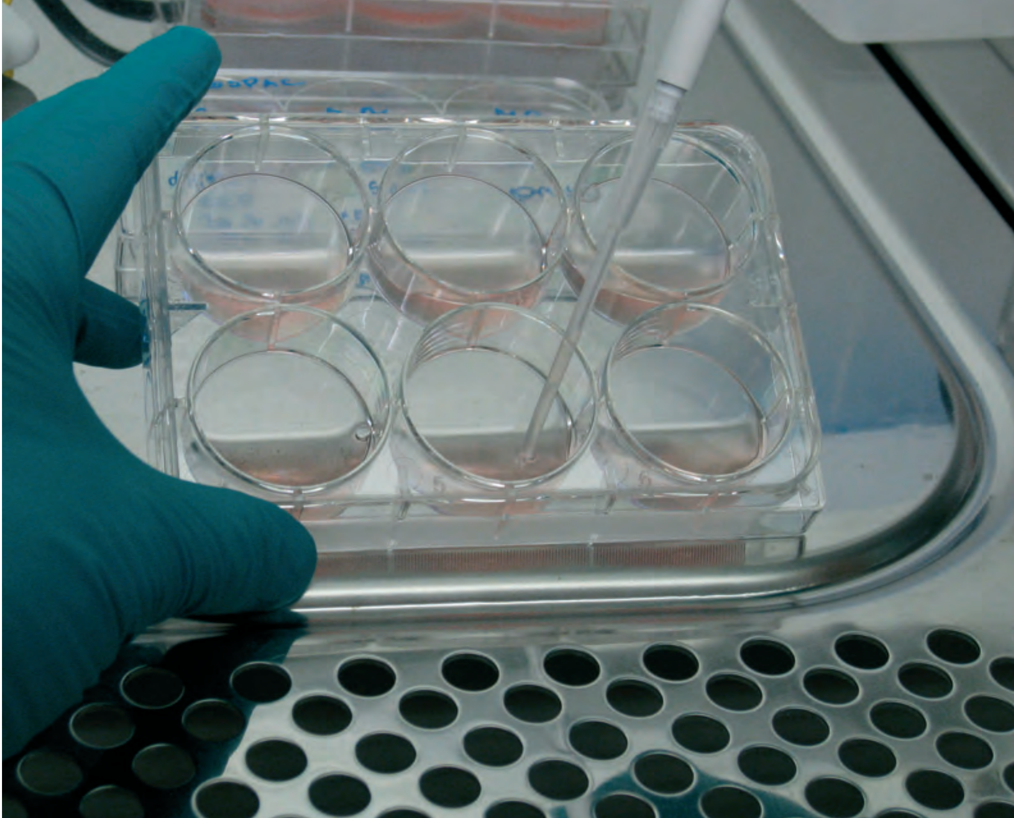

Piia Markkanen

Immunotoxic Responses Induced by *Streptomyces californicus* and *Stachybotrys chartarum* – The Role of Microbial Interactions

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Helsinki, Finland 2008

Piia Markkanen

IMMUNOTOXIC RESPONSES INDUCED BY *STREPTOMYCES CALIFORNICUS* AND *STACHYBOTRYS CHARTARUM*

– THE ROLE OF MICROBIAL INTERACTIONS

ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Natural and Environmental Sciences, University of Kuopio, for public examination in auditorium ML3, Medistudia building, on November 21st 2008, at 12 o'clock noon.

Department of Environmental Health, National Public Health Institute, Kuopio, Finland

and

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To my family

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ABSTRACT

Adverse health effects have been associated with dampness and microbial exposure in buildings, but the possible pathophysiological mechanisms behind these effects are still poorly understood. Although previous studies have shown that certain microbes and microbial components have clear inflammatory and cytotoxic potentials, the complex mixture of microbial species, their spores, metabolites and components in indoor air inevitably leads to interactions which may change the toxic characteristics of the microbes. However, little is known about the importance of microbial interactions in the activation of the cellular mechanisms which may cause varying health outcomes in different exposure situations.

The present study assessed interactions between two microbes isolated from moisture damaged buildings, the actinobacterium *Streptomyces californicus* and the fungus *Stachybotrys chartarum*, during co-exposure or co-cultivation. The main interest was to study how these microbial interactions affect the ability of their spores to activate important cellular mechanisms i.e. cytotoxicity, inflammation, genotoxicity and oxidative stress in mouse RAW264.7 macrophages.

The results of these studies indicated that the spores of *S. californicus* have cytotoxic, cytostatic, genotoxic and inflammogenic properties, whereas the spores of *S. chartarum* caused significant cytotoxicity only at relatively high concentrations, but no cytostatic, genotoxic or inflammogenic activity was observed in macrophages. In simultaneous exposure, the mutual proportion of these microbes influenced the nature of cellular responses, leading to increased or suppressed inflammatory response in macrophages.

Interestingly, the microbial interactions during co-cultivation were capable of stimulating or potentiating the production of highly toxic compound(s), and thus the spores of co-cultivated microbes evoked stronger immunotoxic responses in macrophages than the respective spore-mixture of separately cultivated microbes. Compound(s) produced during co-cultivation had strong cytotoxic, cytostatic and genotoxic properties, and the mechanism of cell death resembled the triggering of the apoptotic pathway by the cytostatic drugs, doxorubicin and actinomycin D,

which both originate from streptomycetes. Furthermore, simultaneous exposure to an antioxidant, N-acetyl-L-cysteine, with the spores of co-cultivated microbes inhibited these responses indicating that oxidative stress was involved in the cascade leading to the detected cellular damages caused by the co-culture.

In conclusion, the present findings showed clearly that the toxic mechanisms activated in macrophages during microbial exposure include cytotoxicity, oxidative stress, genotoxicity and inflammation associated injury. In addition, microbial interactions may significantly change the immunotoxic characteristics of the inhaled particles and this may explain, at least in part, the adverse health effects observed in damp indoor environments where there may be relatively low microbial concentrations. These kinds of interactions should be carefully considered when evaluating the health effects experienced by occupants of moisture-damaged buildings.

Keywords: Cell death, Oxidative stress, DNA damage, Inflammation, *in vitro*, Microbial interaction, Indoor air, *Streptomyces californicus*, *Stachybotrys chartarum*

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TIIVISTELMÄ

Rakennusten kosteusvaurioihin liittyvän mikrobialtistuksen yhteys erilaisiin terveyshaittoihin on osoitettu lukuisissa väestötutkimuksissa ympäri maailmaa, mutta terveyshaittojen mekanismit tunnetaan toistaiseksi huonosti. Aiemmat kokeelliset tutkimukset ovat osoittaneet, että osa kosteusvauriorakennuksista eristetyistä mikrobeista pystyy aiheuttamaan voimakkaita tulehdusreaktiota ja solukuolemaa. Ihmiset altistuvat kuitenkin aina samanaikaisesti useille mikrobilajeille, niiden itiöille, aineenvaihduntatuotteille ja muille sisäilman epäpuhtauksille. Nämä vuorovaikutukset voivat muuttaa yksittäisten mikrobien toksisia ominaisuuksia. Toistaiseksi ei kuitenkaan tiedetä miten mikrobien väliset vuorovaikutukset muuttavat solutason mekanismien käynnistymistä ja edelleen terveyshaittojen syntymistä.

Tässä tutkimuksessa selvitettiin kahden kosteusvauriorakennuksista eristetyn mikrobin *Streptomyces californicus* aktinobakteerin ja *Stachybotrys chartarum* homesienen yhteisvaikutuksia käynnistyviin immunotoksisiin soluvasteisiin. Erityisesti haluttiin verrata sekä yhdessä että erikseen kasvatettujen mikrobi-itiöiden kykyä aiheuttaa solukuolemaa, tulehdusreaktioita, perimävauriota ja oksidatiivistä stressiä hiiren RAW264.7 makrofagisolulinjassa.

Tutkimusten tulokset osoittivat, että *S. californicus* bakteerin itiöt pystyivät aiheuttamaan solukuolemaa, sytostaattisia vaikutuksia, perimävaurioita ja käynnistämään tulehdusvälittäjäainetuotannon makrofageissa. *S. chartarum* homesienen itiöt puolestaan aiheuttivat merkittävää solukuolemaa vasta suhteellisen korkealla annoksella, mutta ne eivät käynnistäneet tulehdusvälittäjäainetuotantoa, aiheuttaneet perimävaurioita tai sytostaattisia vaikutuksia. Altistettaessa makrofageja samanaikaisesti näiden kahden erikseen kasvatetun mikrobin itiöille havaittiin, että tulehdusvälittäjätuotanto joko nousi tai laski riippuen näiden mikrobi-itiöiden suhteellisista osuuksista altistuksen aikana.

Mikrobien yhteiskasvatus stimuloi erittäin toksisen, toistaiseksi tuntemattoman yhdisteen/yhdisteiden tuotantoa. Tämän vuoksi yhteiskasvatettujen mikrobien itiöt aiheuttivat voimakkaammat immunotoksiset vaikutukset makrofageissa kuin

erikseen kasvaneiden mikrobien itiöseos. Yhteiskasvatuksen aikana muodostunut yhdiste/yhdisteet aiheutti perimävaurioita, sytostaattisia vaikutuksia ja solukuolemaa. Sen käynnistämä apoptoottisen solukuoleman mekanismi oli samankaltainen streptomykeetti-peräisten syöpälääkeaineiden, doxorubisiinin ja aktinomysiini D:n, kanssa. Altistamalla soluja samanaikaisesti yhteiskasvatettujen mikrobien itiöille ja antioksidatiiviselle N-asetyyli-L-kysteiinille, pystyimme osoittamaan että oksidatiivinen stressi oli kaikkien havaittujen solutason vaikutusten takana.

Nämä tutkimustulokset osoittivat selkeästi solukuoleman, oksidatiivisen stressin, perimävaurioiden ja tulehdusreaktioiden olevan juuri niitä mekanismeja, jotka aktivoituvat makrofageissa, kun ne altistuvat näille kosteusvauriorakennuksista eristetyille mikrobeille. Lisäksi tulokset viittaavat siihen, että mikrobien yhteisvaikutukset voivat merkittävästi muuttaa näiden hengitettävien altisteiden immunotoksisia ominaisuuksia. Tämä voi osittain selittää terveyshaittojen syntymistä kosteusvauriorakennuksissa altistuvilla ihmisillä, vaikka sisäilman mikrobipitoisuudet näissä kohteissa ovatkin varsin alhaisia. Kosteusvauriorakennuksissa esiintyvän monimuotoisen mikrobilajiston väliset yhteisvaikutukset pitäisi ottaa huomioon arvioitaessa kosteusvauriokohteissa altistuneiden ihmisten terveyshaittoja ja riskejä.

