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Inflammatory potential in relation to the microbial content of settled dust samples collected from moisture-damaged and reference schools: results of HITEA study

Abstract Aiming to identify factors causing the adverse health effects associated with moisture-damaged indoor environments, we analyzed immunotoxicological potential of settled dust from moisture-damaged and reference schools in relation to their microbiological composition. Mouse RAW264.7 macrophages were exposed to settled dust samples (n = 25) collected from moisture-damaged and reference schools in Spain, the Netherlands, and Finland. After exposure, we analyzed production of inflammatory markers [nitric oxide (NO), tumor necrosis factor-α (TNF-)α, interleukin (IL)-6, and macrophage inflammatory protein (MIP)2] as well as mitochondrial activity, viability, apoptosis, and cell cycle arrest. Furthermore, particle counts, concentration of selected microbial groups as well as chemical markers such as ergosterol, 3-hydroxy fatty acids, muramic acid, endotoxins, and glucans were measured as markers of exposure. Dust from moisture-damaged schools in Spain and the Netherlands induced stronger immunotoxicological responses compared to samples from reference schools; the responses to Finnish samples were generally lower with no difference between the schools. In multivariate analysis, IL-6 and apoptosis responses were most strongly associated with moisture status of the school. The measured responses correlated with several microbial markers and numbers of particles, but the most important predictor of the immunotoxicological potential of settled dust was muramic acid concentration, a marker of Gram-positive bacteria.

K. Huttunen[1,2], J. Tirkkonen[1], M. Täubel[3], E. Krop[4], S. Mikkonen[5], J. Pekkanen[3,6], D. Heederik[4], J.-P. Zock[7,8,9], A. Hyvärinen[3], M.-R. Hirvonen[1,3]

[1]Department of Environmental Science, University of Eastern Finland, Kuopio, Finland, [2]School of Bioscience, Cardiff University, Cardiff, UK, [3]Living Environment and Health Unit, National Institute for Health and Welfare, Kuopio, Finland, [4]Division Environmental Epidemiology, Institute for Risk Assessment Sciences, Utrecht University, Utrecht, the Netherlands, [5]Department of Applied Physics, University of Eastern Finland, Kuopio, Finland, [6]Department of Public Health, University of Helsinki, Helsinki, Finland, [7]Centre for Research in Environmental Epidemiology (CREAL), Barcelona, Spain, [8]Universitat Pompeu Fabra (UPF), Barcelona, Spain, [9]CIBER Epidemiología y Salud Pública (CIBERESP), Madrid, Spain

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K. Huttunen
Department of Environmental Science
University of Eastern Finland (UEF)
Yliopistonranta 1 E, D
PO Box 1627
FI-70211 Kuopio
Finland
Tel.: +358 40 7069197
Fax: +358 17 162131
e-mail: kati.huttunen@uef.fi

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Practical Implications

Biological activity of particulate matter collected from moisture-damaged school buildings relates strongly to microbial composition of the sample. However, significant geographical differences and high variance suggest that the immunotoxicological potency of settled dust does not unambiguously separate the moisture-damaged and reference schools, although the trend for higher potency of samples from moisture-damaged environments was visible in two of three studied countries. The effect of Gram-positive bacteria on the immunotoxicological potency requires further attention.

Introduction

Exposure to moisture-damaged indoor environments has been associated with increased occurrence of respiratory infections and upper respiratory tract symptoms, cough, wheeze, and dyspnoea (WHO Regional Office for Europe, 2009). Children are considered to be particularly susceptible to the effects of air pollution, because of their immature physiology and relatively high exposure entering via lungs compared to adults (Farhat et al., 2005). Available moisture in building materials enables the growth of microorganisms, resulting in potentially higher exposure to spores, spore fragments, secondary metabolites, and cellular components of microbes.

Poor indoor air quality is particularly problematic in public buildings such as schools, where inadequate ventilation or indoor source of harmful agents may affect a vast number of children and adults regularly and for extended periods of time. In recent years, the burden of disease caused by poor indoor air quality in schools has gained attention of health authorities (CDC 2013). However, even though there are international guidelines covering both dampness-related exposures and specifically chemical pollutants in indoor air (WHO Regional Office for Europe, 2009, 2010), there are only few studies concerning exposure to biological pollutants in schools, and the information on immunotoxicological potential of the exposure is even scarcer. Disconcertingly, the accumulating evidence does not support the association of any specific indoor microbiological factors with adverse health effects (Jacobs et al., 2014), although both dampness and mold indices are consistently and positively associated with multiple allergic and respiratory effects (Mendell et al., 2011).

Toxicological studies showing inflammatory, cytotoxic, and immunosuppressive responses after exposure to microbial agents provide information about the involved mechanisms and give support to the biological plausibility. The microbial components known to have immunostimulatory effects include (1-3)-βD-glucans, ergosterol (Rylander and Lin, 2000; Park et al., 2008), and endotoxins (Park et al., 2001; Sheehan et al., 2012). Such studies on single exposures show variety of effects, indicating that instead of one mechanism, there are multiple routes leading from exposure to the adverse health effect. Furthermore, several research groups have found that exposure to multiple exposure agents simultaneously may lead to synergistic interaction or 'priming effect', where the presence of another agent potentiates the effect of the exposure (Zhou et al., 2000; Huttunen et al., 2004; Kankkunen et al., 2009). Keeping in mind that the exposure in a moldy building consists of a combination of spores, microbial components, and metabolites as well as various chemicals and particles, interactions between different exposure agents are highly likely. As detailed information about the toxicity of each of the individual exposure agents is not available, testing the toxicological properties of sample material such as dust or indoor air particulate matter has been used to describe the overall potency of the exposure. Indeed, previous studies have shown that the potential to induce production of inflammatory mediators and toxicity in cells appears to indicate a presence of a source of biological contamination such as moisture damage or handling of waste, although a positive result is only semiquantitative (Huttunen et al., 2010a,b). Both mouse and human macrophages as well as human lung epithelial cells have been shown to produce comparable results in toxicological studies, although it is clear that the sensitivity of different cell types varies significantly (Huttunen et al., 2003).

