

Characterization of oak and birch dust-induced expression of cytokines and chemokines in mouse macrophage RAW 264.7 cells

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Abstract

Occupational exposure to wood dust is related to several respiratory diseases, such as allergic rhinitis, chronic bronchitis, and asthma. However, virtually nothing is known about molecular mechanisms behind wood dust-induced pulmonary inflammation. To elucidate the effects of wood dust exposure on cytokine and chemokine expression in murine macrophage cell line cells, mouse RAW 264.7 cells were exposed to two selected hardwood dusts, oak and birch. TiO₂ and LPS were used as controls. Expression patterns of several cytokines, chemokines, and chemokine receptors were assessed by real-time quantitative PCR system and by ELISA. Exposure to birch dust caused a major increase in TNF- α and IL-6 protein levels whereas a weaker induction of TNF- α protein was found after exposure to oak dust. Inorganic TiO₂ dust did not induce significant cytokine expression. With respect to the chemokines, a dose-dependent, about 10-fold induction of CCL2 mRNA and protein was found after exposure to birch dust. Oak dust induced weakly CCL2 protein. Similarly, birch dust induced a strong expression of CCL3, CCL4, and CXCL2/3 mRNA whereas only moderate levels of these chemokine mRNAs were detected after oak dust exposure. In contrast, expression of CCL24 mRNA was inhibited by more than 40-fold by both oak and birch dusts. TiO₂ dust induced about five-fold expression of CCL3 and CCL4 mRNA but did not affect significantly other chemokines. These results suggest that exposure to birch or oak dusts may influence the development of the inflammatory process in the airways by modulating the expression of macrophage-derived cytokines and chemokines.

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Keywords: Wood dust; Macrophage; Inflammation; Cytokine; Chemokine; Gene-expression

1. Introduction

Many individuals come into contact with wood dust due to worldwide use of wood as a fuel and construction material (Demers et al., 1997). It has been estimated that approximately 2.6 million workers in the European Union (EU15) are exposed annually to wood

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dust (Kauppinen et al., 2000). Repeated exposure to airborne wood dust particles has long been associated with an increased risk of many adverse health effects, including respiratory effects, skin symptoms, and even cancer (Goldsmith and Shy, 1988; Enarson and Chan-Yeung, 1990; Flechsig and Nedo, 1990; Demers et al., 1995, 1997; Estlander et al., 2001). The most frequently reported respiratory symptoms include asthma (Chan-Yeung, 1994; Malo et al., 1995; Quirce et al., 2000; Higuero et al., 2001), allergic rhinitis (Hytönen et al., 1997), and chronic bronchitis (Hessel et al., 1995; Li et al., 1990; Mandryk et al., 1999; Zock et al., 2001). Epidemiological studies have shown remarkably increased risk for sino-nasal adenocarcinoma among workers exposed to wood dust (Demers et al., 1995). Indeed, wood dust has been classified by the International Agency for Research on Cancer (IARC) as being carcinogenic to humans (IARC, 1995).

According to the current literature, hardwoods (angiosperms) seem to be more hazardous to human health than softwoods (gymnosperms). The highest risk for sino-nasal cancer has been among workers exposed mainly to hardwood dusts (IARC, 1995). The most common species identified in the studies on cancer have been beech and oak (Mohtashamipur et al., 1989; Nylander and Dement, 1993; Wolf et al., 1998; Klein et al., 2001). The link between softwood dusts and sino-nasal cancer is less evident. However, it has been reported that exposure to softwood dust can cause irritation, bronchial hyperresponsiveness, and airway inflammation (Demers et al., 1997).

The first line defense against airway exposure to organic dust, mineral fibers, or fungal spores is mounted primarily by alveolar macrophages (Castranova and Vallyathan, 2000; Dörger and Krombach, 2002; Gordon and Read, 2002). These professional phagocytes ingest and destroy intruding pathogens but also secrete a variety of cytokines and chemokines that are involved in the development and maintenance of inflammatory response (Borish and Steinke, 2003). A number of studies have shown that inhalation of bioaerosols results in the activation of macrophages leading to secretion of various inflammatory mediators and finally to recruitment of leukocytes to the site of the inflammation (Vanhée et al., 1995; Dörger and Krombach, 2002; Mansour and Levitz, 2002; Yucesoy et al., 2002). At present, virtually nothing is known about the molecular and cellular mechanisms behind the airway inflammation induced by wood dusts.

In the present study, we developed an in vitro model to elucidate the effects of wood dust exposure on cytokine and chemokine expression in murine macrophage cell line RAW 264.7 using two selected hardwoods, oak

and birch. Oak dust was selected because of its adverse health effects, especially because of its carcinogenicity, and birch dust because there is a lack of evidence for any carcinogenicity associated with birch dust in the current literature (Mohtashamipur et al., 1989; Nylander and Dement, 1993; Wolf et al., 1998; Klein et al., 2001). Both oak and birch are widely used, and both species are of huge economical importance. Inorganic TiO₂ dust was used as control to study whether particles per se could induce cytokine and chemokine expression in RAW 264.7 cells. In addition, low lipopolysaccharide (LPS) concentrations (10 and 100 pg/ml) were used as a negative control to make sure that the trace amounts of endotoxins detected from the wood dusts could not induce any significant cytokine and chemokine expression by themselves.

2. Materials and methods

2.1. Wood dust, TiO₂, and LPS

Oak (*Quercus robur*) and birch (*Betula pendula*) dust was produced using a dust collecting face-grinding machine with 400-grit sanding paper.

For particle size distribution analyses, wood dust and TiO₂ dust specimens, gold-coated for 170 s with BAL-TEC SCD 005 Sputter Coater (BAL-TEC AG, Liechtenstein), were examined on a JEOL JSM-6400 scanning electron microscope (JEOL Inc., Peabody, MA) at an acceleration voltage of 20 kV. More than 1000 particles for each dust were analyzed from electron micrographs. The particle size and shape distribution was similar in both wood dust examined (Fig. 1). More than 93% of both oak and birch dust particles had diameters less than 5 µm (Fig. 1). TiO₂ powder (Aldrich Chem. Co., Milwaukee, WI), predominantly rutile, had a particle size less than 5 µm (Fig. 1).

The wood dusts were tested for endotoxins with a kinetic limulus amebocyte lysate (LAL) test. The endotoxin concentrations were 70 pg/mg for oak dust and 220 pg/mg for birch dust. Lipopolysaccharides (LPS) from *Shigella flexneri* serotype 1A were purchased from Sigma (St. Louis, MO) and were sterile-filtered after dissolving them in PBS.

2.2. Cell culture

RAW 264.7 cells were grown at 37 °C in 5% CO₂ in RPMI supplemented with 2 mM L-glutamine, 100 U of penicillin, 100 U of streptomycin sulfate per ml, and 10% (v/v) fetal bovine serum (Gibco/Invitrogen, Carlsbad, CA).