Asiasanat: Solukuolema, oksidatiivinen stressi, perimävaurio, tulehdus, solututkimus, mikrobien yhteisvaikutus, sisäilma, *Streptomyces californicus*, *Stachybotrys chartarum*

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ABBREVIATIONS

ALS	Alkali-labile sites
AMD	Actinomycin D
ANOVA	Analysis of variance
APAF-1	Apoptosis proteinase activating factor 1
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
Bid	BH3 interacting domain
BSA	Bovine serum albumin
CD	Clusters of differentiation
CO_2	Carbon dioxide
Co-culture	The spores of co-cultivated *Streptomyces californicus* and *Stachybotrys chartarum*
cfu	Colony forming unit
DCF^+	2’, 7’-dichlorofluorescein
DED	Death effector domain
DEVD	Synthetic peptide Asp-Glu-Val-Asp
DISC	Death inducing signaling complex
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
ELISA	Enzyme-linked immunosorbent assay
FADD	Fas-associated death domain
Fas	Fibroblast-associated cell surface
FBS	Fetal bovine serum
FL	Fluorescence channel
FS	Forward scatter
G_1	Cells with no DNA synthesis in process

G_2	Cells with duplicated DNA, phase before mitosis
HBSS	Hank's balanced salt solution
H_2DCFDA	2', 7'-dichlorodihydrofluorescein diacetate
H_2O_2	Hydrogen peroxide
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LPS	lipopolysaccharide
M	Cells undergoing mitosis
MEA	Malt extract agar
MIP2	Macrophage inflammatory protein 2
Mixture	The spore-mixture of separately cultivated *Streptomyces californicus* and *Stachybotrys chartarum*
MMC	Mitomycin C
MMS	Methanesulphonate
mtDNA	Mitochondrial DNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NAC	N-Acetyl-L-cysteine
NO	Nitric oxide
NO_2^-	Nitrite
NOS	Nitric oxide synthases
$^{\bullet}O_2^-$	Superoxide
$OH^{\bullet}$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
p53	Tumor suppressor protein
PBS	Phosphate buffered Saline
PHLEO	Phleomycin

PI	Propidium iodide
RAW264.7	Mouse macrophage cell line, RAW264.7
RNS	Reactive nitrogen species
RPMI	Roswell Park Memorial Institute
ROS	Reactive oxygen species
S	Cells with DNA synthesis in process
SCG	Single cell gel
SDS	Sodium dodecyl sulphate
SEM	Standard error of mean
SSB	Single-strand breaks
SS	Side scatter
Sta	The spores of *Stachybotrys chartarum*
Stre	The spores of *Streptomyces californicus*
Sub G_1	Apoptotic cells
tBid	Truncated Bid
TNFα	Tumor necrosis factor alpha
TRAIL	Tumor necrosis factor related apoptosis inducing ligand
TYG	Tryptone yeast glucose agar
$\Delta\Psi m$	Mitochondrial membrane permeability

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I **Penttinen P.**, Huttunen K., Pelkonen J., Hirvonen M.-R. (2005) The proportions of *Streptomyces californicus* and *Stachybotrys chartarum* in simultaneous exposure affect inflammatory responses in mouse RAW264.7 macrophages. Inhalation Toxicology 17:79-85.

II **Penttinen P.**, Pelkonen J., Huttunen K., Toivola M., Hirvonen M.-R. (2005) Interactions between *Streptomyces californicus* and *Stachybotrys chartarum* can induce apoptosis and cell cycle arrest in mouse RAW264.7 macrophages. Toxicology and Applied Pharmacology 202:278-288.

III **Penttinen P.**, Pelkonen J., Huttunen K., Hirvonen M.-R. (2006) Co-cultivation of *Streptomyces californicus* and *Stachybotrys chartarum* stimulates the production of cytostatic compound(s) with immunotoxic properties. Toxicology and Applied Pharmacology 217:342-351.

IV **Penttinen P.**, Tampio M., Mäki-Paakkanen J., Vähäkangas K., Pelkonen J., Hirvonen M.-R. (2007) DNA damage and p53 in RAW264.7 cells induced by the spores of co-cultivated *Streptomyces californicus* and *Stachybotrys chartarum*. Toxicology 235:92-102.

V **Markkanen P. (Penttinen P.)**, Pelkonen J., Tapanainen M., Mäki-Paakkanen J., Jalava P.I., Hirvonen M.-R. (2008) Co-cultivated damp building related microbes *Streptomyces californicus* and *Stachybotrys chartarum* induce immunotoxic and genotoxic responses via oxidative stress. Inhalation Toxicology in press.

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1 INTRODUCTION

Moisture damage and microbial growth in buildings have been associated with adverse health effects in several epidemiological studies (Bornehag et al., 2001, 2004; IOM, 2004; Peat et al., 1998). Airborne microbial particles consisting of spores, cells, structural components as well as biologically active metabolites produced by microbes have been suggested to be possible causative agents of these health effects (Górny, 2004). Although there is a great diversity in the species of microbes in different damp environments, certain microbes e.g. fungal *Stachybotrys* spp. and the actinobacteria, streptomycetes, are often isolated from moisture damaged buildings (IOM, 2004, Nevalainen & Seuri, 2005; Nevalainen et al., 1991). However, the concentrations of microbes and other bioaerosols are relatively low in these indoor environments, and their levels correlate poorly with the detected adverse health effects (Bornehag et al., 2004).

The wide variety of reported adverse health effects attributed to microbial exposure cannot be explained by one single mechanism. Recent *in vitro* and *in vivo* studies have indicated that the toxic mechanisms activated in airways during bioaerosol exposure include cytotoxicity, inflammation associated injury, oxidative stress and genotoxicity (Hirvonen et al., 1997; Huttunen et al., 2003; Jussila et al., 2002, 2003; Wang & Yadav, 2006). In addition, previous studies have shown that some of the microbes isolated from moisture damaged buildings display strong inflammatory potency e.g. gram positive bacteria *Streptomyces californicus* and gram negative bacteria *Pseudomonas fluorescens*, whereas some others are extremely cytotoxic e.g. fungi *Stachybotrys chartarum* and *Aspergillus versicolor* (Hirvonen et al., 1997; Huttunen et al., 2003; Wang & Yadav, 2006).

It has been difficult to establish a causal relationship between human exposure and the adverse health effects, since occupants of moisture damaged buildings are exposed to a complex mixture of bioaerosols (Górny, 2004; Hyvärinen et al., 2002). The complexity of the microbial ecosystem leads inevitably to interactions between microbes which are competing for a limited living space and available nutrients. These interactions may change the characteristics of the microbes and the inhaled particles (Huttunen et al., 2004; Meyer & Stahl, 2003; Murtoniemi et al., 2005). Microbial interactions may also explain the various outcomes of apparently similar microbial exposures in different exposure situations. However, there is only a limited amount of toxicological data available on the possible mechanisms accounting for mold-related health effects. In particular, little is known about the role of microbial interactions.

2 REVIEW OF THE LITERATURE

2.1 Adverse health effects associated with indoor air dampness and microbes

The respiratory system is the primary route of entry for gases and particles suspended in the indoor air. Determination of exposure to air contaminants is complicated because indoor air contains a mixture of substances and the concentration of individual toxicants changes with time and location in the exposure mixture. In moisture damaged buildings, individuals can be exposed to a complex mixture of microbial spores, cells, structural components, biologically active metabolites produced by the microbes as well as other bioaerosols during a single breath (Górny, 2004; Hyvärinen et al., 2002). Some parts of the bioaerosol are small enough to be inhaled all the way down to the alveolar level of the lungs, where macrophages become activated and attempt to destroy this foreign material. As a result, the production of inflammatory mediators and the amount of activated cells in the airways increase triggering a local inflammatory reaction (Sibille & Marchandise, 1993). Protracted or excessive inflammation may damage surrounding tissues and lead to the unspecific symptoms typically encountered in occupants living in buildings with mold contamination.

An association between dampness or moisture damage in buildings, mold, microbial growth and adverse health effects has been shown in several epidemiological studies (Bornehag et al., 2001, 2004; IOM, 2004; Peat et al., 1998). Adverse health effects can occur in children and adults, but the health outcomes in buildings with moisture damage vary greatly. Most of the detected adverse health effects are irritation symptoms, recurrent respiratory infections and unspecific neurological or general symptoms (Bornehag et al., 2001; Husman, 1996; IOM, 2004). In addition, certain diagnosable diseases e.g. an increased risk of asthma has been connected with indoor mold exposure (Bornehag et al., 2001; IOM, 2004; Jaakkola et al., 2005; Kilpeläinen et al., 2001; Pekkanen et al., 2007; Zock et al., 2002). Recently, clusters of autoimmune diseases have been also associated with staying in moisture damaged buildings (Luosujärvi et al., 2003).

Thorough renovation of mold and moisture damaged buildings has been shown to decrease the frequency of reported symptoms in the occupants (Meklin et al., 2005). A previous study on teachers working in a moisture and mold damaged school building showed that both the reported symptoms and the levels of inflammatory markers in nasal lavage fluid were higher compared to a control group, and both

symptoms and markers decreased significantly during absence from the moldy environment (Hirvonen et al., 1999). However, there is only a limited amount of toxicological data available on the possible mechanisms accounting for mold-related health effects.

2.2 Microbial exposure agents in moisture damaged buildings

Indoor air quality is important to human health, since the majority of people in the western world spend most of their time indoors. Therefore poor indoor air quality due to microbial growth that is associated with dampness or moisture damage is a common problem in buildings all over the world. Thus, if there are wet building materials and structures, it is only a matter of time before microbes will start to grow, since moisture is often the growth-limiting factor for these micro-organisms. Microbial growth on moisture damaged materials may result in the release of the microbes themselves, their spores, other cell fragments as well as toxins and microbial volatile compounds into the indoor air, and this may impact negatively on the health of subjects living in these environments. This view is supported by indoor exposure data concerning non-infectious bioaerosols both at home and work environments, highlighting their critical importance to many of today's most relevant public health problems.

Previous studies have demonstrated that microbial diversity is larger in moisture damaged buildings than in reference buildings (Hyvärinen et al., 2001a). Although there is no general international consensus about which micro-organisms should be regarded as indicators of the presence of mold, several microbial species are often isolated from moldy areas (IOM, 2004, Nevalainen & Seuri, 2005; Nevalainen et al., 1991). Table 1 shows examples of such microbes.

Table 1. Examples of fungi and other micro-organisms often associated with dampness or mold growth in buildings (Adapted from IOM, 2004).

Aspergillus fumigatus	*Phialophora* spp.	*Wallemia* spp.
Aspergillus versicolor	*Stachybotrys chartarum*	Actinomycetes
Aspergillus penicilloides	*Trichoderma* spp.	Gram-negative bacteria
Exophiala spp.	*Ulocladium* spp.	

The concentrations of viable fungi in indoor air correlate poorly with the detected adverse health effects (Bornehag et al., 2004). Airborne concentrations of viable microbes are usually higher in moisture damaged buildings than in reference buildings, but there are quite considerable spatial and temporal variations (Hyvärinen et al., 2001a, 2001b; Lignell et al., 2005; O'Connor et al., 2004). In many cases, microbial concentrations in moisture damaged and reference buildings can overlap and hence no absolute level can be said to unequivocally indicate the existence of moisture damage (IOM, 2004). However, Nevalainen and Seuri (2005) presented a rough estimation based on previously published data and they suggest that indoor concentrations of viable fungi under 10^2 colony forming unit (cfu)/m^3 can be considered 'low' and those over 10^3 cfu/m^3 can be regarded as 'high'.

In the following section the characteristics of the two microbes, *Stachybotrys chartarum* and *Streptomyces californicus*, investigated in the present thesis will be reviewed more detailed.

2.3 Stachybotrys chartarum

Stachybotrys chartarum is one of the most thoroughly investigated toxigenic fungus found in the indoor air (Hossain et al., 2004; Kuhn & Ghannoum, 2003; Nielsen, 2003). It also goes by the older names *Stachybotrys atra* and *Stachybotrys alternans* and is popularly known as "black mold". The spores of *S. chartarum* are relatively large, aerodynamic diameter 4.6 µm, and they do not readily become airborne due to their slimy structure (Seo et al., 2008). However, airborne secondary metabolites, mycotoxins, produced by *S. chartarum* can be found attached onto smaller particles (Brasel et al., 2005). In fact, the capability of *S. chartarum* to produce a diverse spectrum of highly toxic mycotoxins has been a cause for great public health concern.