One of the essential parts of a multinational research project 'Health Effects of Indoor Pollutants: Integrating microbial, Toxicological, and Epidemiological Approaches (HITEA)' was a cross-sectional and longitudinal study in school buildings. The HITEA school study included moisture-damaged and reference schools from three European countries (Spain, the Netherlands, and Finland) representing different climate zones. The selected schools were sampled for measurement of various microbial markers as well as other indicators of indoor air quality, along with detailed surveys of the health of pupils and teachers working in the buildings. Analyses of health data in the HITEA school study so far have shown no adverse effects on lung function, but higher prevalence of respiratory symptoms such as nocturnal cough in moisture-damaged schools, particularly in Finnish children who more often had wheeze, nasal symptoms as well as respiratory-related school absence. Levels of different microbial markers were found to be higher in moisture-damaged schools, but these findings were not consistent between countries. Microbial markers did, however, not explain associations of respiratory symptoms with moisture damage. Both microbial marker levels and adverse respiratory health effects varied between the countries studied (Borràs-Santos et al., 2013; Jacobs et al., 2014).

The aim of this study was to relate the concentration of microbial components in settled dust from HITEA schools with toxicity and inflammatory responses induced by these samples in a cell culture model. The results were assessed in relation to moisture damage status and geographical differences between the studied schools to recognize the factors explaining the immunotoxicological potential of the sample.

Methods

Selection and categorization of schools

Altogether, 14 moisture- and mold-damaged schools and 11 reference schools in three European countries

(Spain, the Netherlands, and Finland) were included in the study. The participating schools were selected according to criteria described in detail earlier (Borràs-Santos et al., 2013; Haverinen-Shaughnessy et al., 2012). The final sample of schools was selected and categorized as 'damaged' and 'non-damaged' schools based on walk-through building inspections that generated detailed data on severity, extent, and location of moisture damage observations, including visible mold, mold odor, surface moisture, and other indicators of moisture damage and dampness (Haverinen-Shaughnessy et al., 2012). Finally, twenty-five school buildings representing clear moisture-damaged and reference buildings were selected to participate in the detailed exposure assessment; seven from Spain (five cases + two controls), twelve in the Netherlands (5 + 7), and six in Finland (4 + 2).

Sampling

Settled dust samples were collected in two ways, by placing a polyethylene (PE)-coated cardboard boxes and electrostatic dustfall collectors (EDCs; Jacobs et al., 2013) at a height of 1.5–2.5 m on a top shelf or a similar place for 8 weeks. On average, 15 sampling locations per school were selected with emphasis on covering the whole school building and study population (pupils and teachers). The samples were collected in the spring of 2009; during April–June in Spain, May–July in the Netherlands, and March–May in Finland. Timings of the sampling campaigns were parallelized as much as possible between the three countries, however, taking into account the local school schedules and holidays.

Sample preparation

At the local study center, the accumulated dust was vacuumed from the settled dust boxes on mixed cellulose ester (MCE)-filters and suspended by placing the filter in 5 ml of dilution buffer (43 mg/ml KH_2PO_4, 250 mg/ml $MgSO_4 \times 7H_2O$, 8 mg/ml NaOH, 0.02% (v/v) Tween-80), sonicating for 15 min and shaken for another 15 min. Suspension was stored in −20°C and sent to central laboratory for further processing. A selection of 15 samples from each school was pooled together to a core sample. For the immunotoxicological studies, one portion of the pooled dust suspension was filtered through a 70-μm filter to remove the largest particles and resulting in more homogenous sample. The filtered suspension was further aliquotted and stored frozen (−20°C) until the experiments.

The EDC cloths were extracted as described earlier (Noss et al., 2008) by incubating them for 60 min in 20 ml of pyrogen-free water (B. Braun NPBI, Oss, the Netherlands) in an end-over-end roller. The extract was centrifuged (15 min, 1000 g), aliquotted, and stored frozen (−20°C) in pyrogen-free glass tubes until analysis in central laboratory.

Analysis of microbial components

Concentration of selected microbial groups was measured from the unfiltered settled dust box sample with quantitative PCR assays targeting the fungal group *Penicillium* spp/*Aspergillus* spp./*Paecilomyces variotii*, the bacterial genus *Mycobacterium spp*., Gram-positive bacteria, and Gram-negative bacteria. DNA extraction, qPCR primers, and probes used have been published earlier (Haugland et al., 2004; Torvinen et al., 2010; Kärkkäinen et al., 2010). The qPCR laboratory analyses and calculations were performed as described by Kaarakainen et al., 2009; using the ABI Prism 7000 (Applied Biosystems) and the RotorGene 3000 (Corbett Life Science) equipment.

