

MECHANISMS OF PARTICLE-INDUCED PULMONARY INFLAMMATION IN A MOUSE MODEL: EXPOSURE TO WOOD DUST

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ABSTRACT

Repeated airway exposure to wood dust has long been known to cause adverse respiratory effects such as asthma and chronic bronchitis and impairment of lung function. However, the mechanisms underlying the inflammatory responses of the airways after wood dust exposure are poorly known. We used a mouse model to elucidate the mechanisms of particle-induced inflammatory responses to fine wood dust particles. BALB/c mice were exposed to intranasally administered fine (more than 99% of the particles had a particle size of $\leq 5 \mu\text{m}$, with virtually identical size distribution) birch or oak dusts twice a week for three weeks. PBS, LPS and titanium dioxide were used as controls. Intranasal installation of birch or oak dusts elicited influx of inflammatory cells to the lungs in mice. Enhancement of lymphocytes and neutrophils was seen after oak dust exposure, whereas eosinophil infiltration was higher after birch dust exposure. Infiltration of inflammatory cells was associated with an increase in the mRNA levels of several cytokines, chemokines, and chemokine receptors in lung tissue. Oak dust appeared to be a more potent inducer of these inflammatory mediators than birch dust. The results from our in vivo mouse model show that repeated airway exposure to wood dust can elicit lung inflammation which is accompanied by induction of several pro-inflammatory cytokines and chemokines. Oak and birch dusts exhibited quantitative and qualitative differences in the elicitation of pulmonary inflammation, suggesting that the inflammatory responses induced by the wood species may arise via different cellular mechanisms.

Key Words: *Chemokines; cytokines; gene expression; leukocytes*

INTRODUCTION

Wood dust, generated in the processing of wood, can induce several diseases of the respiratory track. Reported non-malignant diseases and symptoms associated with wood dust exposure include allergic rhinitis, chronic bronchitis, asthma, allergic alveolitis and organic dust toxic syndrome (ODTS) (Bohadana *et al.*, 2000; De Zotti and Gubian, 1996; Douwes *et al.*, 2001; Enarson and Chan-Yeung, 1990; Goldsmith and Shy, 1988). The evidence from epidemiological studies reveal that most wood species can induce occupational asthma (SCOEL, 2003). However, in some cases, the symptoms can be attributable, at least in part, to bacteria and fungi that can be abundant in wood dust especially in warm, humid conditions (Weber *et al.*, 1993).

Increase in blood eosinophilia as well as elevated prevalence of positive methacholine bronchial challenge have been reported in workers handling hard and soft woods (Bohadana *et al.*, 2000; De Zotti and Gubian, 1996; Gripenback *et al.*, 2005). Wintermeyer *et al.* detected increase in IL-8 level from bronchoalveolar lavage (BAL) fluid and an elevation in neutrophils percentage after exposure of healthy volunteers to wood chip mulch dust (Wintermeyer *et al.*, 1997). Also increased numbers of T-lymphocytes and eosinophils in BAL fluid have been reported in the airways when healthy people were exposed to pine dust (Gripenback *et al.*, 2005). Plicatic acid has been identified to be the causative agent of western red cedar asthma (Chan-Yeung, 1994). However, western red cedar asthma and the release of histamine induced by plicatic acid do not appear to be dependent on plicatic acid-specific IgE (immunoglobulin E) antibodies in most individuals (Frew *et al.*, 1993). Therefore, unidentified cell-dependent immunologic mechanisms have been suggested.

Despite a few reports in the literature, our knowledge of cellular mechanisms underlying wood dust-induced airway symptoms is surprisingly poor.

Our recent findings demonstrate that exposure to wood dust can modulate the expression of macrophage-derived cytokines and chemokines (Maatta *et al.*, 2005). Birch and oak dust exposure induced several pro-inflammatory cytokines and chemokines in mouse macrophage RAW 264.7 cell line cells. In the present study, we utilized a murine model to examine cellular mechanisms by which exposure to fine wood dust particles induces inflammatory responses in the airways.

MATERIALS AND METHODS

Study design

Due to the sparse literature data on the nature and molecular mechanisms of the airway inflammation associated with occupational wood dust exposure, we decided to investigate expression of a relatively wide range of cytokines, chemokines, and chemokine receptors. The overall aim of the study was to narrow down molecular events behind the inflammatory response, in order to generate more accurate hypotheses. To have an approach feasible to carry out in practice, and, simultaneously, sensitive enough to elucidate crucial changes in the expression levels, we examined gene expression as mRNA induction in the murine lungs. In our murine *in vivo* model, we investigated expression of major proinflammatory (TNF- α , IL1 β), Th2 (IL-4, IL-5, IL13), Th1 (IFN- γ , IL12p40) and regulatory (TGF- β 1) cytokines using real time PCR. These cytokine groups give information about immunological status of the airways regarding, for example, development of allergic response (Th2-cytokines), or imbalance of the regulatory system. To investigate further the mechanisms of pulmonary inflammation, we measured several chemokines (CCL2-4, CCL8, CCL11-12, CCL17, CCL20, CXCL2/3, CXCL5) and chemokine receptors (CCR1-5, CCR8, CXCR2) which critically regulate recruitment of distinct leukocyte subsets to the site of the inflammation (Murphy *et al.*, 2000; Onuffer and Horuk, 2002; Zlotnik and Yoshie, 2000). In addition, airway responses to inhaled methacholine were investigated in order to have functional information about the development of bronchial hyperreactivity. Finally, we measured antibody responses for receiving essential information about B-cell mediated immune reactions (IgE was studied as a surrogate marker for Th2, and IgG2a as a surrogate marker for Th1). Prior to these investigations, pulmonary inflammation was analyzed using histological techniques and differential cell counts in broncho-alveolar lavage samples.

Animals

Six- to 8-week old female BALB/c mice were purchased from Taconic M&B A/S (Ry, Denmark). All experiments were approved by the Social and Health Services of the Provincial Office of Southern Finland.