2.3.1 Biological effects of *Stachybotrys chartarum*

A previous *in vitro* study revealed that the spores of *S. chartarum* isolated from a moisture damaged building could cause direct cytotoxic responses in mouse RAW264.7 macrophages (Huttunen et al., 2003). Due to potent acute cytotoxicity in most cases, these cells are not able to stimulate the significant production of inflammatory markers such as cytokines, and nitric oxide (NO) *in vitro* (Huttunen et al., 2003). In line with these results, the spore extracted toxins of *S. chartarum* could also cause cytotoxicity, inhibition of cell proliferation and cell death in an alveolar macrophage cell line, but the apoptotic dose of these toxins did not induce any detectable production of inflammatory mediators (Wang & Yadav, 2006). In

addition, these toxins could evoke genotoxic effects such as DNA damage and p53 accumulation. However, it is important to bear in mind that the growth of *S. chartarum* and the subsequent bioactivity of spores (e.g. cytokine production) are highly strain-specific and they are significantly dependent on the composition of their growth environment (Murtoniemi et al., 2003; Ruotsalainen et al., 1998). It is well known that the tendency of environmental microbes to synthesize toxic secondary metabolites to combat other organisms can be altered by different growth factors (Marin et al., 1998; Picco et al., 1999).

S. chartarum is able to produce a diverse spectrum of mycotoxins including two highly toxic trichothecenes i.e. deoxynivalenol (DON) and roridin A (Hossain et al., 2004). The mechanisms explaining the actions of these mycotoxins have been studied more precisely than those induced by the entire spores of *S. chartarum* including all the toxins contained in a single spore. Previous *in vitro* studies demonstrate that mycotoxins produced by *Stachybotrys* spp. can suppress immune function by inducing apoptosis via both the mitochondrial pathway and the death receptor pathway (Miura et al., 2002; Nasage et al., 2002; Yu et al., 2006; Zhou et al., 2005). Certain mycotoxins e.g. DON and citrinin, were shown to be capable of inducing apoptosis by stimulating cytochrome *c* release from mitochondria after mitochondrial membrane depolarization, which was followed by the activation of multiple caspases (Yu et al., 2006; Zhou et al., 2005). Previous *in vivo* studies indicate that *S. chartarum* and its products have the potential to be allergenic, inflammogenic, and cytotoxic, suggesting that this mold indeed has unique bioactivities compared with some of the other fungi encountered in damp buildings (Pestka et al., 2008).

2.4 Streptomyces californicus

Streptomycetes are gram-positive bacteria, which belong to the class actinobacteria (Stackebrandt et al., 1997). Their spores are relatively small, usually below 1 µm (Anderson & Wellington, 2001; Reponen et al., 1998). In moisture damaged buildings, streptomycetes have been isolated from air samples, building materials, and house dust samples (Andersson et al., 1997; Hyvärinen et al., 2002; Nevalainen et al., 1991; Rintala et al., 2004). These bacteria also have important clinical implications, since streptomycetes are capable of producing several biologically active secondary metabolites (Behal, 2000; Demain, 1999; Lazzarini et al., 2000). In fact, it has been estimated that streptomycetes produce more than half of the antibiotics known in 1995 (Demain, 1999).

2.4.1 Biological effects of *Streptomyces californicus*

Previous *in vitro* studies have demonstrated that the spores of *Streptomyces californicus* isolated from a damp indoor environment were able to evoke significant inflammatory reactions such as cytokine production and consequent generation of reactive nitrogen and oxygen species and also they evoke extensive cytotoxicity (Hirvonen et al., 1997; Huttunen et al., 2003; Jussila et al., 1999). In addition to immunostimulation, the spores of *S. californicus* have been shown to decrease the numbers of splenocytes after repeated intratracheal instillation in mice (Jussila et al., 2003). It is well known that streptomycetes are capable of producing several compounds with immunosuppressive properties (Behal, 2000; Demain, 1999; Lazzarini et al., 2000), and they can cause a rapid and massive depletion of lymphocytes, especially in the spleen and lymph nodes (Ferraro et al., 2000). Also in this case, the mechanism of action of the individual compounds produced by streptomycetes has been studied more precisely than those induced by the entire spores of *S. californicus*.

The secondary metabolites produced by streptomycetes include many well known cancer chemotherapeutic agents e.g. doxorubicin (DOX), actinomycin D, mitomycin C and phleomycin, which all are capable of damaging DNA by different mechanisms of action (Chabner et al., 2001). A number of anticancer drugs including DOX exert their effects by inducing apoptosis, which appears to be initiated in most cases through the loss of mitochondrial integrity (Kaufmann & Earnshaw, 2000). Mizutani et al. (2005) showed that DOX induced apoptosis was mainly initiated by oxidative DNA damage, which caused indirect H_2O_2 generation leading to an increase in mitochondrial membrane permeability ($\Delta\Psi m$) and subsequent caspase-3 activation. In addition, DOX induced apoptosis has been reported to involve topoisomerase II inhibition (Mizutani et al., 2005).

2.5 Microbial interactions

Interactions and competition between micro-organisms are inevitable in moisture damaged material, since it forms a habitat for more than one microbial species (Hyvärinen et al., 2002). In moisture damage situations, the environmental conditions as well as the dominant microbial species will vary with time, leading to the appearance and disappearance of different microbes and subsequent changes in the microbial population. Streptomycetes are frequently found in different kinds of damp building materials simultaneously with many other microbes, including *Stachybotrys*, which is most commonly found in gypsum board and also in paper materials (Hyvärinen et al., 2002). Thus, it is obvious that microbial agents may

interact either during the exposure or already during the growth of microbes, and this may lead to marked changes in the characteristics of the inhaled particles (Huttunen et al., 2004; Meyer & Stahl, 2003; Murtoniemi et al., 2005).

2.5.1 Interactions modifying biological effects

Interactions of *S. californicus* together with other microbes frequently isolated in moistured building materials have been studied *in vitro* by using a simultaneous exposure model (Huttunen et al., 2004). A low dose of spores of *S. californicus* with the spores of *S. chartarum* induced a synergistic increase in inflammatory responses such as interleukin-6 (IL-6) production in RAW264.7 macrophages. A similar synergistic effect was found when the metabolites typically produced by *S. chartarum* were tested together with the same low dose of the spores of *S. californicus*.

These results with microbial spores are supported by several studies demonstrating interactions between mycotoxins and the biologically active component of gram-negative bacteria, lipopolysaccharide (LPS). *In vivo* studies indicate that LPS can interact with mycotoxins to modulate the proliferative, cytotoxic and apoptotic processes in a tissue-specific manner, mainly toward the immune system (Islam et al., 2002, 2003; Uzarski et al., 2003; Zhou et al., 2000). Co-exposure of mice to subtoxic doses of LPS and the mycotoxin DON markedly upregulated the proinflammatory cytokine expression and subsequently induced apoptosis (Islam & Pestka, 2003, 2005; Islam et al., 2002, 2003). In addition, roridin A -induced proinflammatory gene expression, apoptosis and inflammation in nasal airways was intensified by simultaneous exposure to LPS (Islam et al., 2007). Both *in vivo* and *in vitro* studies have demonstrated that not only the doses but also the proportions of co-exposures can influence the nature of interactions i.e. they can cause either increased or suppressed inflammatory responses (Zhou et al., 1999; Sugita-Konishi & Pestka, 2001). A synergistic increase in cytokine production was detected after a co-exposure containing more fungal DON than LPS, but no synergistic interactions were seen in cases where LPS was present at high concentrations (Chung et al., 2003; Sugita-Konishi & Pestka, 2001; Zhou et al., 1999). In all these studies, the most potent synergistic interaction occurred when co-exposure contained the lowest amount of the bacterial component, which evoked relatively low cytokine production. Interestingly, both potentiating and suppressive inflammatory responses have also been seen in a human macrophage cell line after co-exposure to fungal DON and bacterial LPS (Sugita-Konishi & Pestka, 2001). While tumor necrosis factor alpha (TNFα) production was synergistically increased in macrophages, the IL-6 response was bidirectional, i.e. causing significantly suppressed IL-6

production when the DON concentration was higher than LPS, and significantly increased IL-6 production in cases where bacterial LPS was prevalent (Sugita-Konishi & Pestka, 2001).

Currently, very little is known about the effects of microbial co-cultures on the potential harmfulness of the microbial population and their associations with detected adverse health effects in moisture damaged buildings. Mayer & Stahl (2003) demonstrated that microbial interactions during co-cultivation could alter the protein expression of micro-organisms. In addition, the influence of co-cultivation was clearly dependent on the growth conditions. It is known that the nutritional conditions provided by the moistured building materials can affect the composition of microbial flora which may lead to the accumulation of certain microbes with high immunotoxic potential such as *Streptomyces* spp. (Murtoniemi et al., 2005). That *in vitro* study also demonstrated that microbial interactions during co-cultivation could modify the inflammatory and cytotoxic potential of the microbial spores even at relatively low concentrations (Murtoniemi et al., 2005). Furthermore, it has been shown that free-living amoebae found in moisture damaged building materials were able to potentiate the cytotoxic and inflammatory properties of *S. californicus* if the species were allowed to grow together (Yli-Pirilä et al., 2007).

In real moisture damage situations, the occupants are simultaneously exposed to multiple microbial agents. In addition, they are exposed to the microbial components or products, which may have been modified by interactions during the growth of the microbes in this microscopic multi-cultural environment. Microbial interactions may explain why there can be such varying outcomes to similar exposures even at the same rather low microbial concentrations in different exposure situations. However, currently rather little is known about the importance of microbial interactions and their role in the development of adverse health effects in the occupants of moisture damaged buildings.

2.6 Important immunotoxic mechanisms activated by non-infectious microbial exposures

Exposure to various fungal products including mycotoxins and substances produced by bacteria that grow in damp environments has been implicated in a variety of immunotoxic responses in experimental settings as well as suspected of damaging the health of occupants of moldy buildings. Mechanisms of inflammation, cytotoxicity, genotoxicity and oxidative stress will be described in the following short overview more thoroughly, since these are crucial to understanding the results of this study.

2.6.1 Inflammation

Inflammation is a normal protective response that is triggered by noxious stimuli and conditions, such as infection and tissue injury (Medzhitov, 2008). Inflammation attempts to destroy, dilute, or isolate foreign agents and to promote the repair of injured tissue. A controlled inflammatory response is beneficial, but it can become detrimental if dysregulated. The typical signs of inflammation are redness, swelling, pain and warmth in the inflamed area. Acute inflammatory response involves the coordinated delivery of blood components to the site of infection or injury. Cells located at the site of exposure are in a crucial position in the generation of inflammation.

Macrophages (a name derived from two Greek words – *macros* 'big' and *phagos* 'eater') are large phagocytic cells derived from the monocytes in the bloodstream. Alveolar macrophages are found on the alveolar walls, and help to defend the lung against inhaled bacteria and other particles. In addition to killing phagocytosed microbes, macrophages possess many other functions in the host defense against infections with many of these functions being mediated by cytokines. Activated macrophages can also convert molecular oxygen into reactive oxygen species (ROS). In addition to ROS, macrophages can produce reactive nitrogen species (RNS), mainly nitric oxide (NO). (Abbas et al., 2007) The inflammatory response is coordinated by a large range of mediators that form complex regulatory networks. The inflammatory mediators measured in this study will be discussed below in more detail.