Chemical markers ergosterol and muramic acid were analyzed both from the filtered and unfiltered settled dust box sample, and 3-hydroxy fatty acids (3-OH FAs) from the unfiltered sample. The sample preparation of the freeze-dried settled dust suspension for ergosterol was carried out by modifying the method published by Axelsson et al. (1995), whereas 3-OH FAs were prepared by slightly modifying the method presented by Sebastian and Larsson (2003) and muramic acid by modifying the method presented by Sebastian et al. (2004). As a modification in our methods, hexane was used instead of heptane, and the ergosterol and muramic acid samples were diluted to 100 ml of hexane and to 150 ml of chloroform, respectively, before analysis by gas chromatography–tandem mass spectrometry (GC–MS–MS). In the preparation of 3-OH FAs, no water was used in the first extraction. The analyses of ergosterol and muramic acid were performed with a PolarisQ ion trap mass spectrometer (MS–MS) from Thermo Scientific (Austin, TX, USA) equipped with a Trace GC-ultra gas chromatograph (GC) (Milan, Italy) with a DB-5MS fused-silica capillary column from J&W Scientific.

The EDC extracts were tested for endotoxin and glucan content. Endotoxins were measured using a limulus amoebocyte lysate (LAL) assay (Lonza Group, Basel, Switzerland) according to the manufacturer's protocol and expressed in endotoxin units (EU/m^2). Glucans were analyzed with a specific β-(1,3)-glucan sandwich enzyme immunoassay (EIA) as previously described by Noss et al. (2010).

Analysis of particle counts

Particle counts in sixteen size classes between 0.5 and >20 μm were measured with particle counter PAMAS SVSS (PAMAS GmbH, Rutesheim, Germany) with sensor SLS-25/25 (0.5 detection limit, max 13 000 particles/ml) and PMA analyzing software. Before

measuring particle counts, filtered settled dust samples were diluted in ultra clean water (1:5000). The particle count of clean water was subtracted from results of all samples. The measured sixteen size classes were combined to form four size categories (0.5–1 μm, 1–3 μm, 3–10 μm, and >10 μm).

Cell culture

A mouse macrophage cell line RAW264.7 obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) was cultured at 37°C and 5% CO_2 atmosphere in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine (2 mM), and antibiotics (penicillin–streptomycin, 100 U/ml) (Gibco BRL, Paisley, UK). The cells were cultured in flasks and refreshed every 2–3 days when confluent. Prior to the exposure experiment, the cells were transferred to 6-well plates (Costar, Corning, NY, USA), 1×10^6 cells/well in 2 ml of culture medium. After 24 h, the detached cells were removed by refreshing the cell culture medium.

The dust samples were melted in a water bath (37°C) and sonicated for 15 min before preparing a dilution series in dilution buffer. The cells were allowed to stabilize for one hour before exposing the cells to four doses of the dust suspension (1:1, 1:2, 1:4, and 1:10), carrier buffer (negative control), lipopolysaccharide (LPS, positive control), or etoposide (positive control). After 24 h, the exposure was stopped by resuspending the cells into cell culture medium. A 200 μl sample was taken for measuring the viability of the cells with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. The rest of the cells were separated from the cell culture medium and divided in two portions; one for analyzing the permeability of the cell membrane [propidiumiodide (PI) staining] and one for analyzing the DNA content of the permeabilized cells. The supernatants were stored (−70°C) for later analysis of pro-inflammatory mediators [nitric oxide (NO), tumor necrosis factor-α (TNF-)α, interleukin (IL)-6, and macrophage inflammatory protein (MIP)2]. Three independent repetitions of the experiment were performed to describe the biological variance of the results.

Analysis of cell viability and cell cycle

Viability of the cells was analyzed with MTT test (Hansen et al., 1989) for detecting mitochondrial activity. The absorbance of each sample was measured with the multilabel counter Victor[3] and compared to absorbance obtained from control cells.

The proportion of dead cells was determined with propidium iodide (PI) exclusion. The cells were washed with phosphate-buffered saline (PBS), resuspended into PBS, and incubated with PI (f.c. 1 μg/ml) for 15 min at room temperature in the dark before flow cytometric analysis using CyAn ADP equipped with a 488 ± 10 nm argon laser as the excitation source with an emission wavelength of 613 ± 20 nm. A total of 10,000 cells were analyzed per sample using Summit software version 4.3 (Dako, Glostrup, Denmark).

DNA content of the cells was analyzed by PI staining of permeabilized cells, which enables the detection of abnormalities in the cell cycle and assessing the amount of apoptotic cells with hypodiploid DNA content. After exposure, the cells were resuspended into PBS, fixed with ice-cold ethanol, and stored at +4°C until staining with PI. Subsequently, the cells were pelleted, suspended into PBS, and incubated with 0.15 mg/ml of ribonuclease A for one hour at +50°C. PI was added to a final concentration of 8 μg/ml, and the incubation was continued for 2 h at +37°C in the dark before flow cytometric analysis. Flow cytometric analysis was performed using CyAn ADP equipped with a 488 ± 10 nm argon laser as the excitation source with an emission wavelength of 613 ± 20 nm. A total of 10,000 cells were analyzed per sample by using Summit software version 4.3. Etoposide (1 μM) was used as a positive control.

Analysis of pro-inflammatory mediators

Concentration of nitric oxide (NO) was measured with Griess method (Green et al., 1982). First, the cell suspension was centrifuged to separate the cells from the culture medium. The nitrate produced by the cells was reduced to nitrite with Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2% phosphoric acid). Subsequently, the absorbance (450 nm) of each sample was measured with a multilabel counter Victor[3] and compared to absorbance obtained from standard curve of sodium nitrite. LPS (0.01 μg/ml) was used as a positive control.