Wood dusts, TiO₂, and LPS

Two carefully prepared wood dusts, namely from oak (*Quercus alba*) and birch (*Betula pendula*), were used in the experiments. Physicochemical characteristics of the both wood materials are given in Table 1. The starting material for oak and birch dusts were industrially dried, knotless birch and oak board obtained from a carpenter company (Puusepäntiike Pöntinen KY, Lappeenranta, Finland). Wood dusts were generated using a band saw. The exhaust air of the saw was led to a vertical elutriator (diameter 1 m, height 3.5 m), where slow upward airflow separated the fine dust from chips and coarse particles. The coarse dust fell down and the fine dust was ducted from the upper part of the vertical elutriator. The size distribution of wood dust particles was monitored in real time with a laser particle monitor (Hiac/Royco 5000, Pacific Scientific, Silver Spring, MD) in the output duct of the vertical elutriator. More than 99% of birch and oak dust particles had a particle size $\leq 5 \mu\text{m}$ (Fig. 1). After the particle size monitoring, the wood dust was collected on membrane filters at the end of the output duct. The endotoxin concentrations were 400 pg/mg for birch dust and 140 pg/mg for oak dust (Limulus Amebocyte Lysate test). Titanium dioxide (TiO₂) powder (Aldrich Chem. Co., Milwaukee, WI), predominantly rutile, had a particle size less than 5 μm (scanning electron microscopy). LPS from *Shigella flexneri* serotype 1A (Sigma, St. Louis, MO) was dissolved in PBS and sterile-filtered before use.

Study protocol, measurement of airway hyperreactivity and collection of samples

Intranasal installation was selected as the route of exposure since it is widely used, easy to standardize and simple to perform. The wood dusts, TiO₂, LPS or PBS suspensions were administered intranasally under light anaesthesia using isoflurane (Abbott Laboratories Ltd, Queenborough, England) two times a week for three weeks. Mice received either 0.5 or 50 µg wood dust or TiO₂ in 50 µl of PBS into the nostrils. Control mice were given 50 µl PBS or LPS at the same concentration as LPS in birch dust (20 pg/50 µl). On day 19 the airway hyperreactivity to methacholine (Sigma-Aldrich Co, St.Louis, MO) was measured using whole-body plethysmography (Buxco Electronics, Inc., Sharon, CT) as previously described (Hamelmann *et al.*, 1997). The enhanced pause (PenH) values were averaged and expressed as the percentage of baseline PenH values (% baseline PenH).

The mice were then sacrificed with CO₂ and samples (blood, bronchoalveolar lavage (BAL) fluids and lung biopsies) collected. BAL samples were prepared by lavaging lungs once with 0.8 ml of PBS via trachea, cytocentrifugated (Shandon Scientific Ltd., Runcorn, UK), and stained with May-Grünwald-Giemsa. Cells were counted in 40 high-power fields at x 1000 under light microscopy (Nikon EclipseE800, Leica Microsystems GmbH, Wetzlar, Germany). The left lung was removed for RNA isolation, quick-frozen and kept at -70°C. For histological examination, the right lung was perfused with 10% formalin (500µl) and stored in formalin for 24 hours. Formalin-fixed lungs were embedded in paraffin and cut into 5µm-thick sections. The slides were stained with haematoxylin and eosin and the amount of lung inflammation was examined under light microscopy.

RNA isolation, reverse transcription and real-time quantitative polymerase chain reaction (PCR)

RNA isolation, cDNA synthesis and real-time quantitative PCR was performed as earlier described (Lehto *et al.*, 2003). The primers and probes used were obtained from Applied Biosystems as predeveloped reagents or they were self-designed (Maatta *et al.*, 2005). The primer and probe sequences for self-designed CCR3 and CCR4 were as follows: CCR3 forward primer 5'-TTG CAG GAC TGG CAG CAT T-3', CCR3 reverse primer 5'-TTC ATT CTT AGA GCA TGG AAA CGT T-3', CCR3 probe 5'-TGG AGA GTT TTC CTG CAG TCC TCG CTA TC-3', CCR4 forward primer 5'-TCA TGA CTT CCG TGA CGC TTT-3', CCR4 reverse primer 5'-GTT TTC TTC CTC AGA GCC CTG TT-3', and CCR4 probe 5'-TCG CCT TGT TTC AGT CAG GGT GCC-3'. The relative quantity was calculated by the comparative C_T method according to the manufacturer's instructions.

Measurement of serum antibodies

Serum levels of total IgE was measured by ELISA as previously described (Lehto *et al.*, 2003). The same ELISA protocol was used for total IgG2a measurement. Briefly, at first the plates were coated with rat anti-mouse IgG2a monoclonal antibodies (clone R11-89). After blocking samples were added and bound IgG2a was detected with biotin-conjugated rat anti-mouse IgG2a (clone R19-15). Streptavidin horseradish peroxidase followed by substrate was used to detect the bound antibody levels and the optical density was measured at 405 nm. Total IgG2a levels were calculated by comparing with mouse IgG2a (clone G155-178). The standard and antibodies for ELISA were purchased from BD Biosciences (San Diego, CA).

Statistical analysis

The data were analysed with the GraphPadPrism Software version 4.00 (GraphPad Software, Inc., San Diego, CA). Single-group comparisons were performed by the Mann–Whitney test.

The results are expressed as mean $\pm$ standard error of the mean (SEM). *P*-values <0.05 were considered to be statistically significant.

RESULTS

Wood dust instillation induces inflammatory cell infiltration to the lungs

Histological examination revealed modest but clear-cut changes in the lung morphology after the wood dust exposure (Fig. 2A). Eosinophil infiltration was dominating in mice exposed to higher concentration of birch dust (50 μ g/50 μ l) whereas exposure to higher concentration of oak dust caused an influx of neutrophils and lymphocytes into the lungs (Fig. 2A inserts). No detectable histological signs of lung inflammation were found after LPS control or TiO₂ (Fig. 2A) exposure or after exposure to lower wood dust concentration (0.5 μ g/50 μ l) (data not shown).

A clear influx of inflammatory cells into the BAL was observed after instillations of higher concentration of birch and oak dusts (Fig. 2B). In line with the histological findings, lymphocyte infiltration in BAL was significantly higher in mice exposed to oak dust when compared to mice exposed to birch dust. Similarly, oak dust exposure also induced higher infiltration of neutrophils compared birch dust although the difference did not reach statistical significance (Fig. 2B). On the other hand, birch dust caused a significantly higher influx of eosinophils into the BAL compared with oak dust. TiO₂ dust induced a statistically significant, but weak, increase in the amount of neutrophils compared to PBS-treated mice (Fig. 2B).