Cytokines

Cytokines are one class of signaling molecules that regulate inflammatory processes and are important host factors influencing the response to noxious agents. The cytokines are small, nonstructural proteins and they are grouped into different classes by their biological activities (Dinarello, 2000). The cytokines have several roles in the inflammatory response and they are produced mainly by activated macrophages. There are several proinflammatory cytokines, that TNFα and IL-1 are considered as the crucial cytokines of the early response to the inflammatory stimuli (Dinarello, 2000). These compounds are the mediators of acute inflammatory reactions to microbes and are able to induce the production of other important cytokines such as IL-6 and chemokines. Subsequently IL-6 is involved in the initiation and extension of the inflammatory process, since it stimulates the synthesis of acute phase protein and also the growth and differentiation of T and B lymphocytes (Abbas et al., 2007). TNFα and IL-6 are involved in most types of inflammation and appear to amplify the ongoing inflammatory response, whereas

chemokines e.g. macrophage inflammatory protein 2 (MIP2) are chemotactic compounds attracting inflammatory cells such as macrophages and leukocytes to the site of inflammation (Driscoll et al., 1997). While proinflammatory cytokines initiate the cascade of activation of inflammatory mediators, anti-inflammatory cytokines e.g. IL-10 block this process or at least suppress the intensity of the cascade by inhibiting activated macrophages. Therefore, the balance between the effects of proinflammatory and anti-inflammatory cytokines is thought to determine the outcome of disease (Abbas et al., 2007; Dinarello, 2000; Driscoll et al., 1997).

Nitric oxide

Nitric oxide (NO) is a hydrophobic molecule and a highly diffusible free radical generated from l-arginine by a family of constitutive or cytokine-inducible NO synthases (NOS). Neuronal and endothelial NOS are constitutively expressed in selected tissues but these enzymes are able to produce relatively small amounts of NO (Alderton et al., 2001). In contrast, expression of the cytokine-inducible NOS (iNOS) is triggered only by the appropriate stimuli (e.g. TNFα, LPS) but this enzyme can synthesize relatively high amounts of the radical. NO is a biologic effector molecule with a broad range of activities. In macrophages, it functions as a potent microbicidal agent intended to kill any inhaled organisms. The effects of NO are believed to be dose-dependent and cell-type specific. At relatively low concentrations, NO is a useful intercellular messenger, but at high concentrations NO can damage cells in many ways, e.g. through involving oxidative stress, DNA damage, protein modification, disruption of energy metabolism or mitochondrial dysfunction (Li & Wogan, 2005). Depending on the context and severity of the damage, such disturbances may result in cell death either by necrosis or by apoptosis. It has been shown that NO can either promote or inhibit apoptosis depending on the cell line and the NO concentration, i.e. a high NO concentration in general induces while a low concentration inhibits apoptosis (Kim et al., 2001; Mannick, 2006). NO has been shown to inhibit apoptosis in pulmonary epithelial cells, whereas in macrophages it seems to promote apoptosis. NO induced apoptosis is often accompanied by an accumulation of the tumor suppressor protein p53, changes in the expression of proapoptotic and antiapoptotic Bcl-2 family members, caspase-3-like protease activation and cytochrome c translocation (Kim et al., 2001; Li & Wogan, 2005; Meβmer et al., 1994).

2.6.2 Cytotoxicity

There are many types of cell death defined by the morphological or biochemical behaviour of the cell. Severely injured cells may undergo necrosis. However cells can die in a more physiological manner and the best known form of this type of cell death, apoptosis, is described in more detail below. In addition, cells can die by autophagic mechanisms and there are also cell death forms that show some similarities to either necrosis or apoptosis (Lockshin & Zakeri, 2004).

Necrosis versus apoptosis

The route of necrosis is activated when a cell is suddenly confronted with a severe stress and it cannot undergo apoptosis. During necrotic cell death, there is cell and organelle swelling, loss of integrity of mitochondrial, peroxisomal and lysosomal membranes and eventually rupture of the plasma membrane, releasing cell contents into the surrounding area to affect adjacent cells (Lockshin & Zakeri, 2004). Several of the constituents released from cells undergoing necrosis can provoke inflammation. The process is not stepwise and may follow different sequences. In contrast, in apoptosis, the cells's own intrinsic suicide mechanism is activated; apoptosing cells do not release their contents and thus apoptosis does not in general affect the surrounding cells. During apoptosis, there is cell shrinkage, chromatin condensation and fragmentation and also nuclear fragmentation. Eventually the entire cell disintegrates into apoptotic bodies, without rupture of the cell membrane, and these are phagocytosed by neighbouring cells. In fact, apoptotic cells display an "eat me signal" on their surface to promote their recognition and rapid uptake by adjacent healthy cells, and thus prevent inflammation and secondary tissue damage (Halliwell & Gutteridge, 2007, Lockshin & Zakeri, 2004).

Apoptosis

Most of the characteristics of apoptosis are controlled by the activation of proteases called caspases (cysteine-aspartyl-specific proteases), but there are also forms of apoptosis that are considered to be independent of caspase activation. The effector caspases such as caspase-3 and caspase-7 typically exist in a proenzyme form in the cytoplasm and are proteolytically activated by the initiator caspases such as caspase-8 and caspase-9. These two caspases are activated in different ways i.e. caspase-8 via death receptors and caspase-9 via mitochondria (Figure 1).

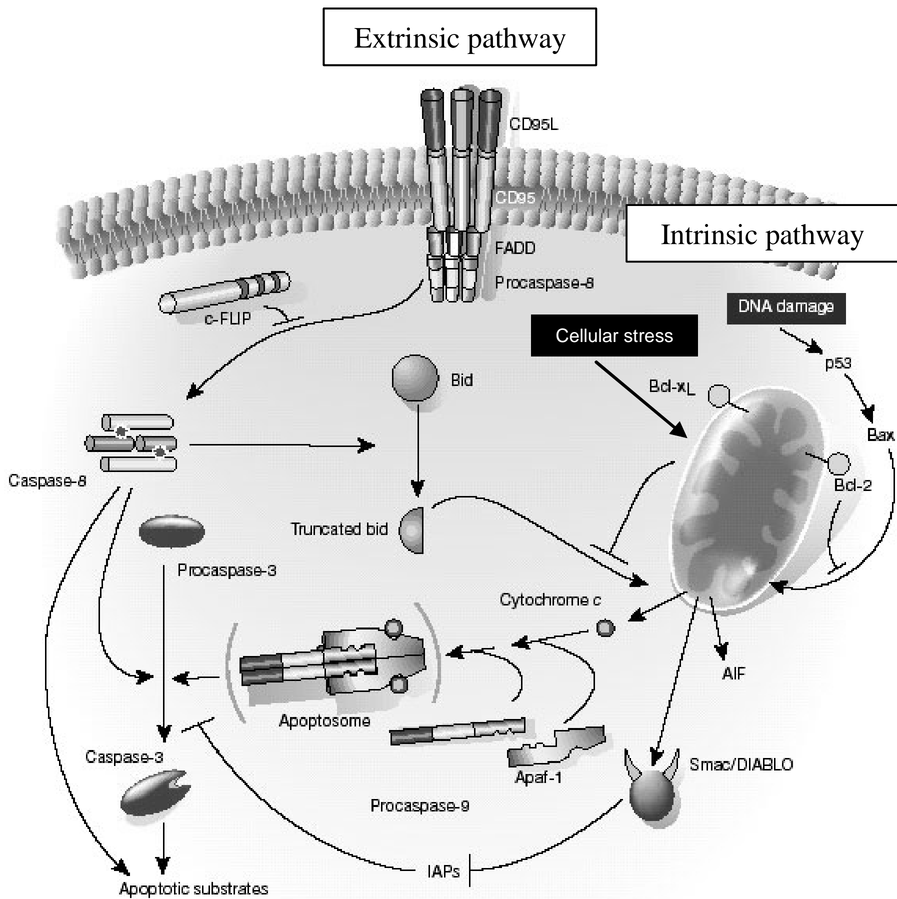

Figure 1. Two major apoptotic pathways in mammalian cells (Modified from Hengartner, 2000; http://users.aber.ac.uk/lum/apoptosis3.htm).

The death receptor pathway 'extrinsic pathway' is initiated when external ligands (e.g. TNFα, Fas/CD95, TRAIL) bind to cell surface death receptors (e.g. TNFα receptor, Fas, TRAIL receptor 1 or 2, respectively) and cause them to aggregate into trimers. The cytoplasmic tails of these receptors recruit other proteins such as Fas-associated death domain (FADD). This carries a death-effector domain (DED) that binds procaspase-8 and incorporates it into the death-inducing-signalling complex (DISC). Caspase-8 is activated and this in turn activates downstream effector caspases such as caspase-3. (Hengartner, 2000; Thorburn, 2004)

Several intracellular death signals including oxidative stress or DNA damage have been shown to activate the mitochondrial 'intrinsic pathway', which is controlled by the Bcl-2 family proteins. Some of these proteins suppress apoptosis (e.g. Bcl-2, Bcl-X_L, Bcl-w, Bcl-B) whereas others promote it (e.g. Bax, Bak, Bcl-X_S). The balance between these various proteins controls the likelihood of mitochondria triggering apoptosis. These proteins can permeabilize the outer mitochondrial membrane and trigger a release of pro-apoptotic molecules such as cytochrome c from the intermembrane space to the cytosol (Hengartner, 2000; Lockshin & Zakeri, 2004). The released cytochrome c interacts with the apoptotic proteinase activating factor-1 (Apaf-1), procaspase-9 and ATP to form a complex called the apoptosome, which activates caspase-9 and subsequently other caspases, including caspase-3 (Li et al., 1997).

In addition, there is a special link between caspase activation triggered by any mechanism and mitochondrial function. The Bcl-2 family protein, Bid, can be responsible for bridging signals from the death receptor pathway to the mitochondrial pathway. Bid is normally present in the cytosol and can be activated by caspase-8. The activated tBid can efficiently translocate to mitochondria and further activate other Bcl-2 proteins and facilitate cytochrome c release (Yin & Dong, 2003). Both pathways are considered to join at the level of caspase-3, which is thought to trigger the final execution of apoptosis and its characteristic morphological manifestation.

2.6.3 Genotoxicity

The cascade leading to DNA damage can be activated by many factors e.g. endogenous reactive oxygen or nitrogen species, stochastic errors in replication, recombination of DNA strands or environmental and therapeutic genotoxic compounds. DNA damage in mammalian cells is associated with cell cycle arrest, a process which activates the DNA repair machinery (Houtgraaf et al., 2006; Lin et al., 1999; Robles et al., 1999). If this process fails to repair the damage, then the cell cycle can be blocked permanently, triggering apoptotic cell death (Houtgraaf et al., 2006). The role of nuclear DNA damage in initiation of cell death has been extensively studied, but some anticancer drugs induce damage also in mitochondrial DNA (mtDNA). Damage to mtDNA, if not repaired, could lead to disruption of the electron transport chain and the production of reactive oxygen species (Norbury & Zhivotovsky, 2004).

