatopic subjects for developing asthmatic responses. On the other hand, atopy was not reported as a risk factor in a follow-up study of 125 subjects with asthma caused by exposure to western red cedar dust (Chan-Yeung *et al.*, 1982). The discrepancies in these results may be attributable to many factors, such as concurrent exposure to varying amounts of other wood species or simultaneous exposure to other harmful inhalants such as airborne dust, endotoxins, formaldehyde, bacteria, or fungi that may be present

in varying concentrations (Alwis *et al.*, 1999; Douwes *et al.*, 2000; Mandryk *et al.*, 1999). The present results suggest that exposure to microbe-free oak dust may not increase Th2-associated airway inflammation and allergic AHR. However, we cannot exclude the possibility that long-term exposure to oak dust may elicit distinct effects compared to our short-term exposure protocol.

In conclusion, our study demonstrates in a murine model that oak dust exposure modulates airway inflammation and asthmatic response depending on the immunological status of the animal. To our knowledge, this is the first study to reveal a differential effect of wood dust exposure in allergic versus nonallergic mice. Our results mimic the real-life situation in which outcome of oak dust exposure may vary considerably depending on immunological status of the exposed individual, i.e., whether he/she is healthy or suffering from allergic asthma. It should be noted, however, that asthmatic symptoms are not always Th2 associated and therefore responses of oak dust exposure in patients with nonallergic asthma may differ from the present study. Further studies are needed to explore the responses of other wood dusts in the elicitation of pulmonary inflammation and AHR using different asthma models. In addition, the effects of long-term exposure to different wood dusts need to be investigated in detail.

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