Microscopic analyses revealed that all particles tested (oak, birch and TiO₂) were readily internalized by alveolar macrophages (Fig. 3). There were no significant differences in the number of ingested particles after oak or birch dust administration (data not shown).

Cytokine expression is induced in wood dust -exposed lungs

Proinflammatory cytokines are produced by activated leukocytes to start a cascade of inflammatory reactions. As shown in the figure 4, there was a significant induction in the mRNA expression of pro-inflammatory cytokines IL-1 β and TNF- α in the mice lungs after repeated instillations of higher concentration of oak dust. The effect of birch dust was weaker. Birch dust induced significantly only TNF- α mRNA expression compared to PBS-control group. TiO₂ dust did not have any significant effect on IL-1 β or TNF- α mRNA expression (Fig. 4). The mRNA expression of regulatory cytokine TGF- β 1 was induced significantly in oak dust -exposed lungs (Fig. 4).

There was no significant increase in the mRNA expression of the major Th1 (IFN- γ and IL-12 p40) or Th2 cytokines (IL-4, IL-5, and IL-13) after exposure to either wood dust (data not shown). The lower dust concentration, LPS control or TiO₂ dust did not induce any of the cytokines studied (Fig 4 and data not shown).

Chemokines and their receptors are significantly induced in wood dust -exposed lungs

Chemokines are small cytokines that critically regulate the recruitment of leukocytes to the site of inflammation (Zlotnik and Yoshie, 2000). Expression of several chemokines was significantly induced in the lung tissue after wood dust instillations as shown in the figure 5. Compared with mice exposed to PBS only, CCL3, CCL17, and CXCL2/3 chemokines were induced significantly by both birch and oak dusts. Oak dust induced significantly also CCL2, CCL4, CCL8, CCL11, CCL12, CCL20, and CXCL5 chemokines. Compared to PBS-treated mice, TiO₂ induced significantly CCL3, CCL4, and CXCL2/3 chemokines. In general, the inducing effect of TiO₂ was weaker than the inducing effect of wood dusts. However, induction of CCL3 mRNA was comparable to TiO₂, birch and oak dust exposures.

The higher concentration of both birch and oak dust induced significantly the expression of CCR1, CCR2, and CCR5 chemokine receptors in the lungs (Fig. 6). Oak dust induced significantly also CCR3, CCR4, CCR8, and CXCR2 chemokine receptors (Fig. 6). TiO₂, the lower wood dust concentration or LPS did not induce significantly any of the chemokines and chemokine receptors studied.

Bronchial reactivity to inhaled methacholine and humoral response were not affected significantly after wood dust exposure

Methacholine-induced bronchial reactivity was not altered after exposures to wood dusts, LPS or TiO₂ (Fig.7). No significant increase in the levels of total IgE or total IgG2a was found after any exposure (data not shown).

DISCUSSION

Repeated airway exposure to wood dust has long been known to cause adverse respiratory effects and impairment of lung function (Demers *et al.*, 1997; Enarson and Chan-Yeung, 1990; Goldsmith and Shy, 1988). However, the mechanisms underlying the inflammatory responses of the airways after wood dust exposure are poorly known.

In the present study we used a murine model to investigate effects of repeated, intranasally administered, oak and birch dust exposures on lung functions and airway inflammation. Oak and birch dust were selected, since oak dust has been reported to induce several malignant and non-malignant diseases, whereas birch dust has appeared to be less hazardous (Klein *et al.*, 2001; Malo *et al.*, 1995; Mohtashamipur *et al.*, 1989; Nylander and Dement, 1993). *In vivo* experiments were performed using very fine birch and oak dusts (more than 99% of the particles had a particle size $\leq 5 \mu\text{m}$). In addition, particle size distribution of birch and oak dusts was virtually identical (see Fig. 1). Therefore, a considerable number of the wood dust particles, administered intranasally to mice under light anesthesia, were expected to reach the alveolar regions of the lungs. Indeed, the macrophages in the BAL contained plenty of wood dust or TiO_2 particles, depending of the exposure. Since alveolar macrophages account for up to 95% of cells in BAL in non-inflammatory conditions (Gordon and Read, 2002), these observations indicate that intranasally administered very fine wood dust (or TiO_2) reaches the alveolar region of the lungs.

Allergic asthma is characterized by infiltration of eosinophils and lymphocytes to the airways (Hamid *et al.*, 2003). Exposure to pinewood dust increases numbers of eosinophils, T cells and mast cells in the BAL fluids in healthy individuals (Gripenback *et al.*, 2005). In the

present study, we observed an increase in the number of macrophages, neutrophils, lymphocytes, and eosinophils in the tissue and BAL fluid after repeated birch or oak dust instillations. Lymphocytes and neutrophils dominated after oak dust exposures and eosinophils after birch dust exposures. Our results indicate that different wood species may induce differences in inflammatory responses although dust particle size distribution between the wood species is identical. TiO₂ dust did not induce macrophages, lymphocytes, or eosinophils, and induced only a weak increase in the neutrophils in the BAL fluid suggesting that the much stronger increase in the inflammatory cells after wood dust instillations is wood dust specific.

In order to study the cytokine milieu in wood dust-exposed lungs, we analyzed the expression of major pro-inflammatory, Th2 and Th1 cytokines. No significant changes in the expression of the Th1 or Th2 cytokines were observed by either of the wood dusts. On the contrary, repeated exposure to oak dust induced IL-1 β and TNF- α mRNA expression in lung tissue whereas birch dust exposure induced significantly only TNF- α mRNA expression. IL-1 β and TNF- α protein levels in the BAL were below the detection limit due to insufficient sensitivity of the ELISA assay (unpublished data). TNF- α has been postulated to be a critical mediator contributing to the airway inflammation in asthma since neutralization of TNF- α reduced the lung inflammation in a murine model of asthma induced by a house dust extract (Kim *et al.*, 2006). Moreover, induction of IL-1 β expression in the lungs of adult mice caused pulmonary inflammation characterized by neutrophil and macrophage infiltrates (Lappalainen *et al.*, 2005). Our findings are consistent with several studies that show that pro-inflammatory cytokines play a central role in the development of the lung inflammation induced by exposure to organic dust, mineral fibers, or fungal spores (Dorger and Krombach, 2002; Driscoll, 2000; Mansour and Levitz, 2002; Vanhee *et al.*, 1995; Yucesoy *et al.*, 2002).