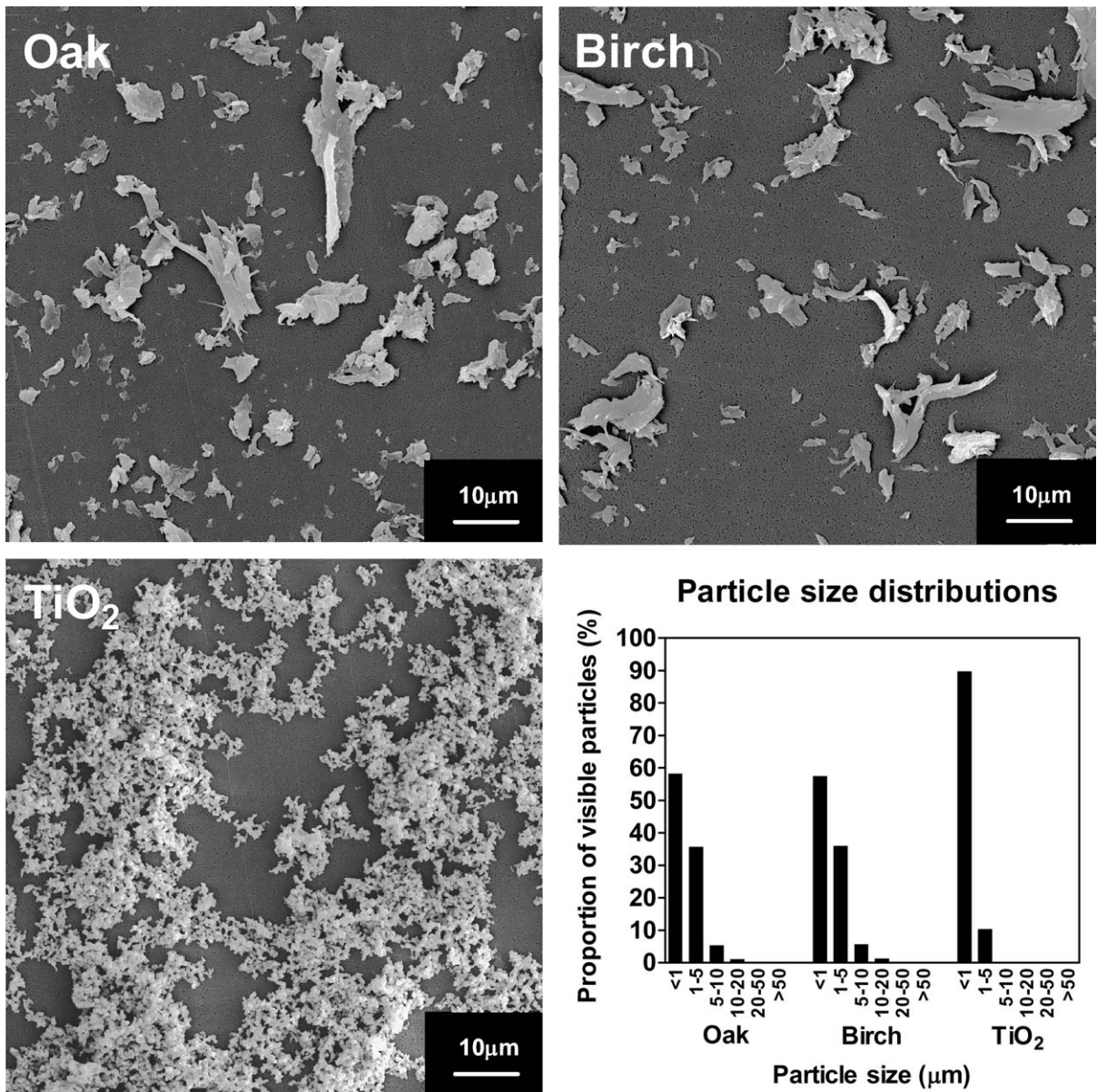


Fig. 1. Scanning electron micrographs of oak, birch, and TiO₂ dusts used in the present study. For particle size distributions, more than 1000 particles for each dust were analyzed from electron micrographs.

2.3. Wood dust, TiO₂, and LPS exposure

Wood dust or TiO₂ was suspended in PBS to prepare a series of dilutions immediately before the exposure. These PBS suspensions were further diluted by adding nine parts of cell culture medium to one part of PBS suspension. The cell culture medium was removed from the RAW 264.7 cells and 5 ml of dust suspension was added to the cells at the wood dust concentra-

tions of 0 (PBS control), 10, 30, 100, or 300 μg/ml. Cells (about 10 million cells per a 25 cm² cell culture flask) were incubated at 37 °C in 5% CO₂ for 2, 6, 24, or 48 h. At the end of the incubation period, the cells were collected and the cell culture supernatants were stored at –20 °C for ELISA analyses. Similarly prepared LPS solution was added to the cells at the LPS concentrations of 0 (PBS control), 10, or 100 pg/ml.

2.4. RNA isolation and cDNA synthesis

After collection, the cells were lysed using total RNA isolation (TRIzol) reagent (Gibco/Invitrogen, Carlsbad, CA). Total RNA was isolated according to the manufacturer's instructions. The RNA samples were then treated with RNase-free DNase (Roche, Branchburg, NJ) to remove residual DNA. One microgram of RNA per sample was reverse-transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Each reverse transcription reaction was performed in a total volume of 50 μ l following the manufacturer's instruction.

2.5. Real-time PCR

PCR primers and fluorogenic probes for murine IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-13, INF- γ , TNF- α , CCL1/TCA-3, CCL2/MCP-1, CCL3/MIP-1 α , CCL5/RANTES, CXCL2/3/MIP-2, CXCL5/LIX, CXCL9/Mig, and CXCL12/SDF-1 were purchased from Applied Biosystems (Foster City, CA). PCR primers and fluorogenic probes for murine TGF- β , CCL4/MIP-1 β , CCL8/MCP-2, CCL11/eotaxin-1, CCL12/MCP-5, CCL17/TARC, CCL20/MIP-3 α , CCL24/eotaxin-2, CCL27/CTACK, CCR1, CCR2, CCR5, and CCR8 (Table 1) were designed using the computer program Primer Express 2.0 (Applied Biosystems) and were purchased from Applied Biosystems. Whenever possible, primer pairs were designed to span exon–exon borders. The probes were 5' end-labelled with 6-carboxyfluorescein (FAM). Each PCR amplification was performed in a total volume of 25 μ l, containing 10.25 μ l RNase-free water, 12.5 μ l 2 $\times$ TaqMan Universal PCR Master Mix (Applied Biosystems), 1.25 μ l 20 $\times$ commercial target primers and probe or self-designed primers at the concentration of 300 nM and probe at the concentration of 200 nM, and 1 μ l cDNA sample (20 ng). PCR reactions were performed with an ABI PRISM 7700 Sequence Detection System (TaqMan; Applied Biosystems) in a 96-well microtiter plate format using the following conditions: 2 min at 50 $^{\circ}$ C and 10 min at 94 $^{\circ}$ C, followed by a total of 40 cycles of 95 $^{\circ}$ C for 15 s and 60 $^{\circ}$ C for 1 min. The PCR product formation was continuously monitored during the PCR reaction using Sequence Detection System software version 1.7 (Applied Biosystems). The FAM signal was standardized to the internal passive reference ROX to exclude non-PCR related fluctuations in the fluorescence and to the endogenous reference rRNA 18S to normalize the quantification of a mRNA target for differences in total RNA. The primers and the VIC-labelled probe for rRNA