The tumor suppressor protein p53

The tumor suppressor protein p53 is widely considered to be the major sensor of genotoxic stress and it represents the critical link between DNA damage, cell cycle arrest and apoptosis (Levine et al., 2006). Cells with wild-type p53 typically respond to genotoxic stress by arresting the cell cycle and repairing damaged DNA, followed by either survival or apoptosis. The decision whether to undergo cell cycle arrest or to trigger apoptosis is related to the extent of the remaining damage, the cellular context, the environment and also the p53 levels. DNA damage may induce the apoptotic response to p53 without altering the p53 protein levels in cells (Chen et al., 1996). A complex network of p53-regulated genes is involved in cell cycle arrest and apoptosis (Chipuk & Green, 2006; Levine et al, 2006). In the nucleus, p53 directly regulates the expression of pro-apoptotic Bcl-2 family proteins, which are essential for mitochondrial membrane permeabilization. In cytoplasm, p53 regulates Bcl-2 proteins by post-transcriptional mechanisms. These pro-apoptotic genes can activate both extrinsic and intrinsic pathways, leading to mitochondrial membrane permeabilization and cytochrome c release. In addition, DNA damage signaling processes in apoptosis can be p53-independent event, but they also result in mitochondrial membrane permeabilization (Norbury & Zhivotovsky, 2004).

2.6.4 Oxidative stress

Oxidative stress is an imbalance between the formation of free radicals and the capacity of the antioxidant defences to remove these reactive molecules. The oxidative stress can result from 1) a diminished amount of antioxidants or 2) an elevated production of reactive oxygen (ROS) or nitrogen species (RNS). Oxidative stress can cause increased proliferation, cell death, senescence and also cell injury including damage to DNA, proteins and lipids. It has been implicated in a number of human diseases e.g. atherosclerosis, diabetes, ischemia-reperfusion, cancer, inflammatory diseases, Parkinson's disease and Alzheimer's disease. (Dröge, 2002; Halliwell & Gutteridge, 2007) Figure 2 illustrates in more detail how cells respond to oxidative stress.

ROS is a collective term that includes both oxygen radicals and certain nonradicals that are oxidizing agents and/or are easily converted into radicals (Halliwell & Gutteridge, 2007). Mitochondria are the major source of ROS, especially superoxide ($^{\bullet}O_2^-$). These highly reactive radicals are generated via aberrant O_2 reactions. Once generated, superoxide is converted both spontaneously and by various forms of superoxide dismutase into hydrogen peroxide (H_2O_2), which may react further, forming the reactive hydroxyl radical ($OH^{\bullet}$). Alternatively, superoxide may react with nitric oxide to form peroxynitrite ($ONOO^-$).

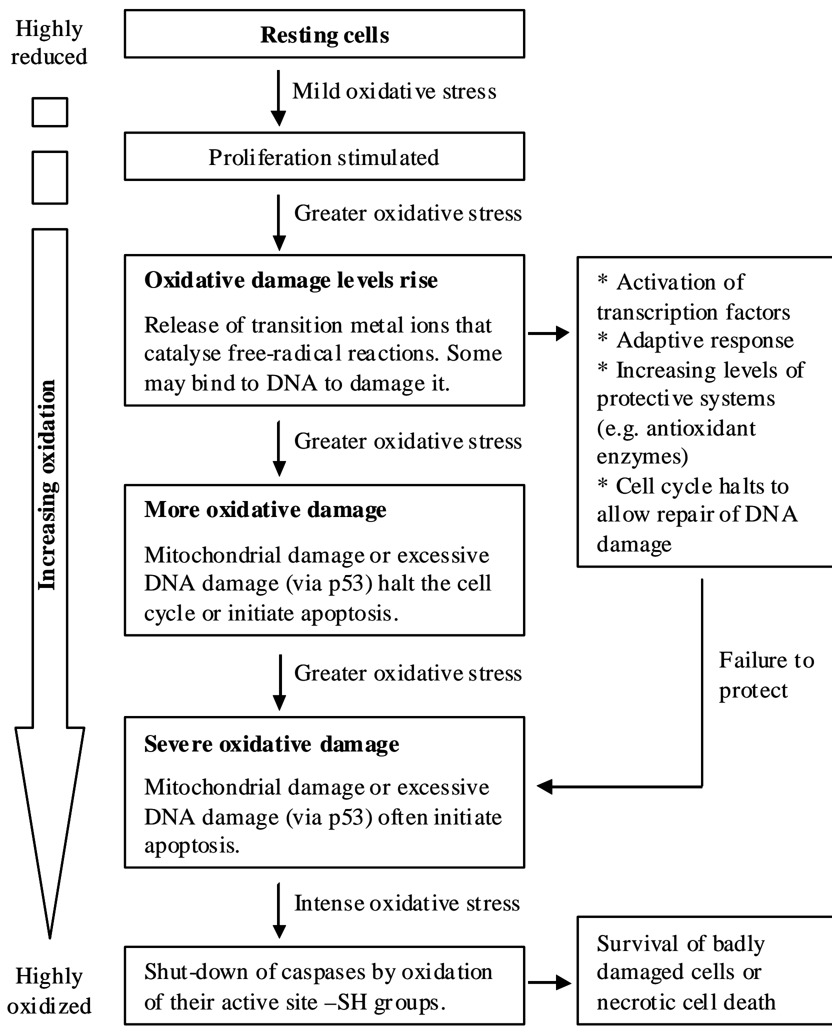

Figure 2. How cells respond to oxidative stress (Modified from Halliwell & Gutteridge, 2007)

Antioxidants are substances that delay, prevent or remove oxidative damage to a target molecule (Halliwell & Gutteridge, 2007). Antioxidants serve to regulate the levels of free radicals, permitting them to perform useful biological functions while minimizing damage. Proteins such as superoxide dismutase, catalase and glutathione peroxidase are enzymatic antioxidants. Many dietary constituents e.g. vitamin C and vitamin E have also been proposed to act as antioxidants. One of the most potent anti-inflammatory and antioxidant agents with several clinical roles is N-acetyl-L-cysteine, NAC (Sochman, 2002).

The productions of ROS and cytokines are closely related with each other. As discussed previously, macrophages are a rich source of proinflammatory cytokines, and these cytokines are often produced in response to oxidative stress. On the contrary, many cytokines affect ROS production by macrophages. For example, the anti-inflammatory cytokine, IL-10, can suppress inflammation by reducing the rate of ROS formation. (Halliwell & Gutteridge, 2007)

Under normal physiological circumstances, there is a balance between the production of ROS/RNS and the levels of the antioxidant defences. At moderate concentrations, ROS play an important role as regulatory mediators in signaling processes and participate directly in the defense against infection (Dröge, 2002; Fialkow et al., 2007; Sauer et al., 2001). Alveolar macrophages are the first line of phagocytic defense against inhaled particles; this is a mechanism that itself evokes an abrupt increase of ROS (Zhang et al., 2000). Macrophages phagocytose not only microorganisms but also dead cells and insoluble material. At the onset of phagocytosis, a marked increase in O_2 uptake during the respiratory burst can be detected. Oxygen uptake is due to the activation of an enzyme complex in that part of the plasma membrane that forms the phagocyte vacuole, and the engulfed particles are exposed to a flux of ROS inside the phagocytic vacuole (Halliwell & Gutteridge, 2007).

3 AIMS OF THE STUDY

The aim of this thesis was to investigate the impact of microbial interactions on induced immunotoxicological responses *in vitro*. The focus of the studies was on cytotoxicity, inflammation, genotoxicity and oxidative stress activated in RAW264.7 macrophages by the spores of actinobacterium *Streptomyces californicus* and the toxic fungus *Stachybotrys chartarum* using both co-exposure and co-cultivated spore mixtures.

The more specific aims were:

1. To develop and apply several new flow cytometric methods for the analysis of the immunotoxic mechanism activated by damp building related microbes: live gate analysis, DNA content analysis, assay for mitochondria membrane depolarization, PI exclusion test and ROS analysis (I-V). In addition, a fluorometric caspase-3 assay was utilized in studies II-III.
2. To study the cytotoxic (II, IV), genotoxic (IV) and inflammatory (II) responses induced by the spores of *S. californicus* or *S. chartarum* alone.
3. To study whether microbial interactions during co-exposure in different proportions affect their abilities to evoke inflammatory and cytotoxic responses (I)
4. To study whether microbial interactions during co-cultivation affect their abilities to evoke cytotoxic (II, III), genotoxic (IV) and inflammatory (II) responses.
5. To investigate the mechanism and kinetics of apoptosis induced by the spores of co-cultivated microbes and to search for plausible microbial metabolites produced during the co-cultivation. (III)
6. To evaluate the involvement of oxidative stress in the detected cytotoxic, genotoxic and inflammatory responses (V)

4 MATERIALS AND METHODS

4.1 Cell line (I-V)

The mouse macrophage cell line RAW264.7 was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cell line was established from a tumor of adult BALB/c mouse induced by Abelson murine leukemia virus. The cells were cultured at +37°C in a moist atmosphere of 5 % CO_2 in RPMI 1640 media supplemented with 10 % of heat inactivated fetal bovine serum (FBS), 2 mM l-glutamine and 100 U/ml penicillin-streptomycin (all from Gibco, Paisley, UK). In the experiments, the cell suspension was diluted to 5×10^5 cells/ml, and 2 ml of the cell suspension was dispensed to each well of 6-well plates. The cells were allowed to adhere for 24 hrs, and fresh complete medium was added 1 h before exposure.

4.2 Exposure agents

4.2.1 Microbial strains (I-V)

Actinobacterium *Streptomyces californicus* (KTL no. A4) was isolated from the indoor air of a building with moisture problems using a six-stage impactor and tryptone yeast-glucose agar (TYG; Bacto Plate Count Agar, Difco Laboratories, Detroit, MI, USA). Fungus *Stachybotrys chartarum* (KTL no. HT580) was isolated from a moisture damaged building material sample on 2 % malt extract agar (MEA; Biokar Diagnostics, Beuvais, France). The identification of *S. californicus* and *S. chartarum* was confirmed by the DSM identification service (DSMZ-Deutsche Sammlung von Microorganismen und Zellkulturen, Germany) and the CBS identification service (Centraalbureau of Schimmelcultures, Utrecht, The Netherlands), respectively.

The microbial strains were stored at -20°C until the experiments. Both strains were cultivated separately as a dense culture on 2 % MEA, although this was not optimal for either of the microbes. However, this compromise had to be made, since *S. californicus* was co-cultivated with *S. chartarum* on the same plate. Based on the results of our preliminary studies, we inoculated microbes by using a ratio of 100:1 (*S. californicus* : *S. chartarum*). The plates were incubated at +25°C in the dark until the microbes sporulated (17-40 days). Subsequently, the spores were collected with a sterile loop and suspended in Hank's Balanced Salt Solution (HBSS) (Gibco, Paisley, UK) containing 0.0001 % Triton X-100. The spore concentrations and the

proportions of each microbe were determined by counting the spores after acridine orange staining using an epifluorescence microscope (Hobbie et al., 1977). At the end of the co-cultivation, the used inoculation ratio (100:1) was determined to be a final proportion of 5:1 of these microbes (*S. californicus* : *S. chartarum*). The mixture of the spores of separately cultivated *S. californicus* and *S. chartarum* (the mixture) was prepared at the same ratio as that determined at the end of the co-cultivation of these microbes (the co-culture), and the spores were stored at -80°C. Before the experiments, the spore suspension was sonicated for 30 min in a water bath sonicator (FinnSonic m03) to ensure a homogeneous spore suspension, and thereafter diluted with HBSS or sterile water.