Present results suggest that oak dust in particular has a major impact on the expression of pro-inflammatory cytokines and that this effect is not significantly affected by the interaction of resident lung cells with particles *per se*. Because both of the tested wood dusts had a practically identical size distribution profile and because their endotoxin content was so low that similar amount of LPS in the control mice did not induce any detectable response, the most likely explanation for the differences between oak and birch dust-induced cytokine expressions seems to be the chemical composition of these wood dusts. Further studies are needed to examine the specific role of various chemical components present in the wood dusts (Table 1) in the elicitation of inflammatory reactions demonstrated in the present study.

We observed that mRNA expression of regulatory cytokine TGF- β was increased in the mice exposed to oak dust. TGF- β has many anti-inflammatory and regulatory functions (Borish and Steinke, 2003; Cerwenka and Swain, 1999). Because it regulates the production of many extracellular matrix components and promotes fibrosis, the wood dust-induced upregulation of TGF- β production, observed in this study, might have some role in the development of cryptogenic fibrosing alveolitis that has been suggested to be induced by wood dust exposure (Hubbard, 2001; Hubbard *et al.*, 1996; Scott *et al.*, 1990; Tatrai *et al.*, 1995). TGF- β has also been reported to modulate extracellular matrix synthesis and fibronectin content in type II pulmonary epithelial cell cultures exposed to coal dust (Lee and Rannels, 1998). Moreover, the alveolar macrophages from the patients that have either chronic bronchitis or asthma release increased amounts of TGF- β and fibronectin - in asthma, however, to a lesser degree (Vignola *et al.*, 1996).

To investigate further the mechanisms of leukocyte infiltration in our murine model, we studied the expression profiles of several chemokines and their receptors in the lungs. In general, oak dust appears to be a stronger inducer of wide variety of chemokines compared to birch dust. TiO₂ induced significantly only CCL3, CCL4, and CXCL2/3 chemokines. The two nonpolarized chemokines, CCL3 and CCL4, are induced also in RAW 264.7 macrophages after TiO₂ dust exposure (Maatta *et al.*, 2005) suggesting that their expression is up-regulated when macrophages come into contact with any foreign particles. The up-regulation of chemokine expression after wood dust exposure was accompanied by the up-regulation of their specific receptors: CCR1 (the receptor of CCL3; present on various lymphocytes), CCR2 (the receptor of CCL2 and CCL12; present on various lymphocytes), CCR3 (the receptor of CCL5 and CCL11; present mainly on eosinophils and mast cells), CCR4 (the receptor of CCL17; present especially on Th2 cells), CCR5 (the receptor of CCL3 and CCL4; present especially on Th1 cells), and CXCR2 (the receptor of CXCL2/3 and CXCL5; present especially on neutrophils). These receptors are expressed on inflammatory cells (e.g. macrophages, neutrophils, Th2 and Th1 lymphocytes, and eosinophils (Murphy *et al.*, 2000; Onuffer and Horuk, 2002) which are also increased in the lung tissue and in BAL-fluid after wood dust instillations. It is of interest, that mRNA expression of CCL20, CCR4 and CCR8 was significantly higher after oak dust exposure compared to birch dust or TiO₂ exposure. CCL20 has been shown to be important in the recruitment of immature dendritic cells to the airways whereas CCR4 and CCR8 have been reported to be expressed especially on Th2 cells (Onuffer and Horuk, 2002; Zlotnik and Yoshie, 2000). Whether these mediators play an important role in the pathobiology of oak dust induced airways symptoms should be explored in the further studies.

Airway hyperresponsiveness and increased levels of Th2 cytokines in the lungs together with the production of IgE antibodies are characteristics of allergic asthma (Oettgen and Geha, 2001). However, bronchial responsiveness to inhaled methacholine was not altered significantly between different mice groups in the present study. Moreover, there was no significant increase in the expression of Th2 cytokines in the lungs and total IgE remain at the baseline level after wood dust exposure. Present findings therefore suggest, that rather than induction of Th2 dominated allergic response, wood dust exposure favor the development of pulmonary inflammation in which macrophages, neutrophils and nonpolarized lymphocytes as well as proinflammatory cytokines are playing central role. It is of interest; however, that exposure to birch dust elicited also recruitment of eosinophils into the airways, the phenomenon which is usually associated with allergic asthma and Th2 type inflammation. On the other hand, exposure to oak dust elicited selectively increased expression of Th2 associated chemokine receptors (CCR4 and CCR8). It should be noted, however, that in addition to expression on Th2 lymphocytes these chemokine receptors are also expressed on the surface of several other cells (Murphy *et al.*, 2000; Onuffer and Horuk, 2002; Zlotnik and Yoshie, 2000). Further studies are needed to examine whether longer exposure time with wood dust would induce development all classical signs of allergic asthma.

Taken together, our results shows that repeated airway exposure to wood dust can elicit lung inflammation which is accompanied by induction of several pro-inflammatory cytokines and chemokines in mice. Oak and birch dust exposure elicited quantitative and qualitative differences in pulmonary inflammation suggesting that different wood species may also have differences in elicitation of inflammatory responses. Further studies are needed to characterize and purify bioactive components from the wood dusts and examine their effects in the mouse model.