18S synthesis were purchased from Applied Biosystems. Finally, the expression levels of cytokines, chemokines, or chemokine receptors in the exposed cells were compared to the expression levels of the same cytokines, chemokines, or chemokine receptors in the unexposed control cells at each collection time point using the comparative C_T -method (Applied Biosystems, 1997; Johnson et al., 2000). In this method, the threshold cycle (C_T) was the PCR cycle at which the amplification plot passed 10 standard deviations above the mean of baseline emission, representing the background level. We determined the C_T values for ten interesting cytokines or chemokines in unexposed control cells collected at the beginning of the experiment (0 h). These C_T values were the following: IL-1 β 30.3 $\pm$ 0.4, IL-6 34.1 $\pm$ 0.7, IL-10 34.8 $\pm$ 0.5, TNF- α 19.9 $\pm$ 0.3, CCL2 24.9 $\pm$ 0.3, CCL3 20.5 $\pm$ 0.3, CCL4 23.2 $\pm$ 0.4, CCL5 34.7 $\pm$ 0.1, CCL24 29.8 $\pm$ 0.4, and CXCL2/3 30.18 $\pm$ 0.2 (mean $\pm$ S.E.M., n = 5). However, to compare the expression of exposed cells to the expression of unexposed cells, this automatically set threshold was manually adjusted to the region of exponential amplification across all of the amplification plots, as described by the manufacturer. The relative quantity of a target mRNA, normalized to the endogenous reference rRNA 18S and to a relevant unexposed control, was given by the equation: relative quantity = $2^{-\Delta\Delta C_T}$, whereas $\Delta\Delta C_T$ = $\Delta C_T - \Delta C_{T_{\text{unexposed control}}}$ and ΔC_T = $C_T - C_{T_{\text{unexposed control}}}$. These results were expressed as fold differences compared to a relevant unexposed control at each collection time point.

2.6. ELISA

The cell culture supernatants were collected and analyzed for TNF- α , IL-6, and CCL2 proteins using commercial ELISA kits Mouse TNF α Module set (Bender MedSystems, Vienna, Austria), Mouse IL-6 ELISA Ready-SET-Go! (eBioscience, San Diego, CA), and OptEIA Mouse MCP-1 Set (BD Biosciences, San Diego, CA). The protocols provided by the manufacturers were followed to the detail. The data were acquired using an automated ELISA reader Multiskan MS with Multiskan Transmit Program version 1.3 (Labsystems, Helsinki, Finland). The concentration of TNF- α , IL-6, and CCL2 for each sample was deduced from the relevant standard curves. All samples were analyzed in duplicate.

2.7. Measurement of cell viability

The effect of different treatments on cell survival was determined by counting the viable cells by means of a

Table 1
Sequences of self-designed primer pairs and fluorogenic probes for real-time PCR

TGF- β	Forward	5'-CAA GGG CTA CCA TGC CAA CTT-3'
	Reverse	5'-ATG GGC AGT GGC TCC AAA G-3'
	Probe	5'-CAC ACA GTA CAG CAA GGT CCT TGC CCT CT-3'
CCL4	Forward	5'-TGC TCG TGG CTG CCT TCT-3'
	Reverse	5'-CAG GAA GTG GGA GGG TCA GA-3'
	Probe	5'-TGC TCC AGG GTT CTC AGC ACC AAT G-3'
CCL8	Forward	5'-CCC TTC GGG TGC TGA AAA G-3'
	Reverse	5'-TCT GGA AAA CCA CAG CTT CCA-3'
	Probe	5'-TAC GAG AGA ATC AAC AAT ATC CAG TGC CCC-3'
CCL11	Forward	5'-ATG CAC CCT GAA AGC CAT AGT C-3'
	Reverse	5'-CAG GTG CTT TGT GGC ATC CT-3'
	Probe	5'-AGC ACA GAT CTC TTT GCC CAA CCT GGT-3'
CCL12	Forward	5'-CCG GGA AGC TGT GAT CTT CA-3'
	Reverse	5'-TGG AAT TCT TAA CCC ACT TCT CCT T-3'
	Probe	5'-ACC ATA CTG GAT AAG GAG ATC TGT GCT GAC CC-3'
CCL17	Forward	5'-CAG GAA GTT GGT GAG CTG GTA TAA G-3'
	Reverse	5'-TGG CCT TCT TCA CAT GTT TGT CT-3'
	Probe	5'-TGT CCA GGG CAA GCT CAT CTG TGC-3'
CCL20	Forward	5'-GGC AGA AGC AAG CAA CTA CGA-3'
	Reverse	5'-CCC ACA ATA GCT CTG GAA GGA A-3'
	Probe	5'-TGT TGC CTC TCG TAC ATA CAG ACG CCA-3'
CCL24	Forward	5'-CGG CCT CCT TCT CCT GGT A-3'
	Reverse	5'-TGG CCA ACT GGT AGC TAA CCA-3'
	Probe	5'-CCC TCA TCT TGC TGC ACG TCC TTT ATT TC-3'
CCL27	Forward	5'-CAG CCT CCC GCT GTT ACT G-3'
	Reverse	5'-TGC TTG GGA GTG GCT GTC TA-3'
	Probe	5'-TCT GCC CTC CAG CAC TAG CTG CTG TAC-3'
CCR1	Forward	5'-AGG CCC AGT GGG AGT TCA C-3'
	Reverse	5'-TCC ACT GCT TCA GGC TCT TGT-3'
	Probe	5'-CAC CGT ACC TGT AGC CCT CAT TTC CCC-3'
CCR2	Forward	5'-CCT TGG GAA TGA GTA ACT GTG TGA-3'
	Reverse	5'-TGT GTC ATT CCA AGA GTC TCT GTC A-3'
	Probe	5'-TGA CAA GCA CTT AGA CCA GGC CAT GCA-3'
CCR5	Forward	5'-TTG CAA ACG GTG TTC AAT TTT C-3'
	Reverse	5'-TCT CCT GTG GAT CGG GTA TAG AC-3'
	Probe	5'-AGC AAG ACA ATC CTG ATC GTG CAA GCT C-3'
CCR8	Forward	5'-CCA TTC AAC GTG GCC CTT T-3'
	Reverse	5'-GCC TCG TGG CAC ATC CA-3'
	Probe	5'-CGT CCC TGC ACG ACC TGC ACA TC-3'

hemocytometer and the trypan blue dye exclusion test following the instruction of the manufacturer of the dye (Sigma, St. Louis, MO).

2.8. Statistical analysis

General linear models (ANOVA) were used to examine whether the effects of wood dusts on cytokine or chemokine expression are dependent of the dose of wood dust and whether the effects induced by oak or birch dusts

differ significantly from each others. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Cytokine expression

Exposure of RAW 264.7 cells to birch dust caused a dose-dependent increase in IL-6 and TNF- α mRNA and protein levels compared to the unexposed control