4.2.2 Chemotherapeutic drugs (III)

Doxorubicin (DOX), actinomycin D (AMD), mitomycin C (MMC) and phleomycin (PHLEO) (Sigma-Aldrich Corp., St. Louis, MO, USA) were used as model cytostatic compounds produced by streptomycetes. The stock solutions were stored at the appropriate temperature (DOX 0.1 mM -20°C; AMD 0.4 mM +4°C; MMC 1.5 mM +4°C; PHLEO 1 mM -20°C, in sterile water) and diluted with HBSS before the experiments.

4.2.3 Other chemicals (I-V)

Etoposide (DNA content, $\Delta\psi_m$ and Caspase-3 analyses), menadione (ROS analysis), lipopolysaccharide (LPS, cytokine analysis) and methyl methanesulphonate (MMS, Comet assay) were used as reference agents for biological responses (all from Sigma-Aldrich Corp., St. Louis, MO, USA). HBSS or sterile water was used as a negative control in these experiments. N-acetyl-L-cysteine (NAC, Sigma-Aldrich Corp., St. Louis, MO, USA) was used as a ROS scavenger in study V.

4.3 Experimental design (I-V)

In study I, the microbial interactions during co-exposure were studied by exposing RAW264.7 cells simultaneously to the spores of separately cultivated *S. californicus* and *S. chartarum* at five different proportions (*S. californicus* : *S. chartarum* 10:1, 5:1, 1:1, 1:5 and 1:10, total dose 3×10^5 spores/ml). In addition, cells were exposed to the spores of these microbes alone using the same amount of spores as in the respective combination of microbes.

In studies II-V, the microbial interactions during co-cultivation were studied by exposing cells in a dose- and time-dependent manner to the spores of co-cultivated

S. californicus and *S. chartarum* and also their separately cultivated spore-mixture (the spore ratio was 5:1 in both combinations). Since the co-culture and the mixture contain five times more bacterial spores of *S. californicus* than the fungal spores of *S. chartarum*, the cells were exposed to spores of either *S. californicus* or *S. chartarum* alone by using the same amount of spores as in the respective combination of microbes (Table 2). In addition, the cells were exposed to graded doses of chemotherapeutic drugs produced by streptomycetes in study III. After exposure, cellular responses were studied as presented in Figure 3 by using the analyzing methods summarized in Table 3. The involvement of oxidative stress in the detected responses was evaluated by using the ROS scavenger NAC simultaneously with the microbial exposure in study V.

Table 2. The microbial co-culture and the spore-mixture used in the studies II-V. The microbial co-culture and the spore-mixture contained five times more spores of *S. californicus* than spores of *S. chartarum* (5:1). Exposures to the spores of either *S. californicus* or *S. chartarum* alone contained the same amount of spores than the respective combination of microbes. The two lowest spore-doses examined in study II are excluded, since they did not induce any significant immunotoxic responses.

			***S. californicus* and *S. chartarum* (5:1)**	
Dose	***S. californicus*** (spores/ml)	***S. chartarum*** (spores/ml)	Mixture (spores/ml)	Co-culture (spores/ml)
1	0.83×10^5	0.17×10^5	1.0×10^5	1.0×10^5
2	2.50×10^5	0.50×10^5	3.0×10^5	3.0×10^5
3	0.83×10^6	0.17×10^6	1.0×10^6	1.0×10^6
4	2.50×10^6	0.50×10^6	3.0×10^6	3.0×10^6

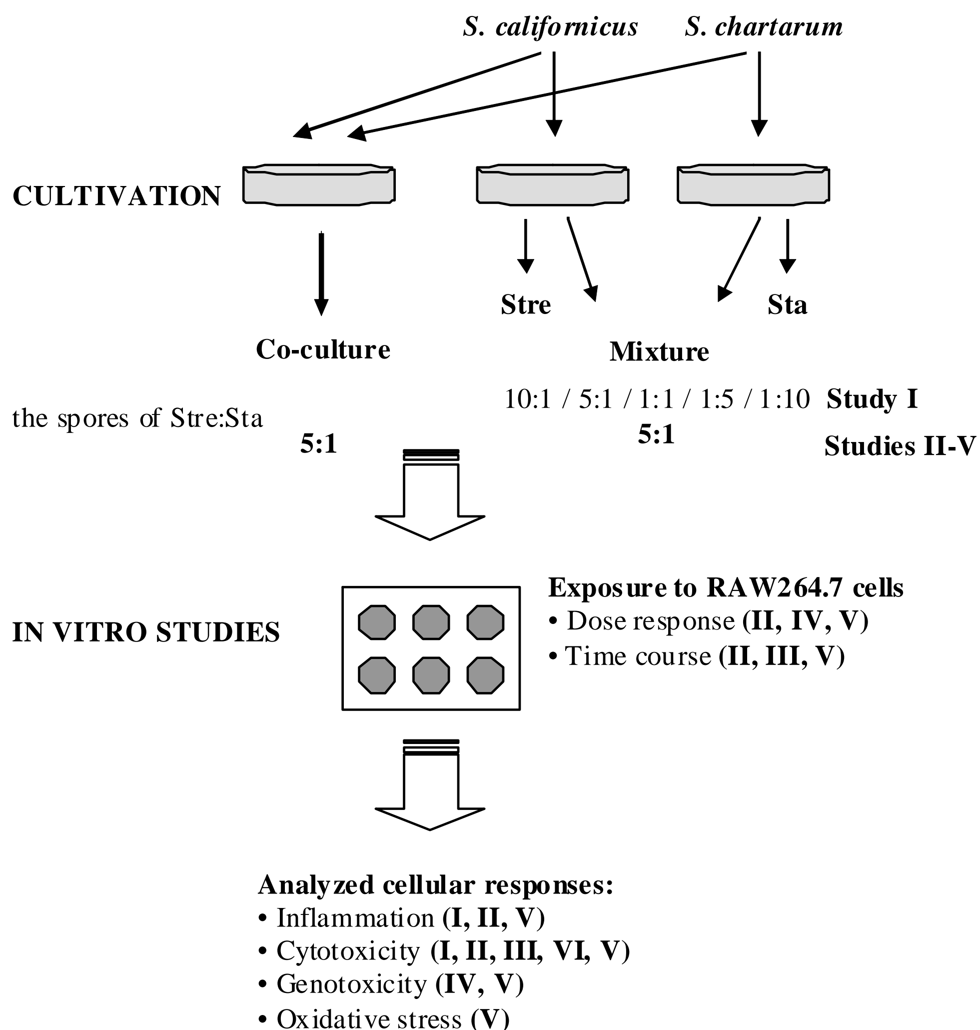

Figure 3. Summary of the experimental design of studies I-V. Mouse RAW264.7 macrophages were exposed to the spores of co-cultivated *Streptomyces californicus* (Stre) and *Stachybotrys chartarum* (Sta), their separately cultivated spore-mixture and the spores of these microbes alone.

Table 3. Measured parameters and methods used in studies I-V.

Parameter	Method	Study
Overall cytotoxicity		
-Cell size	-Live gate analysis	III, IV, V
-Functional mitochondria	-MTT test	I-V
-Cell membrane permeability	-Trypan blue staining	IV
Apoptotic cell death		
-Fragmented DNA	-DNA content analysis	I, II, III, V
-Mitochondria membrane permeability, $\Delta\psi_m$	-Assay for $\Delta\psi_m$	III
-Caspase-3 enzyme activity	-Caspase-3 assay	II, III
Necrotic/late apoptotic cell death		
-Cell membrane permeability	-PI exclusion test	IV, V
-Cell membrane permeability	-Trypan blue staining	IV
Growth arrest		
-Changes in cell cycle	-DNA content analysis	I, II, III, V
Genotoxicity		
-DNA damage	-Comet assay	IV, V
-Cellular p53 level	-p53 by immunoblotting	IV
Oxidative stress		
-Intracellular peroxide production	-ROS analysis	V
-Nitric oxide production	-Nitric oxide analysis	I, II, III, V
Inflammation		
-Cytokine production	-Cytokine analysis	I, II, V
-Nitric oxide production	-Nitric oxide analysis	I, II, III, V

4.4 Cytotoxicity analyses (I-V)

Cytotoxicity was analysed by using several flow cytometric methods: live gate analysis, DNA content analysis, assay for mitochondria membrane depolarization and PI exclusion test.

4.4.1 Live gate analysis (III, IV, V)

Flow cytometric analysis of size and granularity of the cells reveals changes in cell viability (Eray et al., 2001). After exposure, fresh cells were analyzed by selecting normal sized living cells in the live gate based on their forward (FS) and side scatter (SS) profiles. The gate was standardized with control cells and the exposed samples were analyzed by using the same gate. A total of 10,000 cells per sample were collected with a CyAn ADP flow cytometer (Dako, USA) and analyzed by Summit software.

4.4.2 DNA content analysis (I, II, III, V)

DNA content was analyzed by PI staining of permeabilized cells, where apoptotic cells can be identified as the cells containing fragmented DNA (Sub G_1) (Darzynkiewics et al. 1992). This method also provides information about the cell cycle of the nonapoptotic cells. In brief, after exposure, the cells were resuspended into phosphate-buffered saline (PBS Dulbecco's, Gibco, Paisley, UK), fixed with ice cold ethanol (70 % v/v) 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 RNase A (Ribonuclease A, Sigma-Aldrich Corp., St. Louis, MO, USA) for 1 h at +50°C. PI (Sigma-Aldrich Corp., St. Louis, MO, USA) was added at a final concentration of 8 µg/ml and the incubation was continued for 2 hrs at +37°C in the dark before flow cytometric analysis [FACSCalibur, Becton Dickinson, USA, Cell Quest software (papers I, II)/CyAn ADP, Dako, USA, Summit software (papers III, V)].

4.4.3 Mitochondria membrane depolarization, $\Delta\psi_m$ (III)

ApoAlert Mitochondrial Membrane Sensor Kit (Clonetech Laboratories, Inc., CA, USA) was used for detecting changes in $\Delta\psi_m$ during apoptosis. The kit contains a cationic dye MitoSensor™ that fluoresces differently in apoptotic and nonapoptotic cells, because of changes in mitochondrial membrane permeability. The cells were stained and analyzed according to the manufacturer's instructions. Briefly, immediately after the exposure, the cells were collected and resuspended in 500 µl of incubation buffer containing 1 µl MitoSensor™ reagent/ml and incubated for 15

min at +37°C in a 5 % CO_2 atmosphere. After staining, cells were washed with and resuspended in Hepes buffer (10 mM Hepes, 140 mM NaCl, 5 mM $CaCl_2$, pH 7.4). The fluorescence signal of early apoptotic cells inside the live gate was analyzed by flow cytometry (CyAn ADP, Dako, USA) using Summit software with an emission wavelength of 530 ± 40 nm (FL1) and 613 ± 20 nm (FL3).

4.4.4 PI exclusion test (IV, V)

Late apoptotic or necrotic cells with a low cell membrane potential are not capable of excluding propidium iodide (PI). The total amount of dead cells can be detected by flow cytometry. Briefly, the cell suspension was centrifuged (5 min at 370 × g) to separate the cells from the culture medium and washed once with 1 ml phosphate-buffered saline (PBS, Gibco, Paisley, UK). The cells were resuspended into 0.5 ml PBS and PI (f.c. 1 µg/ml, Sigma-Aldrich Corp., St. Louis, MO, USA) was added. The samples were incubated for 15 min at room temperature in the dark before flow cytometric analysis (CyAn ADP, Dako, USA) with an emission wavelength of 613 ± 20 nm (FL3). A total of 10,000 cells per sample were analyzed by Summit software.