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REFERENCES

- Bohadana, A. B., Massin, N., Wild, P., Toamain, J. P., Engel, S., and Goutet, P. (2000). Symptoms, airway responsiveness, and exposure to dust in beech and oak wood workers. *Occup Environ Med* **57**, 268-273.
- Borish, L. C., and Steinke, J. W. (2003). 2. Cytokines and chemokines. *J Allergy Clin Immunol* **111**, S460-475.
- Cerwenka, A., and Swain, S. L. (1999). TGF-beta1: immunosuppressant and viability factor for T lymphocytes. *Microbes Infect* **1**, 1291-1296.
- Chan-Yeung, M. (1994). Mechanism of occupational asthma due to western red cedar (*Thuja plicata*). *Am J Ind Med* **25**, 13-18.
- De Zotti, R., and Gubian, F. (1996). Asthma and rhinitis in wooding workers. *Allergy Asthma Proc* **17**, 199-203.
- Demers, P. A., Teschke, K., and Kennedy, S. M. (1997). What to do about softwood? A review of respiratory effects and recommendations regarding exposure limits. *Am J Ind Med* **31**, 385-398.
- Dorger, M., and Krombach, F. (2002). Response of alveolar macrophages to inhaled particulates. *Eur Surg Res* **34**, 47-52.
- Douwes, J., McLean, D., Slater, T., and Pearce, N. (2001). Asthma and other respiratory symptoms in New Zealand pine processing sawmill workers. *Am J Ind Med* **39**, 608-615.
- Driscoll, K. E. (2000). TNFalpha and MIP-2: role in particle-induced inflammation and regulation by oxidative stress. *Toxicol Lett* **112-113**, 177-183.
- Enarson, D. A., and Chan-Yeung, M. (1990). Characterization of health effects of wood dust exposures. *Am J Ind Med* **17**, 33-38.

- Frew, A., Chan, H., Dryden, P., Salari, H., Lam, S., and Chan-Yeung, M. (1993). Immunologic studies of the mechanisms of occupational asthma caused by western red cedar. *J Allergy Clin Immunol* **92**, 466-478.
- Goldsmith, D. F., and Shy, C. M. (1988). Respiratory health effects from occupational exposure to wood dusts. *Scand J Work Environ Health* **14**, 1-15.
- Gordon, S. B., and Read, R. C. (2002). Macrophage defences against respiratory tract infections. *Br Med Bull* **61**, 45-61.
- Gripenback, S., Lundgren, L., Eklund, A., Liden, C., Skare, L., Tornling, G., and Grunewald, J. (2005). Accumulation of eosinophils and T-lymphocytes in the lungs after exposure to pinewood dust. *Eur Respir J* **25**, 118-124.
- Hamelmann, E., Schwarze, J., Takeda, K., Oshiba, A., Larsen, G. L., Irvin, C. G., and Gelfand, E. W. (1997). Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am J Respir Crit Care Med* **156**, 766-775.
- Hamid, Q., Tulic, M. K., Liu, M. C., and Moqbel, R. (2003). Inflammatory cells in asthma: mechanisms and implications for therapy. *J Allergy Clin Immunol* **111**, S5-S12; discussion S12-17.
- Hubbard, R. (2001). Occupational dust exposure and the aetiology of cryptogenic fibrosing alveolitis. *Eur Respir J Suppl* **32**, 119s-121s.
- Hubbard, R., Lewis, S., Richards, K., Johnston, I., and Britton, J. (1996). Occupational exposure to metal or wood dust and aetiology of cryptogenic fibrosing alveolitis. *Lancet* **347**, 284-289.
- Kim, J., McKinley, L., Natarajan, S., Bolgos, G. L., Siddiqui, J., Copeland, S., and Remick, D. G. (2006). Anti-tumor necrosis factor-alpha antibody treatment reduces pulmonary inflammation and methacholine hyper-responsiveness in a murine asthma model induced by house dust. *Clin Exp Allergy* **36**, 122-132.

- Klein, R. G., Schmezer, P., Amelung, F., Schroeder, H. G., Woeste, W., and Wolf, J. (2001). Carcinogenicity assays of wood dust and wood additives in rats exposed by long-term inhalation. *Int Arch Occup Environ Health* **74**, 109-118.
- Lappalainen, U., Whitsett, J. A., Wert, S. E., Tichelaar, J. W., and Bry, K. (2005). Interleukin-1 β causes pulmonary inflammation, emphysema, and airway remodeling in the adult murine lung. *Am J Respir Cell Mol Biol* **32**, 311-318.
- Lee, Y. C., and Rannels, D. E. (1998). Regulation of extracellular matrix synthesis by TNF- α and TGF- β 1 in type II cells exposed to coal dust. *Am J Physiol* **275**, L637-644.
- Lehto, M., Koivuluhta, M., Wang, G., Amghaiab, I., Majuri, M. L., Savolainen, K., Turjanmaa, K., Wolff, H., Reunala, T., Lauerma, A., Palosuo, T., and Alenius, H. (2003). Epicutaneous natural rubber latex sensitization induces T helper 2-type dermatitis and strong prohevein-specific IgE response. *J Invest Dermatol* **120**, 633-640.
- Maatta, J., Majuri, M. L., Luukkonen, R., Lauerma, A., Husgafvel-Pursiainen, K., Alenius, H., and Savolainen, K. (2005). Characterization of oak and birch dust-induced expression of cytokines and chemokines in mouse macrophage RAW 264.7 cells. *Toxicology* **215**, 25-36.
- Malo, J. L., Cartier, A., Desjardins, A., Van de Weyer, R., and Vandenplas, O. (1995). Occupational asthma caused by oak wood dust. *Chest* **108**, 856-858.
- Mansour, M. K., and Levitz, S. M. (2002). Interactions of fungi with phagocytes. *Curr Opin Microbiol* **5**, 359-365.
- Mohtashamipur, E., Norpoth, K., and Luhmann, F. (1989). Cancer epidemiology of woodworking. *J Cancer Res Clin Oncol* **115**, 503-515.
- Murphy, P. M., Baggiolini, M., Charo, I. F., Hebert, C. A., Horuk, R., Matsushima, K., Miller, L. H., Oppenheim, J. J., and Power, C. A. (2000). International union of

- pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol Rev* **52**, 145-176.
- Nylander, L. A., and Dement, J. M. (1993). Carcinogenic effects of wood dust: review and discussion. *Am J Ind Med* **24**, 619-647.
- Oettgen, H. C., and Geha, R. S. (2001). IgE regulation and roles in asthma pathogenesis. *J Allergy Clin Immunol* **107**, 429-440.
- Onuffer, J. J., and Horuk, R. (2002). Chemokines, chemokine receptors and small-molecule antagonists: recent developments. *Trends Pharmacol Sci* **23**, 459-467.
- Scott, J., Johnston, I., and Britton, J. (1990). What causes cryptogenic fibrosing alveolitis? A case-control study of environmental exposure to dust. *Bmj* **301**, 1015-1017.
- Tatrai, E., Adamis, Z., Bohm, U., Meretey, K., and Ungvary, G. (1995). Role of cellulose in wood dust-induced fibrosing alveo-bronchiolitis in rat. *J Appl Toxicol* **15**, 45-48.
- Vanhee, D., Gosset, P., Boitelle, A., Wallaert, B., and Tonnel, A. B. (1995). Cytokines and cytokine network in silicosis and coal workers' pneumoconiosis. *Eur Respir J* **8**, 834-842.
- Weber, S., Kullman, G., Petsonk, E., Jones, W. G., Olenchock, S., Sorenson, W., Parker, J., Marcelo-Baciu, R., Frazer, D., and Castranova, V. (1993). Organic dust exposures from compost handling: case presentation and respiratory exposure assessment. *Am J Ind Med* **24**, 365-374.
- Vignola, A. M., Chanez, P., Chiappara, G., Merendino, A., Zinnanti, E., Bousquet, J., Bellia, V., and Bonsignore, G. (1996). Release of transforming growth factor-beta (TGF-beta) and fibronectin by alveolar macrophages in airway diseases. *Clin Exp Immunol* **106**, 114-119.

- Wintermeyer, S. F., Kuschner, W. G., Wong, H., D'Alessandro, A., and Blanc, P. D. (1997). Pulmonary responses after wood chip mulch exposure. *J Occup Environ Med* **39**, 308-314.
- Yucesoy, B., Vallyathan, V., Landsittel, D. P., Simeonova, P., and Luster, M. I. (2002). Cytokine polymorphisms in silicosis and other pneumoconioses. *Mol Cell Biochem* **234-235**, 219-224.
- Zlotnik, A., and Yoshie, O. (2000). Chemokines: a new classification system and their role in immunity. *Immunity* **12**, 121-127.

FIGURE LEGENDS

Figure 1. Size distributions of wood dusts measured by optical particle monitoring.

Figure 2. (A) Representative micrographs of haematoxylin and eosin stained lungs (scale bar= 200 μ m). Eosinophilic infiltration appears after birch dust exposure whereas neutrophils and lymphocytes dominate after oak dust (scale bar= 20 μ m). (B) Inflammatory cells in BAL fluids 24 hrs after intranasal exposure. The columns and the error bars represent means $\pm$ SEMs. * $p<0.05$, ** $p<0.01$, *** $p<0.001$; n=8 mice per group.

Figure 3. Representative micrographs of BAL macrophages after exposure to PBS, TiO₂ or birch and oak dusts (50 μ g/50 μ l) (May Grünwald-Giemsa staining). Arrows point to dust particles or aggregates of several particles.

Figure 4. The mRNA expression of IL-1 β , TNF- α , and TGF- β in the lung tissue after repeated PBS, TiO₂, birch dust or oak dust (50 μ g/50 μ l) instillations are shown as relative quantity units (Rel.Quant.). The columns and the error bars represent means $\pm$ SEMs. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns = not significant, n= 8 mice per group.

Figure 5. The induction of chemokine mRNA expression in the lung after exposure to PBS, TiO₂, birch dust or oak dust (50 μ g/50 μ l) are shown as relative quantity units (Rel.Quant.). The columns and error bars represent means $\pm$ SEMs. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns = not significant, n=8 mice per group.

Figure 6. The induction of chemokine receptor mRNAs after exposure to PBS, TiO₂, birch dust or oak dust are expressed as relative quantity units (Rel.Quant.). The columns and error bars represent means $\pm$ SEMs. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant; n=8 mice per group.

Figure 7. Bronchial responsiveness in mice 24 h after the last exposure to PBS, TiO₂, birch dust or oak dust. The bronchial responsiveness was assessed as a change in the Penh value (% increase) after challenge with increasing amounts (mg/ml) of aerosolized methacholine. The columns and error bars represent means $\pm$ SEMs; n=8 mice per group.

TABLE 1.**Physical and chemical characteristics of wood materials**

	American White Oak <i>Quercus alba</i>	European Birch <i>Betula pendula</i>
Physical properties:		
Specific gravity, green, fresh (kg/dm ³)	1,0 ^{a, b}	
Specific gravity, air dry (kg/dm ³)	0,75 ^{a, b}	0,75 ^{a, b}
Specific gravity, humidity 15% (kg/dm ³)		0,64 ^{a, b}
Relative hardness (kg)	604 ^{a, b}	546 ^{a, b}
Crushing strength (max) (kg/cm ²)		574 ^{a, b}
Chemical properties:		
Identified toxic compounds in wood dust (%)	Tannins (3-13 %) ^c	
Identified toxic compounds in wood bark	Tannins (4-16 %) ^c	
Identified toxic extractive compounds (%)	Grandinin/roburin E, Vescalagin/castalagin, Gallic acid, Valoneic acid bilactone, Digalloyl	Betuloside pentoside, Catechin, Catechin pentoside, Platyphylloside, 5-

	glucose, Trigalloyl glucose, Monogalloyl glucose, Ellagic acid rhamnoside, Ellagic acid, Quercitrin ^d	Hydroxyplatyphyllone, Agyllone+pentose, Agyllone+hexose ^e Quinones, (1-4 %), Betulachrysoquinone ^c
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^aPuulajit. Internetsivusto, <http://www.webforest.fi/>. Webforest Ky, Espoo.

^bCD-ROM. The Wood Explorer. <http://www.thewoodexplorer.com/>. Timber Solution, Inc. USA.

^cHausen, B.M. (1981). Woods Injurious to Human Health. De Guyter, New York.

^dMämmelä, P., Savolainen, H., Lindroos, L., Kangas, J., and Vartiainen, K. (2000). Analysis of oak tannins by liquid chromatography-electrospray ionisation mass spectrometry. *Journal of Chromatogr A* **891**, 75-83.

^eMämmelä, P. (2001). Phenolics in selected European hardwood species by liquid chromatography-electrospray ionisation mass spectrometry. *Analyst* **126**, 1535-1538.