The concentration of pro-inflammatory cytokines IL-6, TNF-α, and chemokine MIP-2 in the cell culture medium was measured with commercially available enzyme-linked immunosorbent assay (ELISA) kits according to manufacturer's instructions. The absorbance of each sample was measured with a multilabel counter Victor[3], and the concentration of the analyte was calculated by comparing the results to the standard curve. LPS (0.01 μg/ml) was used as a positive control.

Statistical analyses

All the data were analyzed using the IBM Statistics 19.0 (IBM®, New York, NY, USA).

As the variables are not normally distributed, differences between damaged and reference schools as well as the comparison to carrier control were tested with

nonparametric Mann–Whitney *U*-test and the differences between countries were tested with Kruskall–Wallis test. Multivariate analysis of covariance (MANCOVA) was used to identify microbial markers associated strongest to toxicity and inflammatory response. An agnostic approach was followed in the sense that all microbial determinants and particle counts were explored without *a priori* hypotheses as covariates, taking into account the multicollinearities. Almost all possible two variable interactions within the covariates were also tested but found non-significant in our data. Some variables could not be tested simultaneously in the model because of (multi) collinearity. The predictors were tested for potential collinearity with Spearman correlation coefficient. A conservative limit of a correlation $r > 0.5$ for two variables was used to detect collinearity and decide which variables can be included simultaneously in a model. However, these variables were the best predictors for the model also regardless of the co-linearity limitation.

Results

Inflammatory markers

The production of all measured inflammatory mediators (NO, IL-6, TNF-α, MIP-2) increased dose dependently in mouse macrophages after exposure to settled dust samples. The dose-response curve typically leveled out at the highest dose, presumably due to the high toxicity of respective extract. There was a clear difference in the inflammatory potential of samples from different countries: The samples collected from Spain and the Netherlands caused a stronger inflammatory response than the samples collected from Finland ($P < 0.05$). When data from all three countries were pooled, the samples collected from damaged schools appeared to induce a slightly higher production of inflammatory mediators than samples from reference schools (Table 1). When the samples from damaged and reference schools were compared within each country, there was a clear trend for higher inflammatory potential for dust collected from damaged schools in Spain and the Netherlands, but not in Finland (Table 1). Overall, the variation between schools was high, but the difference between the schools was clearest in the Netherlands, reaching statistical significance for NO (doses 1:10, 1:4, and 1:2) and IL-6 (dose 1:4) (Figure 1).

Toxicity

Exposure to settled dust decreased the number of viable cells, increased the number of dead cells, and caused apoptosis, but the effect on cell cycle was minimal. Observed responses were dose dependent, and again, there was a clear difference between countries: Samples collected from Spain and the Netherlands decreased viability and increased the number of damaged and apoptotic cells more than samples from Finland. When comparing schools on the basis of damage status, samples collected from damaged schools were slightly more toxic compared with samples from reference schools, although the difference did not reach statistical significance due to the large variation (Table 2). Samples collected from moisture-damaged schools in

Table 1 Inflammatory markers [median (IQR)] in cell culture medium of mouse RAW264.7 macrophages after exposure to graded doses of settled dust suspension for 24 h

		Spain		the Netherlands		Finland		All countries		
	Dose	Reference ($n = 2$)	Damaged ($n = 5$)	Reference ($n = 7$)	Damaged ($n = 5$)	Reference ($n = 2$)	Damaged ($n = 4$)	Reference ($n = 11$)	Damaged ($n = 14$)	Carrier control ($n = 4$)
NO (μM)	1:10	5.7 (1.5)	5.6 (3.8)**	4.1 (4.1)*,**	9.8 (0.70)*,**	1.6 (0.24)	1.5 (0.11)**	4.1 (4.6)**	5.5 (7.7)**	1.0 (0.20)
	1:4	11 (1.9)	14 (2.1)**	8.8 (5.2)*,**	17 (3.0)*,**	3.2 (1.2)	3.1 (0.67)**	8.7 (6.5)**	14 (11)**	
	1:2	11 (1.3)	16 (5.2)**	10 (4.4)*,**	16 (2.3)*,**	5.3 (2.4)	5.2 (1.4)**	9.7 (4.5)**	13 (7.1)**	
	1:1	9.2 (0.18)	13 (4.2)**	12 (2.0)**	9.9 (4.3)**	9.3 (3.1)	10 (2.1)**	11 (3.2)**	11 (4.1)**	
IL-6 (ng/ml)	1:10	1.3 (0.58)	1.0 (2.6)**	1.1 (1.2)**	3.9 (1.4)**	0.22 (0.13)	0.17 (0.04)**	0.71 (1.4)**	1.0 (3.4)**	0.01 (0.02)
	1:4	7.3 (2.5)	7.4 (6.1)**	5.4 (11)*,**	20 (2.6)*,**	0.78 (0.46)	0.67 (0.25)**	4.8 (9.5)**	7.3 (18)**	
	1:2	16 (3.9)	20 (9.4)**	14 (17)**	33 (6.0)*,**	2.1 (1.5)	1.6 (0.42)**	12 (17)**	17 (25) **	
	1:1	17 (2.1)	25 (9.4)**	22 (21)**	31 (7.9)**	5.0 (3.3)	5.2 (1.5)**	15 (13)**	22 (22)**	
TNF-α (ng/ml)	1:10	5.4 (0.98)	5.9 (3.9)**	5.0 (3.4)**	9.1 (3.0)**	1.4 (0.64)	1.1 (0.22)**	4.4 (4.1)**	5.4 (7.2)**	0.07 (0.02)
	1:4	11 (1.9)	13 (6.6)**	10 (8.9)**	18 (1.9)**	2.7 (1.1)	2.7 (0.83)**	9.4 (8.7)**	12 (13)**	
	1:2	22 (4.5)	23 (8.4)**	17 (16)**	27 (8.9)**	5.0 (2.5)	4.2 (0.74)**	17 (16)**	21 (21)**	
	1:1	29 (4.5)	38 (5.5)**	25 (9.3)**	36 (7.3)**	7.6 (3.1)	8.3 (1.5)**	25 (16)**	32 (26)**	
MIP2 (ng/ml)	1:10	31 (7.7)	28 (23)**	28 (19)**	49 (40)**	6.6 (3.4)	6.2 (0.44)**	23 (24)**	26 (40)**	0.20 (0.02)
	1:4	110 (18)	120 (71)**	68 (93)**	180 (24)**	20 (8.2)	23 (8.3)**	68 (92)**	110 (140)**	
	1:2	200 (50)	270 (67)**	160 (130)**	250 (14)*,**	37 (22)	40 (6.1)**	150 (150)**	230 (210)**	
	1:1	260 (32)	270 (46)**	220 (160)**	230 (45)**	69 (29)	92 (17)**	220 (170)**	230 (160)**	