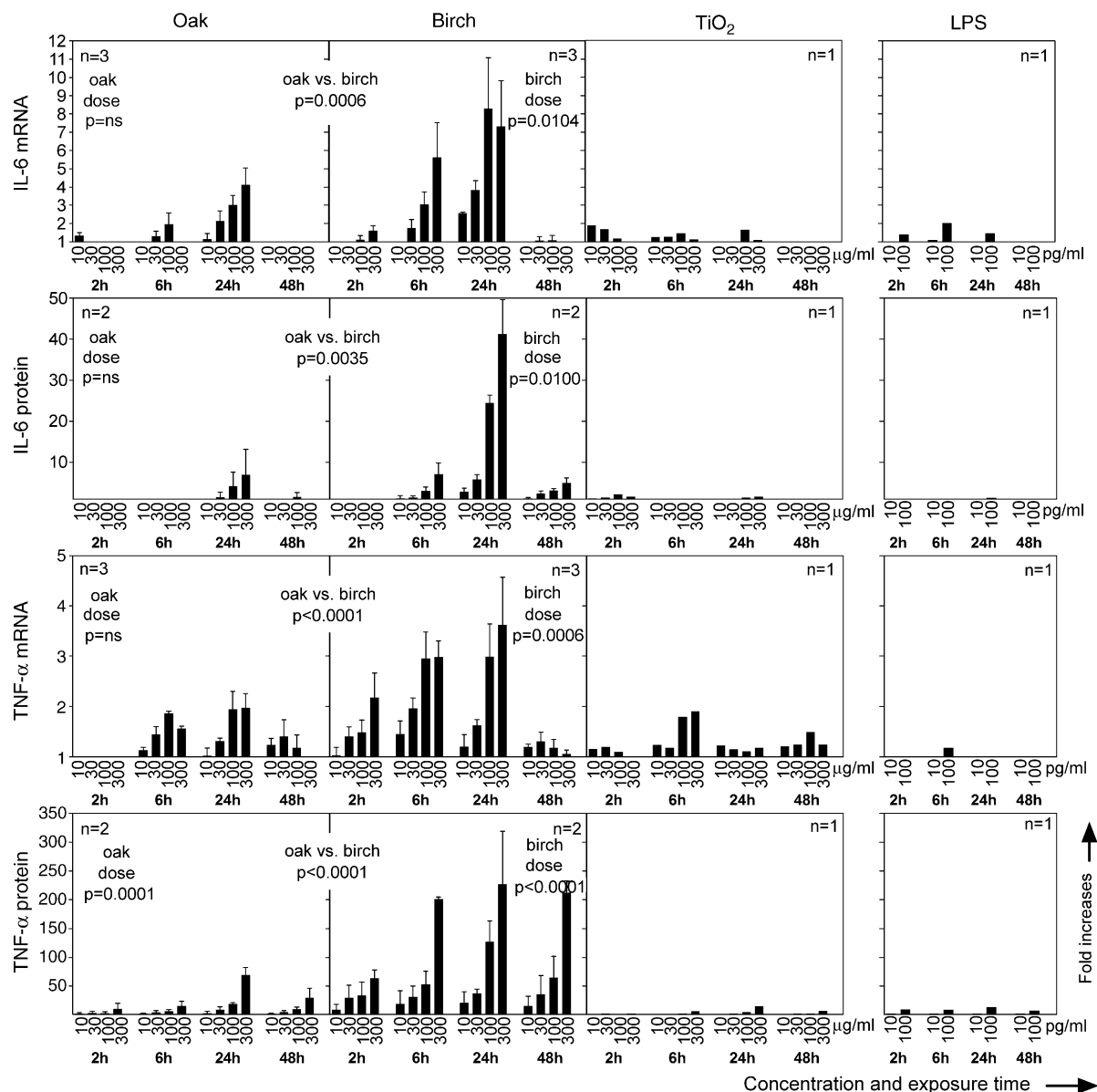


Fig. 2. IL-6 and TNF- α mRNA and protein produced by mouse macrophage RAW 267.4 cells exposed to oak, birch, or TiO₂ dust (10, 30, 100, or 300 μg/ml) or LPS (10 or 100 pg/ml). The samples of cell culture media were collected 2, 6, 24, or 48 h after the beginning of the exposure and analyzed by ELISA. The IL-6 or TNF- α mRNA expression of exposed cells was compared to the expression of unexposed cells at each time point by real-time PCR. The results are expressed as fold differences to the expression level of unexposed cells. The bars represent mean $\pm$ S.E.M. of three experiments (oak or birch dust-induced mRNA expression), or a single experiment (TiO₂ and LPS). The results of the statistical analysis show whether the effects induced by wood dusts are dependent of the dose of wood dust (named "oak dose" or "birch dose") and whether the effects induced by oak or birch dusts differ significantly from each others (named "oak vs. birch"). Statistical significance was defined as $p < 0.05$. ns = not significant.

cells. A maximal, 41-fold induction of IL-6 protein and 227-fold induction of TNF- α protein, was reached at 24 h after exposure (Fig. 2). According to the statistical analysis, the expression of IL-6 and TNF- α mRNA and protein was significantly higher after birch dust exposure

than after oak dust exposure (Fig. 2). Exposure of RAW 264.7 cells to oak dust did not induce significantly IL-6 mRNA and protein or TNF- α mRNA and caused a much weaker increase in TNF- α protein compared with birch dust (Fig. 2). TiO₂ and the low doses of LPS (10 or

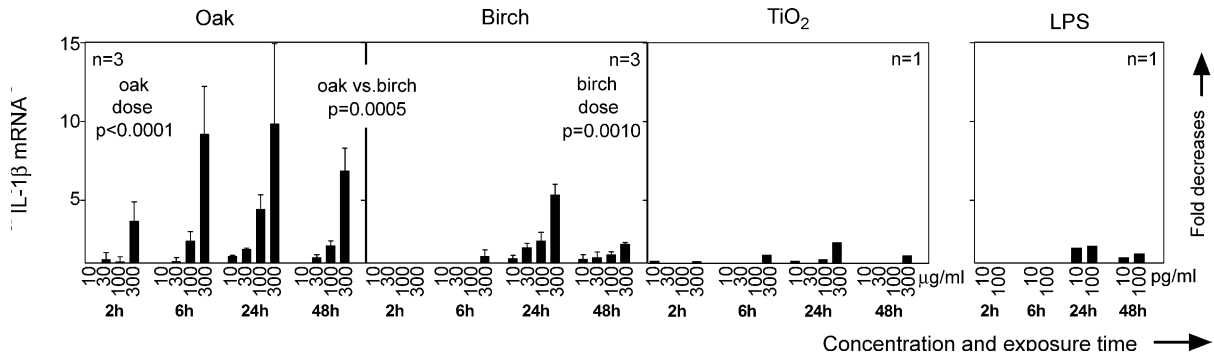


Fig. 3. The IL-1 β mRNA expression profile of RAW 264.7 cells after exposure to oak, birch, or TiO₂ dust, or low LPS doses. The IL-1 β mRNA expression of exposed cells was compared to the expression of unexposed cells at each time point by real-time PCR. Note that the results are shown as fold decrease in expression. The bars represent mean $\pm$ S.E.M. of three experiments (oak and birch) or a single experiment (TiO₂ and LPS). The results of the statistical analysis show whether the effects induced by wood dusts are dependent of the dose of wood dust (named "oak dose" or "birch dose") and whether the effects induced by oak or birch dusts differ significantly from each others (named "oak vs. birch"). Statistical significance was defined as $p < 0.05$.

100 pg/ml), which both served as negative controls, did not induce any significant expression of IL-6 or TNF- α (Fig. 2).

Exposure to oak dust caused a dose-dependent, 10-fold inhibition in IL-1 β mRNA expression at 24 h, and exposure to birch dust a five-fold inhibition at 24 h after exposure (Fig. 3). The inhibition in IL-1 β expression caused by oak dust was significantly stronger than the inhibition caused by birch dust (Fig. 3). The negative controls, TiO₂ and the low doses of LPS (10 or 100 pg/ml), did not have any significant effect on IL-1 β mRNA expression (Fig. 3).

There was no significant mRNA expression of the major Th1 (IFN- γ , IL-2, and IL-12 p40) or Th2 cytokines (IL-4, IL-5, and IL-13) after exposure to either wood dust (data not shown). The baseline expression of regulatory cytokines IL-10 and TGF- β mRNA was not affected significantly by birch or oak dust exposure (data not shown).