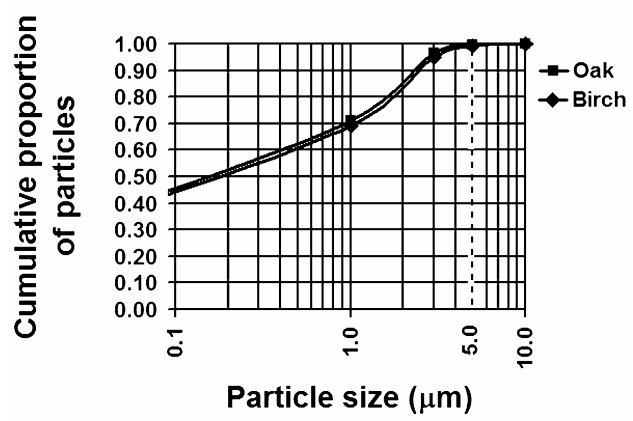


Figure 1

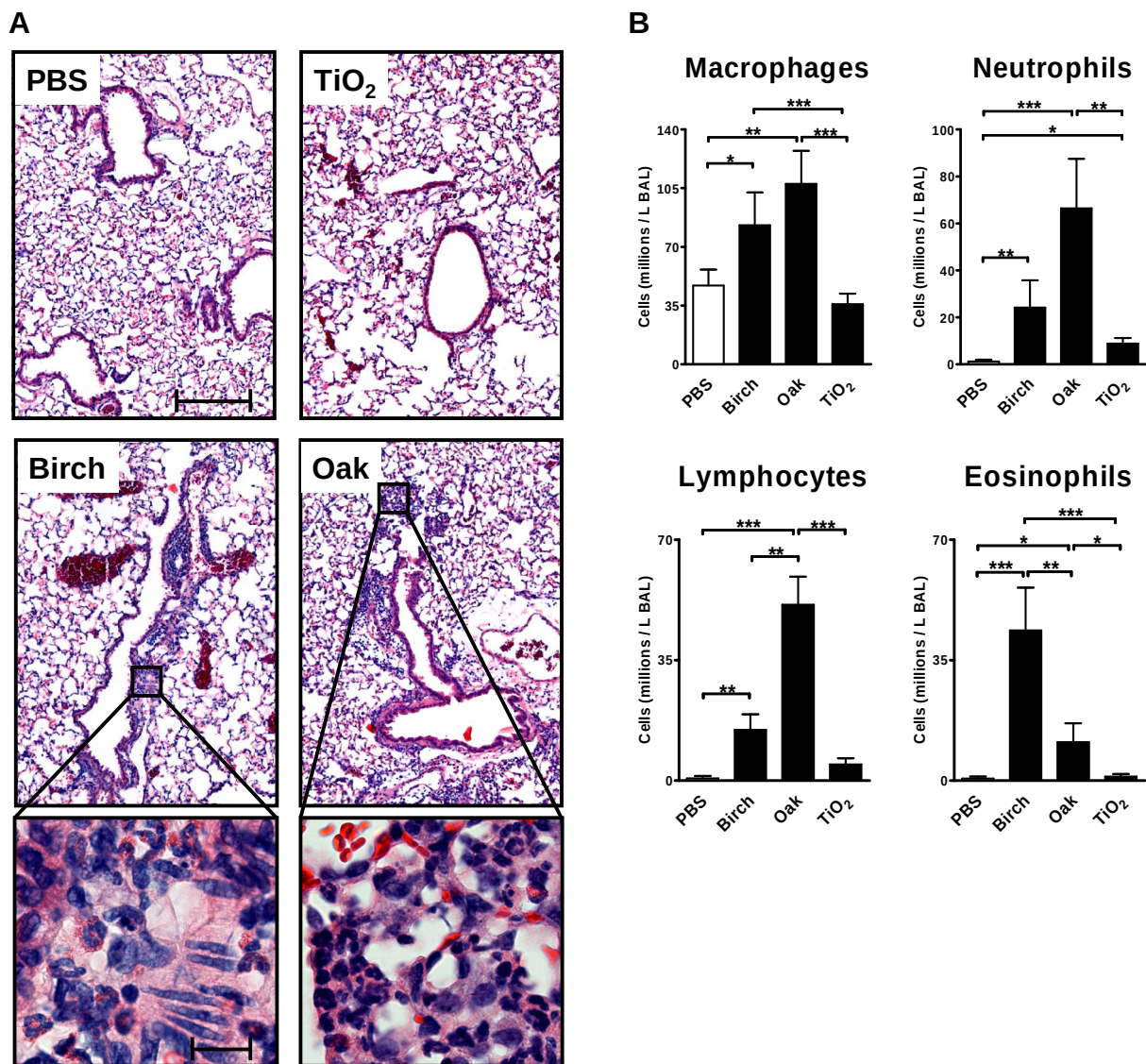


Figure 2

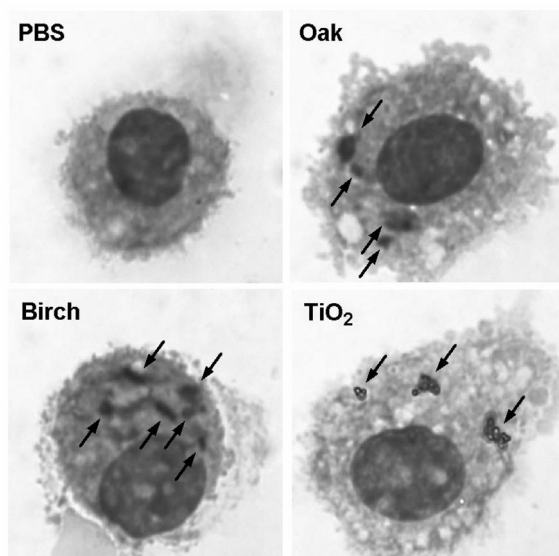


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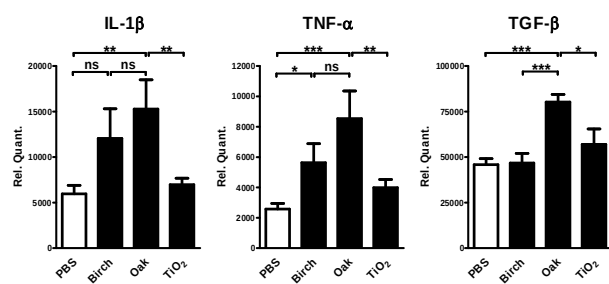