IQR, interquartile range; NO, nitric oxide; IL-6, interleukin 6; TNF-α, tumor necrosis factor-α; MIP2, macrophage inflammatory protein 2; *n*, number of schools.

*Statistically significant ($P < 0.05$) difference between damaged and reference school, Mann–Whitney *U*-test.

**Statistically significant ($P < 0.05$) difference compared to carrier control, Mann–Whitney *U*-test.

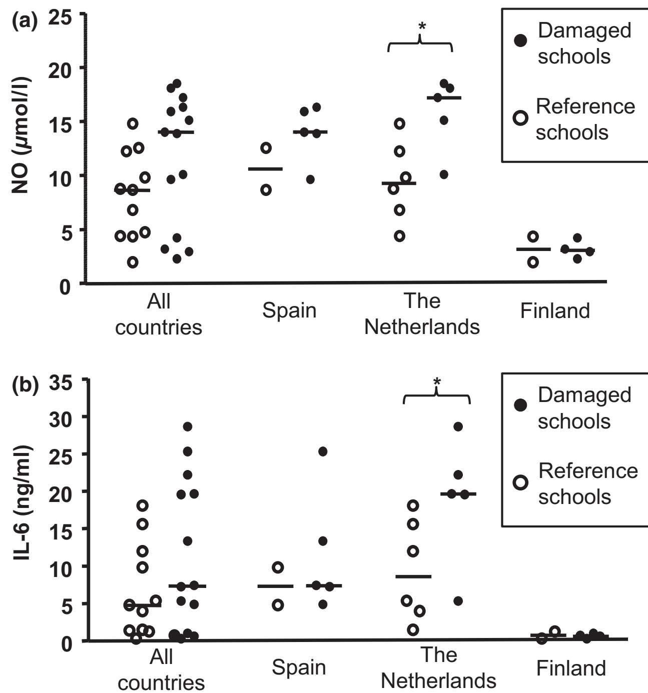

Fig. 1 Concentration of (a) nitric oxide (NO) and (b) interleukin (IL)-6 in the cell culture medium of mouse RAW264.7 macrophages after 24-hour exposure to settled dust collected from moisture-damaged and reference schools in Spain, the Netherlands, and Finland (dose 1:4). Lines represent median values. Statistically significant ($P < 0.05$) difference between schools is marked with an asterisk (*)

Spain and the Netherlands were consistently more toxic than samples from reference schools, but the difference reached statistical significance only for a decreased viability caused by the lowest dose of samples collected from the Netherlands. The Finnish moisture-damaged and reference schools did not differ from each other. Interestingly, the lowest dose of samples collected from Finland slightly increased the number of viable cells and decreased apoptosis compared to carrier control. (Table 2).

Particle counts

As the dose-response curve was based on dilution series of the amount of accumulated dust in each location instead of a fixed mass concentration, the differences between the particle counts in the sample suspensions were analyzed. There were no significant differences in the number of particles in the samples from damaged and reference schools, although a trend for higher particle counts in damaged Dutch schools was seen for size classes 1–3, 3–10, and >10 μm. Numbers of particles in the sample differed between countries; Samples from Dutch and Spanish schools had 2- to 4-fold higher numbers of particles compared to samples from Finland (Table 3).

Correlation between markers

The ability to induce inflammatory responses and cytotoxicity (excluding cell cycle arrest) correlated with each other (data not shown), with particle counts in all size classes (particularly with the largest particles with diameter >10 μm), and with the concentration of several microbial components in the dust. The weakest, mostly non-significant correlations with immunotoxicological potential were found for fungal group *Pencillium/Aspergillus* and *Mycobacterium* spp., whereas the concentration of muramic acid and endotoxins correlated strongly with immunotoxicological potential (Table 4). Only the correlations for dose level 1:4 are presented here, as correlations at other dose levels were similar apart from some observed trends: The correlations between numbers of the smallest particles and immunotoxicological parameters were strengthened along increasing dose levels, and correlations between particle counts and cell cycle arrest were evident only at the lowest dose level. The ergosterol and muramic acid concentrations measured from filtered and unfiltered settled dust sample correlated strongly with each other, although filtering slightly reduced the microbial material in the sample (data not shown).