3.2. Chemokine expression

A strong and dose-dependent induction of CCL2 mRNA and protein was found after exposure to graded doses of birch dust. A maximal eight-fold induction of mRNA expression was reached at 6 h after exposure whereas maximal 18-fold expression of CCL2 protein was reached at 48 h (Fig. 4). CCL2 mRNA was not significantly induced and only a borderline induction of CCL2 protein was found after oak dust or TiO₂ dust exposure (Fig. 4). According to the statistical analysis, birch dust induced significantly higher levels of CCL2 mRNA and protein than oak dust (Fig. 4). Low doses of

LPS (10 or 100 pg/ml) had no significant effect on CCL2 expression (Fig. 4).

Birch dust exposure induced a dose-dependent expression of CCL3, CCL4, and CXCL2/3 mRNA. Maximal over 13-fold expression of CCL3 and CCL4 was reached at 6 h after exposure and 32-fold expression of CXCL2/3 at 2 h after exposure (Fig. 5). Exposure to both oak dust and TiO₂ elicited an approximately five-fold induction of CCL3 and CCL4 mRNA at 6 h after exposure (Fig. 5). CXCL2/3 mRNA was induced by seven-fold by oak dust at 24 h after exposure, whereas TiO₂ did not induce any significant increase in CXCL2/3 mRNA (Fig. 5). Low doses of LPS (10 or 100 pg/ml) did not induce these chemokines (Fig. 5). In each case, the expression caused by birch dust was significantly stronger than the expression caused by oak dust (Fig. 5).

Both oak and birch dust elicited a dose-dependent, over 40-fold, inhibition of CCL24 mRNA expression (Fig. 5). The maximal inhibition was reached at 24 h after exposure. TiO₂ and the low doses of LPS (10 or 100 pg/ml) did not have any significant effect (Fig. 5).

The baseline expression of CCL5 and CXCL9 mRNA was very weak and was not affected significantly by oak or birch dust exposure (data not shown). No significant expression of CCL1, CCL8, CCL11, CCL12, CCL17, CCL20, CCL27, CXCL5, and CXCL12 mRNA was detected after exposure to oak or birch dust in RAW 264.7 cells (data not shown). mRNA for chemokine receptors CCR1, CCR2, and CCR5 was expressed at the baseline level but the level of expression was not significantly affected by the birch or oak dust exposure (data not shown). No significant expression of CCR8 mRNA

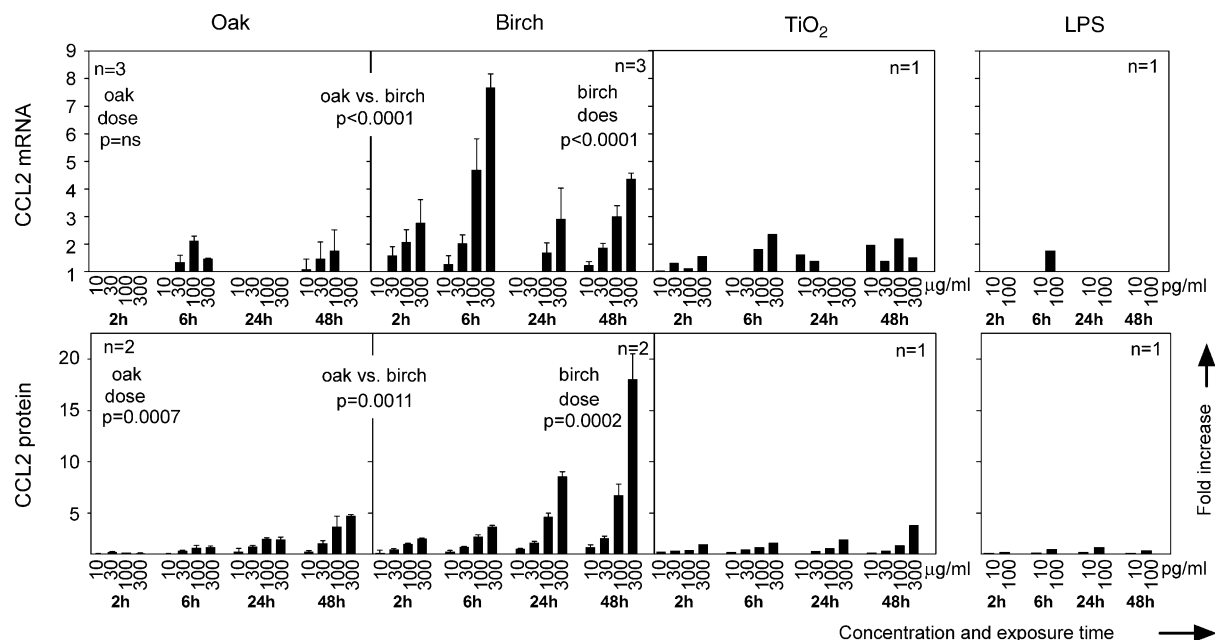


Fig. 4. CCL2 mRNA and protein expression after exposing RAW 264.7 cells to oak, birch, or TiO₂ dust or low LPS doses. The CCL2 mRNA expression of exposed cells was compared to the expression of unexposed cells at each time point by real-time PCR. CCL2 protein was analyzed from the cell culture media by ELISA. The results are expressed as fold differences to the expression level of unexposed cells. The bars represent mean $\pm$ S.E.M. of three experiments (oak or birch dust-induced mRNA expression) or mean $\pm$ S.D. of two experiments (oak or birch dust-induced protein expression), or a single experiment (TiO₂ and LPS). The results of the statistical analysis show whether the effects induced by wood dusts are dependent of the dose of wood dust (named "oak dose" or "birch dose") and whether the effects induced by oak or birch dusts differ significantly from each others (named "oak vs. birch"). Statistical significance was defined as $p < 0.05$. ns = not significant.

was detected after exposure to birch or oak dust (data not shown).

3.3. Cell viability

Both oak and birch dust at the concentrations of 300 μ g/ml decreased only slightly and virtually in an identical manner the viability of the RAW 264.7 cells (Fig. 6). The maximal decrease in cell viability was reached at 2 h after exposure. The viability started to recover gradually 6 h after exposure. No significant effect on cell viability was found after exposure to TiO₂ (300 μ g/ml) or LPS (100 pg/ml) (Fig. 6).

4. Discussion

Repeated airway exposure to wood dust has long been known to cause adverse respiratory effects and impairment of lung function (Goldsmith and Shy, 1988; Enarson and Chan-Yeung, 1990; Flechsig and Nedo, 1990; Demers et al., 1997). At present, however, virtually nothing is known about cellular mechanisms underlying wood dust-induced airway symptoms. In the present study, we developed an in vitro model to elucidate the

effects of exposure to two selected hardwood dusts, birch and oak, on cytokine and chemokine expression of murine macrophage cell line RAW 264.7 cells.