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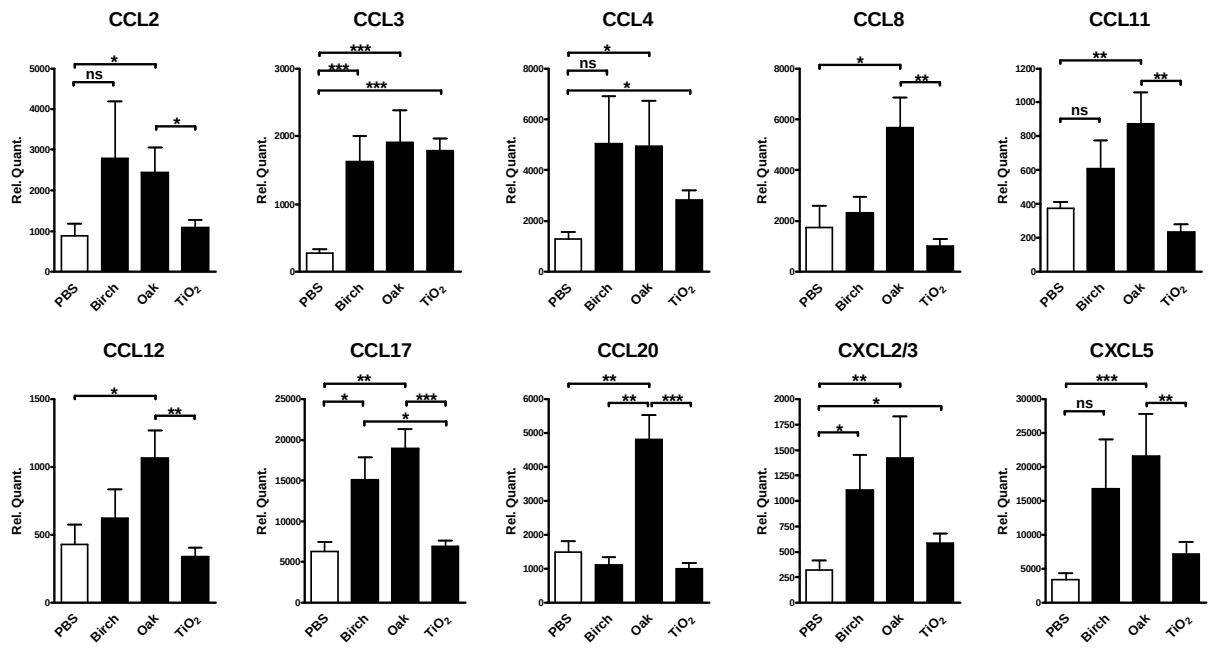


Figure 5

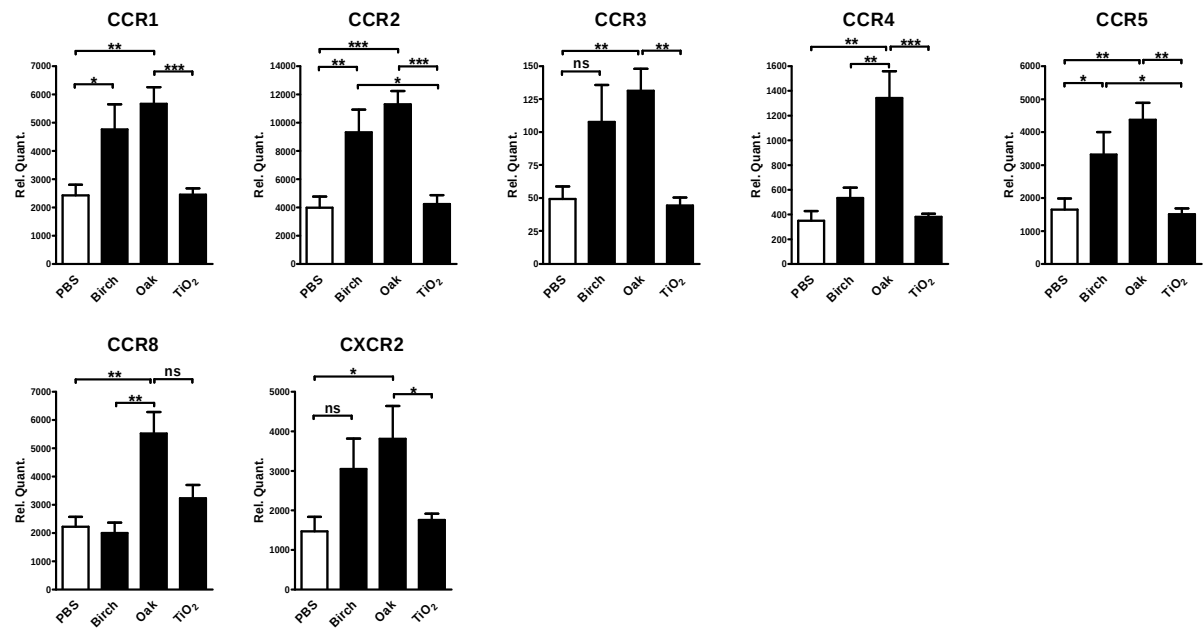


Figure 6

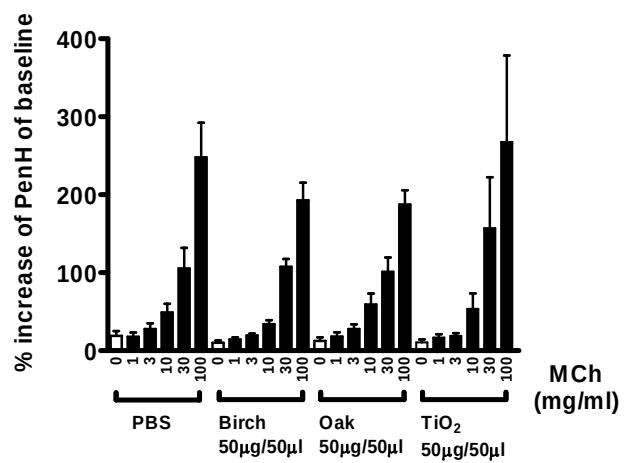


Figure 7