Multivariate analysis of covariance

MANCOVA analysis showed that most important predictor of inflammatory response and toxicity was muramic acid concentration in the sample, which increased the production of cytokines IL-6, TNF-α, and MIP2 as well as affected the viability of the cells and amount of damaged and apoptotic cells. In addition to muramic acid, other significant predictors of immunotoxicological markers were mycobacteria and endotoxins (explaining levels of nitric oxide), Gram-negative bacteria (explaining the number of apoptotic cells), and glucans (explaining the level of cell cycle arrest). Type of school (damaged/reference) was a significant predictor of the levels of one inflammatory mediator (IL-6) and one toxicity marker (apoptosis), whereas geographical location had significant effect on all analyzed immunotoxicity markers except cell cycle arrest. Models with and without particle numbers gave almost as good results; only levels of largest particles (>10 μm) improved the ability of the model to explain the loss of viability, and levels of coarse particles (3–10 μm) improved the ability of the model to explain the amount of damaged and apoptotic cells. The analysis was repeated for each dose level, which showed that the effects were most evident at dose levels 1:4 and 1:2. Best predictors for toxicity and inflammatory response at dose levels 1:4 and 1:2 are listed in Tables S1 and S2, respectively. A summary of the results at the most representative dose level (1:4) is presented in Figure 2.

Table 2 Toxicity [median (IQR)] of the exposure to graded doses of settled dust suspension in mouse RAW264.7 macrophages for 24 h

		Spain		the Netherlands		Finland		All countries		
	Dose	Reference (n = 2)	Damaged (n = 5)	Reference (n = 7)	Damaged (n = 5)	Reference (n = 2)	Damaged (n = 4)	Reference (n = 11)	Damaged (n = 14)	Carrier control (n = 4)
Viability[a] (%)	1:10	72 (5.3)	74 (14)**	95 (27)*	69 (4.8)*	130 (1.7)	140 (7.9)**	95 (41)	75 (56)	100
	1:4	44 (3.6)	50 (12)**	56 (26)**	40 (5.0)**	87 (3.7)	82 (2.9)**	56 (35)**	50 (39)**	
	1:2	29 (1.7)	32 (10)**	45 (24)**	30 (3.3)**	70 (5.9)	76 (7.4)**	45 (32)**	34 (33)**	
	1:1	19 (2.0)	23 (5.1)**	32 (13)**	24 (1.9)**	54 (7.6)	56 (7.9)**	32 (23)**	24 (25)**	
Toxicity[b] (%)	1:10	12 (1.9)	13 (1.8)**	11 (3.8)	16 (2.5)	8.8 (0.43)	7.9 (1.4)	10 (4.3)**	12 (5.4)**	5.6 (5.3)
	1:4	17 (2.3)	19 (5.1)**	17 (8.9)**	23 (7.7)	7.6 (0.20)	7.6 (0.86)	15 (8.6)**	18 (14)**	
	1:2	24 (1.1)	31 (11)**	25 (13)**	32 (7.4)**	10 (0.82)	10 (2.2)	23 (14)**	28 (24)**	
	1:1	37 (2.8)	44 (15)**	28 (17)**	45 (3.2)**	15 (4.2)	13 (2.4)	28 (19)**	37 (26)**	
Apoptosis[c] (%)	1:10	2.0 (0.20)	2.3 (0.55)	2.3 (0.50)	2.1 (0.51)	1.4 (0.05)	1.4 (0.08)**	1.9 (0.56)**	2.1 (0.89)	2.8 (1.6)
	1:4	3.8 (0.15)	3.6 (2.3)	3.0 (1.6)	5.7 (0.50)	2.1 (0.20)	1.5 (0.13)**	3.0 (1.6)	3.5 (3.8)	
	1:2	7.1 (0.10)	7.8 (6.9)**	4.8 (4.3)	11 (2.5)	2.9 (0.44)	2.0 (0.21)	4.8 (4.3)**	7.1 (8.4)**	
	1:1	15 (0.90)	25 (15)**	9.8 (12)**	23 (3.6)**	2.9 (1.0)	2.7 (2.2)	9.8 (11)**	18 (18)**	
Cell cycle arrest[d] (%)	1:10	27 (0.05)	27 (1.3)	27 (0.80)	28 (0.85)	27 (0.30)	27 (0.37)	27 (0.55)	28 (1.3)	28 (1.0)
	1:4	32 (0.25)	31 (1.7)	31 (2.4)**	31 (0.50)**	28 (0.63)	28 (0.70)	31 (2.7)**	31 (2.5)**	
	1:2	27 (1.5)	29 (3.8)	31 (1.5)**	28 (0.76)	29 (0.24)	28 (0.49)	30 (2.1)**	28 (1.5)	
	1:1	27 (1.3)	25 (1.3)	28 (2.7)	29 (1.6)	31 (1.1)	31 (1.6)	28 (2.9)	28 (3.6)	

n, number of schools.
[a]Amount of viable cells compared to carrier control (MTT test, % of viable cells).
[b]Amount of damaged cells (PI exclusion, % of dead cells).
[c]Amount of apoptosis (DNA content analysis, % of hypodiploid cells).
[d]Cell cycle arrest (DNA content analysis, % of cells in G2/M phase).
*Statistically significant ($P < 0.05$) difference between damaged and reference school, Mann–Whitney *U*-test.
**Statistically significant ($P < 0.05$) difference compared to carrier control, Mann–Whitney *U*-test.