Several studies have confirmed that TNF- α , IL-6, and IL-1 β play a central role in the development of the lung inflammation induced by exposure to the organic dust, mineral fibers, or fungal spores (Vanhée et al., 1995; Driscoll, 2000; Dörger and Krombach, 2002; Mansour and Levitz, 2002; Yucesoy et al., 2002). Binding of TNF- α and/or IL-1 β to their specific receptors on endothelial cells is able to induce the expression of several adhesion molecules which contribute to the adhesion of leukocytes to endothelium (Borish and Steinke, 2003). Moreover, TNF- α and IL-1 β act synergistically in stimulating cytokines and chemokines by activating transcription factors NF- κ B and AP-1 (Dinarello, 2000, 2002; Baud and Karin, 2001; Ciesielski et al., 2002). Together they form an important amplification loop of the inflammatory response (Apte and Voronov, 2002). In addition to TNF- α and IL-1 β , IL-6 is one of the major regulators of acute phase response and it also mediates T-cell activation, growth, and differentiation.

In the present study, exposure to birch dust induced a strong and dose-dependent production of TNF- α

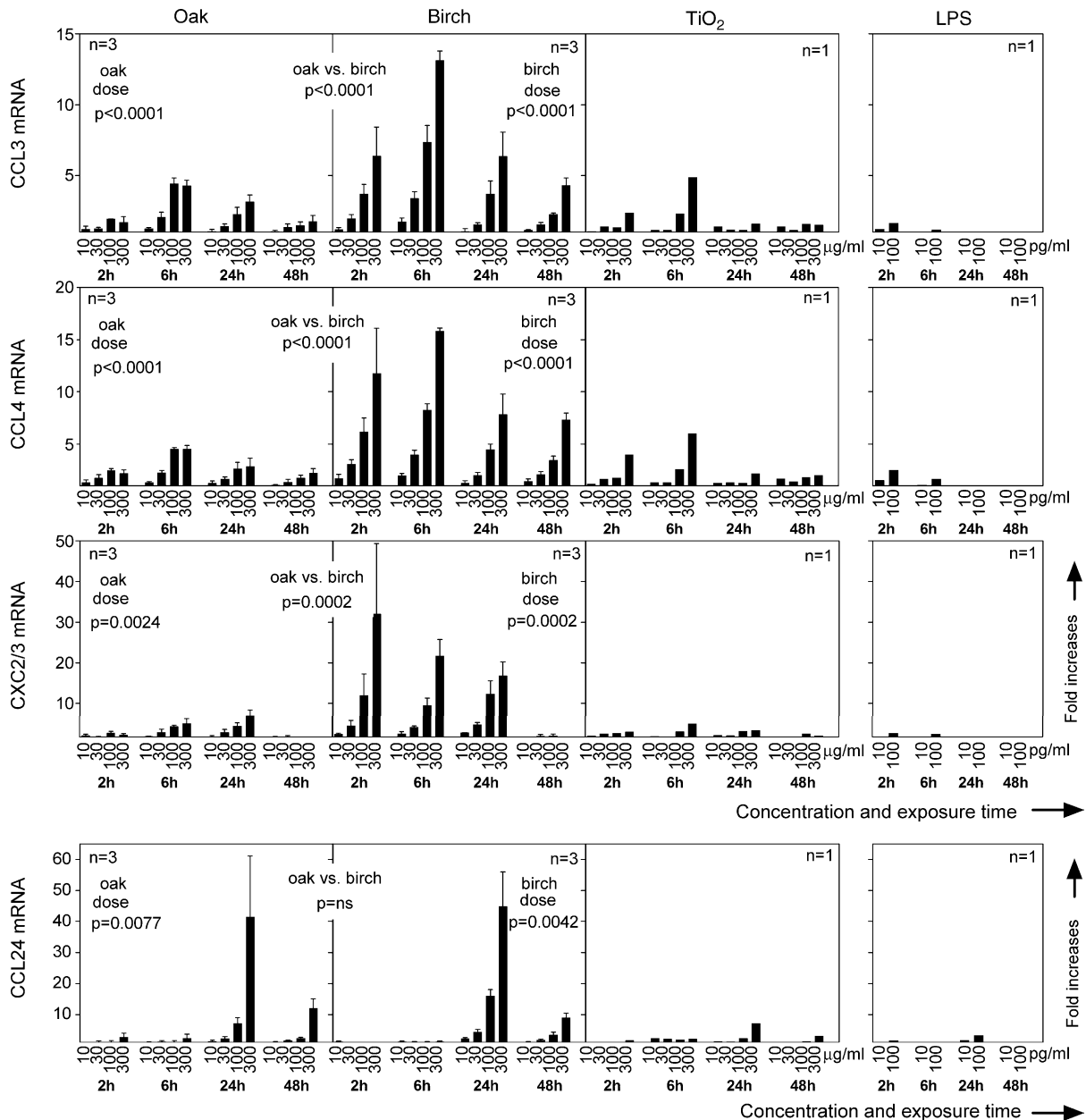


Fig. 5. The CCL3, CCL4, CXCL2/3, and CCL24 mRNA expression profiles of RAW 264.7 cells exposed to oak, birch, or TiO_2 dust or low LPS doses. The CCL3, CCL4, CXCL2/3, or CCL24 mRNA expression of exposed cells was compared to the expression of unexposed cells at each time point by real-time PCR. The results are expressed as fold differences to the expression level of unexposed cells. Note that the results of CCL24 are shown as fold decrease in expression. The bars represent mean $\pm$ S.E.M. of three experiments (oak and birch) or a single experiment (TiO_2 and LPS). The results of the statistical analysis show whether the effects induced by wood dusts are dependent of the dose of wood dust (named "oak dose" or "birch dose") and whether the effects induced by oak or birch dusts differ significantly from each others (named "oak vs. birch"). Statistical significance was defined as $p < 0.05$. ns = not significant.

protein. Compared with birch dust, a significantly weaker induction of TNF- α protein was found after exposure to oak dust. Moreover, oak dust did not induce significantly IL-6 protein whereas birch dust caused a strong and dose-dependent induction of IL-6 protein. No

significant effect on IL-6 and TNF- α protein production was seen after exposure to inorganic TiO_2 dust. These results suggest that birch dust is able to induce higher amounts of IL-6 and TNF- α protein compared with oak dust. Some previous studies suggest that wood dust

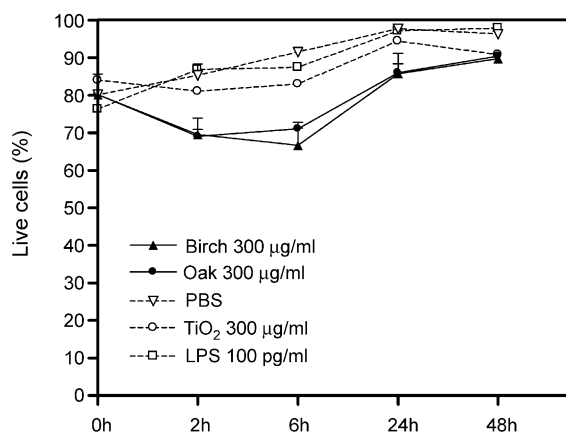


Fig. 6. Viability of RAW 264.7 cells as determined by the trypan blue dye exclusion test. Cells were exposed to birch, oak, or TiO₂ dust at 300 µg/ml, to LPS at 100 pg/ml, or were treated only with PBS. Cell viability was measured at five different time points (0–48 h). The bars represent mean $\pm$ S.D. of two experiments (birch, oak, and PBS) or a single experiment (TiO₂ and LPS).

exposure may increase the expression of pro-inflammatory cytokines in the airways. Healthy volunteers, exposed to Scots pine dust and other air contaminants in a sawmill, have been reported to have increased levels of IL-6 protein in their nasal lavage fluid (Dahlqvist et al., 1996). Moreover, Long et al. (2004) have reported that pine wood dust induces TNF- α expression in rat alveolar macrophages. According to their results, TNF- α expression in rat alveolar macrophages is mediated, at least in part, by reactive oxygen species.