4.4.5 Caspase-3 activity assay (II, III)

Activity of caspase-3 was analyzed by measuring the cleavage rate of the synthetic fluorescent substrate Ac-DEVD-AMC (Calbiochem, CA, USA). Briefly, exposed and frozen cells were lyzed in 100 µl of lysis buffer (10 mM Tris, pH 7.5, 1 % Triton X-100 in PBS). After a 40 minute incubation on ice, the cell lysate was centrifuged (13,684 × g, 30 min, +4°C) and the supernatant was used as a cytosolic extract. Cell lysates were added to the 96-well plates in triplicate and samples were incubated with 20 µM Ac-DEVD-AMC fluorogenic substrate and protease assay buffer (20 mM Hepes, pH 7.5, 10 % glycerol, 2 mM dithiothreitol) for 1 h at +37°C in the dark. The released fluorogenic AMC was measured fluorometrically (Fluoroscan Ascent, Labsystems, Finland) with an excitation wavelength of 390 nm and an emission wavelength of 460 nm. The relative fluorescence units were proportioned to total cell content of each sample, which was counted after exposure in a Bürker chamber under a light microscope after staining the cells with Trypan Blue solution (see 4.4.7).

4.4.6 MTT test (I-V)

Cell viability was measured by the MTT test, which is traditional toxicological cytotoxicity test. In the MTT test, exogenously administered MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] is converted to the colored formazan via intact mitochondria of the cells (Mosmann, 1983). Briefly, after the exposure, the cell suspension and MTT-solution (Sigma-Aldrich Corp., St. Louis, MO, USA) were added in duplicate to the 96-well plate. Sodium dodecyl sulfate (SDS) buffer was added after 2 hrs of incubation at +37°C. Incubation was continued overnight and optical densities were measured with a microplate reader [iEMS Reader MF, Labsystems, Finland (papers I-III)/Victor^3, PerkinElmer, Finland (papers IV-V)] at the wavelength of 570 nm. The proportion of viable cells in exposed samples was compared to a control sample.

4.4.7 Trypan blue staining (I-V)

Trypan blue (Sigma-Aldrich Corp., St. Louis, MO, USA) is a vital stain used to selectively stain dead cells, while live cells with intact cell membranes are not coloured. Briefly, cells were counted in Bürker chamber with a light microscope (Olympus BX40, Olympus Co., Japan) after staining with Trypan blue solution (proportion 1:1). Dead cells were shown as a distinctive blue colour under a microscope, while live cells excluded trypan blue.

4.5 Genotoxicity analyses (IV,V)

4.5.1 Single cell gel (SCG)/Comet assay (IV, V)

The alkaline version of the SCG/Comet assay was used to detect DNA damage. The test is capable of detecting single-strand breaks (SSB), alkali-labile sites (ALS), DNA-DNA/DNA-protein cross-linking, and SSB associated with incomplete excision repair sites (Tice et al., 2000). The test was performed as earlier described (Nieminen et al., 2002), with some modifications. After the exposure, the cells (7.5×10^5 cells/ml) were centrifuged ($264 \times g$, 5 min) and suspended in 0.5 ml PBS. Ten µl of the cell suspension (about 15000 cells) was mixed with 75 µl of 0.5 % low-melting agarose (LMA, Bio-Rad Laboratories, CA, USA) and spread on a microscope slide covered with 0.5 % normal-melting agarose (NMA, Life Technologies, Paisley, UK). The slides were kept on ice for 5 min before the coverslip was removed and the cells were treated in a lysing solution (2.5 M NaCl;

100 mM Na_2EDTA; 10 mM Tris; pH 10; 1 % sodium sarcosinate) for one hour at +4°C. The slides were placed in a horizontal electrophoresis tank, and the DNA was allowed to unwind for 15 min in the electrophoresis buffer (1 mM Na_2EDTA and 300 mM NaOH, pH>13) before the run for 10 min at 24 V/300 mA. Analysis of the cells (nuclei) was done in PI-stained, coded slides (50 cells per slide, 100 cells per dose) using an image analysis system, Komet 4.0.2. from Kinetic Imaging Ltd. The comet parameter used for the statistical analysis was the Olive tail moment ((tail mean - head mean) * tail%DNA/100).

4.5.2 Preparation of cytoplasmic and nuclear protein extracts for immunoblotting (IV)

Protein samples were prepared as described earlier (Serpi et al., 1999) with some modificatios. Briefly, after exposure the adherent cells were suspended into PBS by scraping and triplicate wells were pooled together. After centrifugation (5 min at 270 × g, +4°C), cells were suspended in 200 µl of EMSA A buffer [20 mM Hepes pH 7.6; 20 % glycerol; 10 mM NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA pH 7.6; 0.1 % NP 40; 0.5 mM phenylmethylsulfonyl fluoride (PMSF); 1.7 µg/ml aprotinin; 1 µg/ml antipain; 0.7 µg/ml Pepstatin A and 1 mM dithiothreitol (DTT)]. The cell suspension was incubated on ice for 10 min and centrifuged 4 min at 800 x g at +4°C. Supernatants containing cytosolic fractions were collected. The cell pellets were resuspended in 150 µl of EMSA B buffer (500 mM NaCl, otherwise the same as EMSA A). The suspension was incubated for 30 min on ice and then centrifuged for 15 min at 13,000 x g at +4°C and supernatants containing the nuclear fraction were collected. The protein concentrations of the extracts were determined by using the modified Lowry method, DC Protein Assay (Bio-Rad, Hercules, CA, USA). The BSA standards (Sigma-Aldrich Corp., St. Louis, MO, USA) and samples were pipetted to 96-well plate in duplicate. An alkaline copper tartrate solution and a diluted Folin Reagent were added, the plate was mixed and after a 15 min incubation, the absorbance was measured by Victor^3 (PerkinElmer, Finland) at a wavelength 690 nm. Extracts were stored at -80°C until analyzed.

4.5.3 Detection of p53 by immunoblotting (IV)

Twenty micrograms of protein were separated electrophoretically at 200 V (BioRad Power Pac 200) in 12% polyacrylamide gel containing SDS. Proteins were electroblotted (Trans-Blot® SD, Semi-Dry Transfer Cell, BioRad, Hercules, CA, USA) onto a polyvinylidene difluoride (PVDF) membrane (Immobilon P, 0.45 µm pore size, Millipore). After electroblotting, the membranes were incubated in 5 %

non-fat cow's milk-TBS for 1 hour 15 minutes to block unspecific binding. Next, the membranes were incubated with a primary antibody overnight at +4°C. Primary antibodies used were rabbit polyclonal FL-393 for p53 protein (Santa Cruz Biotechnology, Inc., dilution 1:2000). The membranes were then washed 2 x 2 min, 15 min and 3 x 5 min with washing buffer (TBS-0.05 % Tween) and incubated in secondary Anti-rabbit-IgG (Cell Signaling, dilution 1:2000) antibody for one hour at room temperature and washed in the same way as after the primary antibody treatment. Protein bands were visualized with the ECL+ Plus system (Immunoblotting detection system, Amersham BioSciences, UK) according to the manufacturer's instructions. Prestained Precision Protein standard (BioRad, Broad range) was used to compare the molecular weights of the bands and monoclonal Anti-β-actin-IgG (Sigma-Aldrich Corp., St. Louis, MO, USA) was used as a loading control. Densitometric analysis was done using BIO RAD GS710 (BioRad, Hercules, CA, USA) Quantity One (4.6.1) software.

4.6 Inflammation analyses (I-III,V)

4.6.1 Cytokine analysis (I, II, V)

Cytokines (TNFα, IL-1β, IL-6, IL-10, MIP2) were analyzed from the cell culture medium by using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, the 96-well microtiter plates (MaxiSorp, Nunc A/S, Naperville, IL, Denmark) were coated with a monoclonal capture antibody and the plates were incubated overnight. The wells were washed with washing buffer (0.05 % Tween in PBS, pH 7.4) and nonspecific binding was blocked by blocking buffer (1 % BSA, 5 % sucrose in PBS). After the incubation, the nonbound proteins were washed away and cell culture medium samples and standards were pipetted to the plates in duplicate and incubated with solid phase antibody. The nonbound fraction was washed away and biotinylated second antibody was added. The nonbound second antibody was removed after incubation, the wells were washed and a detection reagent, horseradish peroxidase (HBR)-conjugated streptavidin was added. After incubation, the wells were washed and tetramethyl-benzidine (TMB) substrate solution was added. Reactions were terminated by addition of 1 M H2SO4, which converts the blue color into yellow. The samples were analyzed with a microplate reader [iEMS Reader MF, Labsystems, Finland (papers I, II)/Victor^3, PerkinElmer, Finland (paper V)] at a wavelength of 450 nm. Cytokine concentrations of samples were calculated by interpolating absorbances of samples to the standard curve.

4.6.2 Nitric oxide analysis (I, II, III, V)

Nitric oxide was measured spectrophotometrically as the stable metabolite, nitrite (NO2) according to the Griess method (Green et al., 1982). Briefly, Griess reagent (1 % sulphanilamide and 0.1 % naphthylethylenediamine dihydrochloride in 2 % phosphoric acid) was mixed 1:1 with samples of the fresh cell culture medium. Nitrite forms a colored chromophore with the reagent, with an absorbance maximum at a wavelength of 543 nm, which was measured using a microplate reader [iEMS Reader MF, Labsystems, Finland (papers I-III)/Victor3, PerkinElmer, Finland (paper V)]. The production of nitrite was quantified by comparing the results with absorbances of standard solutions of sodium nitrite.

4.7 Oxidative stress analysis (V)

4.7.1 ROS analysis (V)

The intracellular accumulation of ROS was measured by flow cytometry using the fluorescent probe 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Molecular Probes). H_2DCFDA is a non-polar compound that readily diffuses into the cells where it is enzymatically deacetylated by intracellular esterases to the polar non-fluorescent derivate and in that way, becomes thereby trapped within the cells. In the presence of H_2O_2 or other peroxides, the probe is oxidized to the fluorescent product 2', 7'-dichlorofluorescein (DCF^+) reflecting the intracellular amount of peroxides produced by cells. Briefly, during the last 30 min of incubation, cells were loaded to 1 μM H_2DCFDA in phosphate-buffered saline (PBS, Gibco, Paisley, UK). After the incubation, the cells were washed with PBS, harvested by gently scraping, centrifuged (5 min at 370× g) and washed once more with 1 ml PBS. The pellet was suspended in 1 ml PBS and the fluorescence signal of DCF^+ cells was analyzed by flow cytometry (CyAn ADP, Dako, USA) with an emission wavelength of 530 ± 40 nm (FL1). A total of 10,000 cells were analyzed per sample by using Summit software.

4.8 Statistical analysis

The responses caused by the spores of co-cultivated microbes, the spore-mixture of separately cultivated microbes, the spores of *S. californicus* and *S. chartarum* alone were compared to control and also between each other (studies II-V). The data was statistically analyzed (SPSS) using analysis of variance (ANOVA) and Tukey's test, except for the Comet assay which was statistically analyzed by using the Kruskall-Wallis test. In addition, statistically significant changes caused by the antioxidant, NAC, were detected. Differences were considered to be statistically significant at $p < 0.05$.

To explore the synergistic interactions between the spores of *S. californicus* and *S. chartarum* during simultaneous exposure (study I), the results of the co-exposure were compared with the individual results of both *S. californicus* and *S. chartarum* exposures by using two-way ANOVA. An interaction term was included in the model to assess the possible synergy of the microbes. The difference was considered to be statistically significant at $p < 0.05$.