Table 3 Particle counts in four size classes [median (IQR)] (10^3 particles/ml, dose 1:4)

	Spain		the Netherlands		Finland		All countries	
Particles (μm)	Reference (n = 2)	Damaged (n = 5)	Reference (n = 7)	Damaged (n = 5)	Reference (n = 2)	Damaged (n = 4)	Reference (n = 11)	Damaged (n = 14)
0.5–1	790 (270)	710 (190)	300 (120)	330 (250)	170 (0.22)	160 (120)	300 (270)	400 (420)
1–3	200 (64)	180 (110)	86 (73)	150 (86)	42 (14)	26 (10)	86 (92)	160 (120)
3–10	54 (5.1)	51 (33)	34 (28)	67 (30)	12 (5.4)	7.8 (3.2)	34 (35)	48 (55)
>10	7.1 (1.4)	6.9 (1.4)	4.6 (3.6)	10 (2.2)	1.5 (0.76)	1.1 (0.32)	4.6 (4.5)	6.3 (7.2)
All	1100 (340)	930 (320)	430 (230)	550 (370)	220 (20)	210 (150)	430 (400)	600 (570)

IQR, interquartile range; *n*, number of schools.

Discussion

The present results show that the immunotoxicological potential of settled dust samples differs largely between geographical locations and may be influenced by moisture damage status of the building. We found that the samples collected from Spain and the Netherlands were more toxic *in vitro* and caused higher production of inflammatory mediators than the samples collected from Finland. A clear trend for higher immunotoxicological potency of dust from Spanish and Dutch schools categorized as moisture-damaged buildings was observed, but this difference between moisture-damaged and reference schools was not seen in Finland. This indicates that the effect of moisture damage on immunotoxicological potency of settled dust is not necessarily consistent for different climatic situations, school environments, and the specificities of moisture problems.

Interestingly, a questionnaire survey showed that pupils from Finnish water damage schools had more respiratory symptoms in comparison with children from reference schools, whereas in Dutch and Spanish schools, this difference was not evident (Borràs-Santos et al., 2013). These findings have been confirmed recently in the same sample of schools as considered in our study (Jacobs et al., 2014). This suggests that the biological activity of the settled dust samples may not reflect directly the respiratory effects as observed among children in water-damaged schools. The discrepancy between the health data and immunotoxicological potency of dust suggests that either the applied sampling excludes some of the key exposures (such as volatile compounds) or that other factors (such as genetic susceptibility or lacking innate immunity stimulus) are essential for the adverse health effects to present themselves. Nevertheless, the high correlation

Table 4 Spearman correlations between the analyzed immunotoxicological markers, microbial components, and numbers of particles (dose 1:4)

	NO	IL-6	TNF-α	MIP2	Viability	Toxicity	Apoptosis	Cell cycle arrest
Ergosterol	0.67	0.69	0.68	0.63	−0.68	0.58	0.48	0.49
Glucan	0.57	0.62	0.60	0.56	−0.66	0.46	0.46	0.70
Penicillium/Aspergillus	0.42	0.26	0.33	0.35	−0.36	0.31	0.24	0.19
Endotoxin	0.84	0.84	0.77	0.77	−0.82	0.60	0.38	0.63
Gram-negative bacteria	0.59	0.48	0.61	0.67	−0.62	0.71	0.66	0.07
Muramic acid	0.82	0.97	0.97	0.96	−0.88	0.92	0.87	0.36
Gram-positive bacteria	0.58	0.42	0.51	0.58	−0.56	0.59	0.55	0.16
Mycobacteria	0.51	0.33	0.41	0.44	−0.47	0.38	0.32	0.31
Particles 0.5–1 μm	0.42	0.51	0.68	0.69	−0.63	0.78	0.89	0.16
Particles 1–3 μm	0.53	0.60	0.76	0.75	−0.71	0.83	0.92	0.27
Particles 3–10 μm	0.71	0.78	0.89	0.88	−0.85	0.92	0.94	0.36
Particles >10 μm	0.86	0.89	0.94	0.93	−0.95	0.91	0.81	0.49

NO, nitric oxide; IL-6, interleukin 6; TNF-α, tumor necrosis factor-α; MIP2, macrophage inflammatory protein 2.

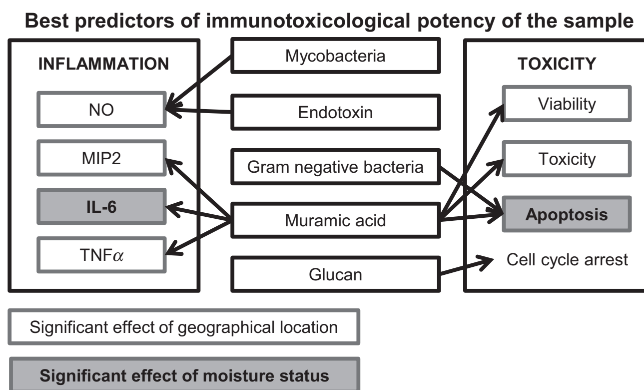

Fig. 2 Best predictors of inflammatory responses and toxicity in mouse RAW264.7 macrophages after exposure to settled dust from moisture-damaged and reference schools. Results of multivariate analysis of covariance at dose level 1:4 are presented (particle numbers are not included in the model). Statistically significant effect of geographical location is presented with gray outline, effect of moisture status of the school with gray fill and bold font, and effect of microbial markers on immunotoxicological parameters with arrows.

between several microbial markers and immunotoxicological potential of the samples as well as differences in the potency of samples collected from moisture-damaged and reference schools in Spain and the Netherlands suggests that the moisture status does affect the microbiological characteristics of the sample, which subsequently is linked with the immunotoxicological potency of the dust.