In the present study, both the tested wood dusts caused a weak but dose-dependent inhibition in IL-1 β mRNA expression. This decrease in IL-1 β mRNA observed after exposure to wood dusts, but not after exposure to TiO₂ dust, could be due to the induction of a transcriptional repressor (Lebedeva and Singh, 1997; Xie et al., 2002) and/or a decrease in mRNA half-life (Fenton et al., 1988; Kern et al., 1997; Sirenko et al., 1997, 2002) by wood dust derived components, or it can be associated with the decreased viability of wood dust treated cells detected at 2–6 h after exposure. However, the exact mechanism of the observed inhibition and its biological significance are unknown and require further studies.

After phagocytosis of inhaled particles, activated alveolar macrophages release chemokines, which attract inflammatory cells into the lung tissue (Driscoll, 2000). A dose-dependent induction of both CCL2 mRNA and protein was found after exposure to birch dust, but only a borderline induction of CCL2 protein was detected after exposure to oak dust. Similarly, birch dust induced a strong and dose-dependent expression of CCL3, CCL4,

and CXCL2/3 mRNA whereas only moderate levels of these chemokines were detected after oak dust exposure. In each case, the chemokine expression was significantly stronger after birch dust exposure than after oak dust exposure. Interestingly, the mRNA expression of CCL24 chemokine was inhibited by more than 40-fold after exposure to both oak and birch dust. TiO₂ dust did not have any significant effect on the expression of chemokines CCL2, CCL24, and CXCL2/3. However, TiO₂ dust induced as strong expression of CCL3 and CCL4 chemokines as oak dust. These findings suggest that CCL2 is almost solely induced by birch dust whereas the expression of CCL3, CCL4, CCL24, and CXCL2/3 is affected also by oak dust. The levels of CCL3 and CCL4 mRNA seen after oak dust exposure may be related to the phagocytosis phenomenon per se, since TiO₂ induced similar expression of these chemokines as oak dust. It should be noted, however, that strikingly higher levels of CCL3 and CCL4 mRNA were found after birch dust exposure compared with exposure to TiO₂. Taken together, these results suggest that birch dust in particular has a major impact on chemokine expression and that this effect is only partially affected by the interaction of macrophage cells with particles per se.

The biological effects of chemokines are mediated by the interaction with their specific receptors, which belong to the superfamily of G protein-coupled receptors (Onuffer and Horuk, 2002). CCL2 acts as a chemoattractant via the CCR2 receptor, CCL3 via the CCR1 and CCR5 receptors, CCL4 via the CCR5 receptor, and CCL24 via the CCR3 receptor. CCR1, CCR2, and CCR5 are expressed on the surface of a variety of cells involved in the host defense such as T cells, monocytes, dendritic cells, and natural killer (NK) cells. CCR3 is primarily expressed on a variety of cells that are associated with allergy, e.g. eosinophils, basophils, and Th2 polarized cells (Onuffer and Horuk, 2002). No detectable effect on the expression of chemokine receptor mRNAs was found after exposure of the cells to wood dusts in the present study. These results should be interpreted with caution since cell surface expression of receptors should be preferably studied by flow cytometry. However, due to the interference of the wood fibers with sensitive and vulnerable optic system of flow cytometry instrument, it was difficult this powerful technique to utilize in the present study.

The effect on cell viability was comparable with both wood dusts examined. In addition, oak and birch dust particles had very similar size and shape distribution. Thus, the observed differences in cytokine and chemokine expression cannot be attributable to the differences in the cell toxicity of the wood dusts or to

the differences in their particle size or shape distribution. During the wood dust exposures in this study, the highest endotoxin level varied from 66 pg/ml (birch dust) to 21 pg/ml (oak dust) at the wood dust concentration of 300 µg/ml. Since LPS at the concentration of 100 pg/ml had no detectable effect on cytokine or chemokine expression, these trace amounts of endotoxins detected from the wood dusts cannot be the cause for the differences seen in the present study. Taken together, these findings suggest that observed differences between the effects of birch and oak dusts may be due to differences in the composition of dust-derived immunomodulatory substances.

In conclusion, our results demonstrate that exposure to wood dust is able to induce a heterogeneous, dose-dependent expression of cytokines and chemokines in mouse macrophage RAW 264.7 cells. They also suggest that there may be differences between different wood dust species in their ability to affect cytokine and chemokine expression in RAW 264.7 cells. Further studies are needed to compare more wood dust species and to compare the effects of softwood dusts vs. hardwood dusts on the cytokine and chemokine expression. Our results suggest that exposure to wood dust can influence the development of the inflammatory process in the airways by modulating the expression of macrophage derived cytokines and chemokines, which are the key molecules in the regulation of leukocyte recruitment to the site of inflammation. To extend our approach to a model closer to the whole immune system, we are currently setting up a murine model to study the effects of wood dusts in vivo.

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References

Applied Biosystems, 1997. Relative quantitation of gene expression. Applied Biosystems ABI PRISM 7700 Sequence Detection System User Bulletin No. 2.

Apte, R.N., Voronov, E., 2002. Interleukin-1—a major pleiotropic cytokine in tumor–host interactions. *Semin. Cancer Biol.* 12, 277–290.

Baud, V., Karin, M., 2001. Signal transduction by tumor necrosis factor and its relatives. *Trends Cell. Biol.* 11, 372–377.

Borish, L.C., Steinke, J.W., 2003. Cytokines and chemokines. *J. Allergy Clin. Immunol.* 111, 460–475.

Castranova, V., Vallyathan, V., 2000. Silicosis and coal workers' pneumoconiosis. *Environ. Health Perspect.* 108 (Suppl. 4), 675–684.

Chan-Yeung, M., 1994. Mechanism of occupational asthma due to western red cedar (*Thuja plicata*). *Am. J. Ind. Med.* 25, 13–18.

Ciesielski, C.J., Andreakos, E., Foxwell, B.M., Feldmann, M., 2002. TNF α -induced macrophage chemokine secretion is more dependent on NF- κ B expression than lipopolysaccharides-induced macrophage chemokine secretion. *Eur. J. Immunol.* 32, 2037–2045.

Dahlqvist, M., Palmberg, L., Malmberg, P., Sundblad, B.M., Ulfvarson, U., Zhiping, W., 1996. Acute effects of exposure to air contaminants in a sawmill on healthy volunteers. *Occup. Environ. Med.* 53, 586–590.

Demers, P.A., Kogevinas, M., Boffetta, P., Leclerc, A., Luce, D., Gérin, M., Battista, G., Belli, S., Bolm-Audorf, U., Brinton, L.A., Colin, D., Comba, P., Hardell, L., Hayes, R.B., Magnani, C., Merler, E., Morcet, J.F., Preston-Martin, S., Matos, E., Rodella, S., Vaughan, T.L., Zheng, W., Vainio, H., 1995. Wood dust and sino-nasal cancer: pooled reanalysis of twelve case-control studies. *Am. J. Ind. Med.* 28, 151–166.

Demers, P.A., Teschke, K., 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.

Dinarello, C.A., 2000. Proinflammatory cytokines. *Chest* 118, 503–508.

Dinarello, C.A., 2002. The IL-1 family and inflammatory diseases. *Clin. Exp. Rheumatol.* 20, S1–S13.