Evidently, the associations between the *in vitro* effects of settled dust and respiratory health of the pupils are not straightforward. The immunotoxicological potency differs by geographical regions and is associated with microbial characteristics and particle numbers present in the sample. In the approach chosen, it is not possible to disentangle which component is directly responsible for the observed toxicological potency, but it is possible to associate microbiological characteristics of the sample with immunotoxicological activity. Studying individual effects of highly intercorrelated variables is challenging, but results of the multivariate analysis of this extensive dataset point to an independent association between moisture damage status of school and inflammatory marker interleukin 6 as well as a measure of programmed cell death, apoptosis. According to our analysis, the microbial markers that were the best predictors of levels of these immunotoxicological markers were muramic acid, an indicator of Gram-positive bacteria, and concentration of Gram-negative bacteria. The importance of exposure to bacterial growth in context of moldy house problems is supported by experimental studies showing the inflammatory potency of certain bacterial species (Hirvonen et al., 2005) and co-exposure to bacteria and fungi (Huttunen et al., 2004). The complexity of the issue of biological contaminants indoors arises from the apparently inverted U-shaped dose-response curve of microbial exposure, possibly resulting in either harmful or protective effect depending on the quantity and diversity of the microbiome (Karvonen et al., 2014). Particularly, bacterial exposure indicated, for example, by muramic acid has been associated earlier also with decreased respiratory symptoms (Zhao et al., 2008; Zhang et al., 2011).

The lower overall biological activity of Finnish samples compared with Spanish and Dutch samples is most likely explained by the significantly lower number of particles accumulated in the sample during the sampling period. However, the multivariate analysis showed that the particle numbers do not explain the difference seen between the Spanish and Dutch moisture-damaged and reference schools nor the lack of difference in Finnish schools. In fact, the lowest dose of dust from Finnish schools seemed to improve the viability of the exposed cells and decrease the number of apoptotic cells, although this finding is most likely explained by the increased metabolism and phagocytosis of the activated macrophages (Pozzolini et al., 2003). Possible explanations behind the lower particle counts in Finnish schools could be the ventilation of the buildings, which was typically mechanical

in Finland, unlike in Spain and the Netherlands, where natural ventilation (i.e., through opening windows) was most common. Moreover, differences can be expected in the amount of particulate matter carried indoors along shoes and clothes and resuspended from floors, etc. due to occupancy and activity in the classrooms (Qian et al., 2012; Meadow et al., 2014). Indoor microbial counts are known to be strongly affected by outdoor air; thus, it is likely that also the different climate and meteorological conditions of the studied countries affect the microbial levels, Finland being significantly colder compared to Spain and the Netherlands. In the study by Jacobs et al. (2014), levels of microbial markers were indeed reported to be lower in Finnish schools as well as affected by season.

A major strength of this study is the presentation of fully comparable dataset from different climatic areas in Europe, combined with extensive list of microbial markers to be evaluated against the measured immunotoxicological potential of the samples. The unified sampling protocols and centralized sample analysis provided us with a coherent dataset, avoiding variation due to differences in sample processing or handling. As the categorization of the schools to moisture-damaged and reference schools was performed based on walk-through building inspections carried out by centrally trained research personnel and unified checklists, misclassification due to alternating criteria has been minimized.

The advantage of collecting settled dust is that it provides an integrated sample of particles that once were airborne, and the sampling time extends typically over several weeks, which improves the representativeness of the sample compared to short time sampling. Microbial concentrations in settled dust samples have been found to reflect the microbial content and composition of the actively collected samples rather well (Noss et al., 2008; Normand et al., 2009). However, settled dust lacks information about both the gaseous phase and the smallest, non-settling particles present in indoor air, which may have led to underestimation of the biological effects of exposure in this study. It should be noted that the collection of settled dust covers also the time when building is unoccupied, which may dilute the concentration of pollutants that are only present during active hours of the school. This should not be a major issue, as substantial part of the airborne dust is assumed to accumulate during the hours of active use. Driven by the necessity to provide sufficient sample volume for parallel analyses and to reduce the number of samples to be included in the labor-intensive immunotoxicological assays, we followed an approach of pooling of the samples within schools, which may have resulted in dilution of effects of single sampling locations. Furthermore, adding an additional filtering step when preparing dust samples for immunotoxicological assays could remove selectively some of the components, but this is not likely as the microbiological data available from both filtered and unfiltered samples showed only a slight reduction in measured markers and a high correlation between the two sample types. To confirm our findings, the responses of *in vitro* models based on human immunocompetent cells or respiratory tissue should be assessed.

Conclusions

The immunotoxicological potency of settled dust differs clearly between schools from different geographical regions, showing also a trend for higher potency of samples from moisture-damaged environments in two of the three studied countries. Geographical differences together with high variance between schools within a country indicate that the immunotoxicological potency of settled dust does not unambiguously link to moisture damage in schools. The effect of bacterial content, particularly Gram-positive bacteria, on the immunotoxicological potency requires further attention.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Best predictors for toxicity and inflammatory response in MANCOVA model (dose level 1:4)

Table S2. Best predictors for toxicity and inflammatory response in MANCOVA model (dose level 1:2)

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