Dörger, M., Krombach, F., 2002. Response of alveolar macrophages to inhaled particulates. *Eur. Surg. Res.* 34, 47–52.

Driscoll, K.E., 2000. TNF α and MIP-2: role in particle-induced inflammation and regulation by oxidative stress. *Toxicol. Lett.* 112–113, 177–183.

Enarson, D.A., Chan-Yeung, M., 1990. Characterization of health effects of wood dust exposures. *Am. J. Ind. Med.* 17, 33–38.

Estlander, T., Jolanki, R., Alanko, K., Kanerva, L., 2001. Occupational allergic contact dermatitis caused by wood dusts. *Contact Dermat.* 44, 213–217.

Fenton, M.J., Vermeulen, M.W., Clark, B.D., Webb, A.C., Auron, P.E., 1988. Human pro-IL-1 β gene expression in monocytic cells is regulated by two distinct pathways. *J. Immunol.* 140, 2267–2273.

Fleischig, R., Nedo, G., 1990. Hazardous health effects of occupational exposure to wood dust. *Ind. Health* 28, 107–119.

Goldsmith, D.F., Shy, C.M., 1988. Respiratory health effects from occupational exposure to wood dusts. *Scand. J. Work Environ. Health* 14, 1–15.

Gordon, S.B., Read, R.C., 2002. Macrophage defences against respiratory tract infections. *Br. Med. Bull.* 61, 45–61.

Hessel, P.A., Herbert, F.A., Melenka, L.S., Yoshida, K., Michaelchuk, D., Nakaza, M., 1995. Lung health in sawmill workers exposed to pine and spruce. *Chest* 108, 642–646.

Higuero, N.C., Zabala, B.B., Villamuza, Y.G., Gómez, C.M., de Gregorio, A.M., Sanchez, C.S., 2001. Occupational asthma caused by IgE-mediated reactivity to *Antiaris* wood dust. *J. Allergy Clin. Immunol.* 107, 554–556.

Hyttönen, M., Kanerva, L., Malmberg, H., Martikainen, R., Mutanen, P., Toikkanen, J., 1997. The risk of occupational rhinitis. *Int. Arch. Occup. Environ. Health* 69, 487–490.

- IARC, 1995. Wood dust. IARC Monogr. Eval. Carcinog. Risks Hum. 62, 35–215.
- Johnson, M.R., Wang, K., Smith, J.B., Heslin, M.J., Diasio, R.B., 2000. Quantitation of dihydropyrimidine dehydrogenase expression by real-time reverse transcription polymerase chain reaction. *Anal. Biochem.* 278, 175–184.
- Kauppinen, T., Toikkanen, J., Pedersen, D., Young, R., Ahrens, W., Boffetta, P., Hansen, J., Kromhout, H., Maqueda Blasco, J., Mirabelli, D., de la Orden-Riviera, V., Pannett, B., Plato, N., Savelle, A., Vincent, R., Kogevinas, M., 2000. Occupational exposure to carcinogens in the European Union. *Occup. Environ. Med.* 57, 10–18.
- Kern, J.A., Warnock, L.J., McCafferty, J.D., 1997. The 3' untranslated region of IL-1 β regulates protein production. *J. Immunol.* 158, 1187–1193.
- Klein, R.G., Schmezer, P., Amelung, F., Schroeder, H.G., Woeste, W., 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.
- Lebedeva, T.V., Singh, A.K., 1997. Constitutive activity of the murine IL-1 β promoter is regulated by a transcriptional repressor. *Biochim. Biophys. Acta* 1353, 32–38.
- Li, D.H., Yuan, L., Yi, S.L., Jiang, Z.H., 1990. Effects of wood dust exposure on respiratory health: cross-sectional study among farmers exposed to wood dust. *Am. J. Ind. Med.* 17, 84–85.
- Long, H., Shi, T., Borm, P., Määttä, J., Husgafvel-Pursiainen, K., Savolainen, K., Krombach, F., 2004. ROS-mediated TNF- α and MIP-2 gene expression in alveolar macrophages exposed to pine dust. *Part. Fibre Toxicol.* 1, 3.
- Malo, J.L., Cartier, A., Desjardins, A., Van de Weyer, R., Vandenplas, O., 1995. Occupational asthma caused by oak wood dust. *Chest* 108, 856–858.
- Mandryk, J., Alwis, K.U., Hocking, A.D., 1999. Work-related symptoms and dose-response relationships for personal exposures and pulmonary function among woodworkers. *Am. J. Ind. Med.* 35, 481–490.
- Mansour, M.K., Levitz, S.M., 2002. Interactions of fungi with phagocytes. *Curr. Opin. Microbiol.* 5, 359–365.
- Mohtashamipur, E., Norpoth, K., Lühmann, F., 1989. Cancer epidemiology of woodworking. *J. Cancer Res. Clin. Oncol.* 115, 503–515.
- Nylander, L.A., Dement, J.M., 1993. Carcinogenic effects of wood dust: review and discussion. *Am. J. Ind. Med.* 24, 619–647.
- Onuffer, J.J., Horuk, R., 2002. Chemokines, chemokine receptors and small-molecule antagonists: recent developments. *Trends. Pharmacol. Sci.* 23, 459–467.
- Quirce, S., Hinojosa, M., Marañón, F., Ferrer, A., Fernández-Caldas, E., Sastre, J., 2000. Identification of obeche wood (*Triplochiton scleroxylon*) allergens associated with occupational asthma. *J. Allergy Clin. Immunol.* 106, 400–401.
- Sirenko, O., Böcker, U., Morris, J.S., Haskill, J.S., Watson, J.M., 2002. IL-1 β transcript stability in monocytes is linked to cytoskeletal reorganization and the availability of mRNA degradation factors. *Immunol. Cell Biol.* 80, 328–339.
- Sirenko, O.I., Lofquist, A.K., DeMaria, C.T., Morris, J.S., Brewer, G., Haskill, J.S., 1997. Adhesion-dependent regulation of an A+U-rich element-binding activity associated with AUF1. *Mol. Cell. Biol.* 17, 3898–3906.
- Vanhée, D., Gosset, P., Boitelle, A., Wallaert, B., Tonnel, A.B., 1995. Cytokines and cytokine network in silicosis and coal workers' pneumoconiosis. *Eur. Respir. J.* 8, 834–842.
- Wolf, J., Schmezer, P., Fengel, D., Schroeder, H.G., Scheithauer, H., Woeste, P., 1998. The role of combination effects on the etiology of malignant nasal tumours in the wood-working industry. *Acta Otolaryngol. Suppl.* 535, 1–16.
- Xie, Y., Chen, C., Stevenson, M.A., Auron, P.E., Calderwood, S.K., 2002. Heat shock factor 1 represses transcription of the *IL-1 β* gene through physical interaction with the nuclear factor of interleukin 6. *J. Biol. Chem.* 277, 11802–11810.
- Yucesoy, B., Vallyathan, V., Landsittel, D.P., Simeonova, P., Luster, M.I., 2002. Cytokine polymorphisms in silicosis and other pneumoconioses. *Mol. Cell. Biochem.* 234–235, 219–224.
- Zock, J.P., Sunyer, J., Kogevinas, M., Kromhout, H., Burney, P., Antó, J.M., The ECRHS Study Group, 2001. Occupation, chronic bronchitis, and lung function in young adults. An international study. *Am. J. Respir. Crit. Care Med.* 163, 1572–1577.