5 RESULTS

5.1 Microbial co-cultivation (II-V)

The final proportion of the microbial spores reached during the co-cultivation was not predictable. According to the results of a pilot study, 2 % malt extract agar provided reasonable growth conditions for both of the microbes, but the growth rate of *S. californicus* was lower than that of *S. chartarum* on this agar. In co-cultivation these microbes were inoculated on the same plate at a ratio of 100:1 (*S. californicus* : *S. chartarum*), and after incubation, this inoculation ratio ended up to a final proportion of 5:1 of these microbes. The immunotoxic responses in macrophages shown in studies II-V were induced by the spores of co-cultivated microbes containing five times more of the spores of *S. californicus* than the spores of *S. chartarum*. The spore-mixture of separately cultivated microbes was prepared at the same proportion (5:1).

5.2 Cytotoxicity (II-V)

It was demonstrated in studies II-V that the spores of co-cultivated *S. californicus* and *S. chartarum*, the spore-mixture of separately cultivated microbes and the spores of *S. californicus* alone induced a significant dose-dependent decrease in the cell viability (e.g. see paper IV, Fig. 1). The cytotoxic responses were measured by using several methods (see Table 3, page 38), which indicated that the spores of co-cultivated microbes were more cytotoxic than the equal spore-mixture of separately cultivated microbes. The spores of co-cultivated microbes induced significant apoptotic cell death detected by the DNA content analysis (Figure 4A, Figure 5) and the caspase-3 activity assay (see paper II, Fig. 2 and paper III, Fig. 5). In addition, necrotic/late apoptotic cell death was detected by using the PI exclusion test (Figure 4B). These responses induced by the microbial co-culture were detected in macrophages already at the relatively low dose, whereas the spore-mixture of separately cultivated microbes induced cell death only at high doses (Figure 4A-B). Furthermore, both the spores of *S. californicus* and *S. chartarum* alone induced significant cell death (Figure 4B), but only the spores of *S. chartarum* alone were capable of activating apoptosis (Figure 4A, Figure 5). Since the spores of *S. californicus* did not trigger caspase-3 activation (see paper II, Fig. 2) or a significant $SubG_1$ peak (Figure 4A, Figure 5), the cell death detected by the PI exclusion test was presumably necrotic (Figure 4B).

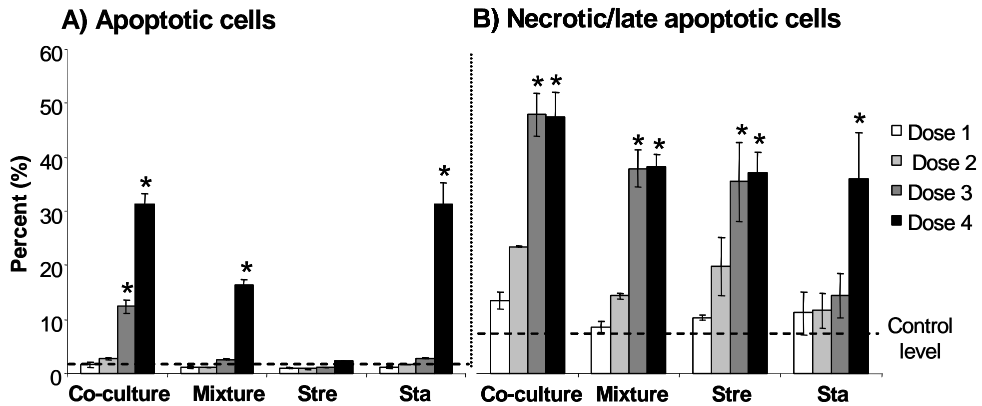

Figure 4. A) Proportion of apoptotic cells (study II, DNA content analysis, Sub G_1) and B) proportion of necrotic/late apoptotic cells (study IV, PI exclusion test) in the RAW264.7 macrophages after exposure for 24 hrs to the spores of co-cultivated *S. californicus* (Stre) and *S. chartarum* (Sta), the spore-mixture of separately cultivated microbes or these microbes separately. The doses used are presented in Table 2. Each column represents mean ± SEM of at least three independent experiments and dashed line express control level. * indicates a statistically significant difference from control (Tukey, $p<0.05$).

5.3 Cytostatic properties (II, III)

It was demonstrated in study II that exposure to the spores of co-cultivated *S. californicus* and *S. chartarum* had a significant influence on the regulation of the cell cycle in mouse RAW264.7 macrophages (Figure 5). The spores of co-cultivated microbes induced cell cycle arrest at G_2/M phase, but this effect was not seen after the exposure to the spore-mixture of separately cultivated microbes. Cell cycle arrest at G_2/M was also detected after the exposure to the spores of *S. californicus* alone, but the response was not significant until treatment with the highest dose. Furthermore, the spores of *S. chartarum* alone did not trigger cell cycle arrest at any tested dose.

In the search for plausible microbial metabolites produced during the co-cultivation of these microbes, similar responses were detected after the exposure to a relatively low dose of certain widely used cytostatic agents, especially doxorubicin and actinomycin D, which are both produced by streptomycetes (study III).

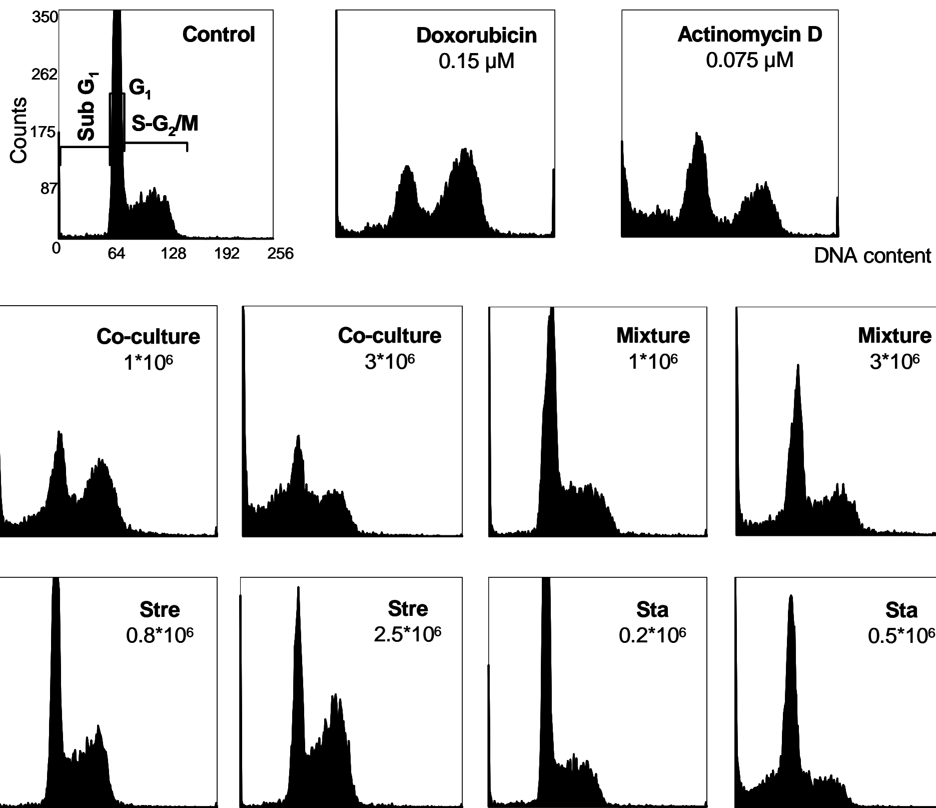

Figure 5. The RAW264.7 macrophages were exposed for 24 hrs to the cytostatic drugs doxorubicin or actinomycin D and also the spores of co-cultivated *S. californicus* (Stre) and *S. chartarum* (Sta), the spore-mixture of separately cultivated microbes or these microbes separately. Example histograms indicate the changes in cell cycle and the proportion of apoptotic cells (Sub G1).

5.4 Genotoxicity (IV)

It was revealed in study IV that the genotoxic capacity of the spores of *S. californicus* was potentiated by interactions occurring during co-cultivation with *S. chartarum* (Figure 6). DNA damage was detected in RAW264.7 cells by the SCG/Comet assay after exposure to even low doses of the spores of microbial co-culture while this kind of effect required high doses of the spores of *S. californicus* alone (Figure 6A). In contrast, no DNA damage was observed at any tested dose after the exposure to the spore-mixture of separately cultivated microbes or the spores of *S. chartarum* alone (Figure 6A). However, the spores of co-cultivated microbes and the separately cultivated spore-mixture as well as the spores of *S. californicus* alone all induced p53 accumulation in both the nucleus and the cytoplasm (Figure 6B), but ,in contrast, cytoplasmic p53 accumulation was not seen after exposure to the spores of *S. chartarum* alone (see paper IV, Fig. 4).

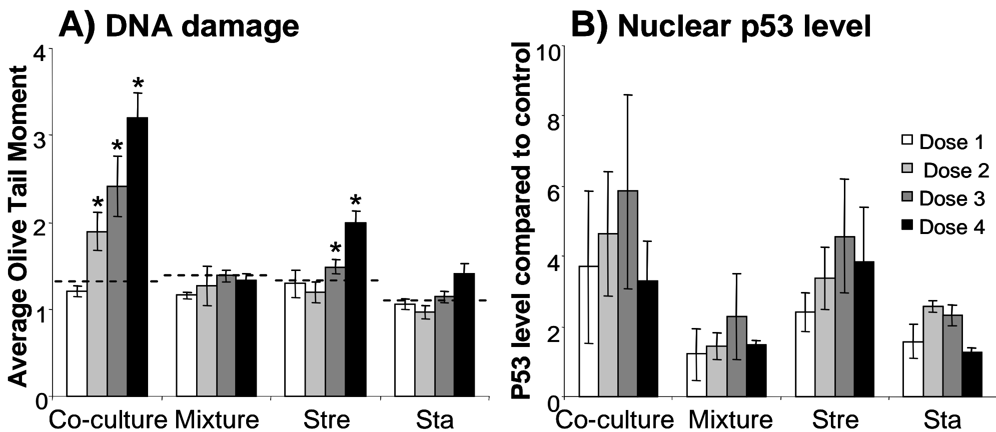

Figure 6. A) DNA damage and B) nuclear p53 level in the RAW264.7 macrophages after exposure for 24 hrs to the spores of co-cultivated *S. californicus* (Stre) and *S. chartarum* (Sta), the spore-mixture of separately cultivated microbes or these microbes separately (study IV). The doses used are presented in Table 2 Each column represents mean ± SEM of at least three independent experiments and the dashed line expresses the control level. * indicates a statistically significant difference from control (Kruskall-Wallis, $p<0.05$).

5.5 Production of inflammatory mediators (I, II)

It was shown in study I that mutual proportions of fungal and bacterial spores in simultaneous exposure affect the nature of cellular responses leading to increased or suppressed production of inflammatory mediators in mouse RAW264.7 macrophage (Figure 7). A significant antagonistic NO response was detected when the co-exposure contained more bacterial (*S. californicus*) than fungal (*S. chartarum*) spores compared to the response induced by the bacterial spores alone (Figure 7A). Although a low number of fungal spores alone did not trigger the NO production in RAW264.7 macrophages, they were able to suppress significantly the response evoked by the bacterial spores in the co-exposure. In contrast to the suppressed NO production, the synergistic interaction in cytokine production (MIP2, TNFα and IL-6) of RAW264.7 macrophages was apparent most clearly when co-exposure contained more of the fungal spores of *S. chartarum* than the bacterial spores of *S. californicus* (Figure 7B).

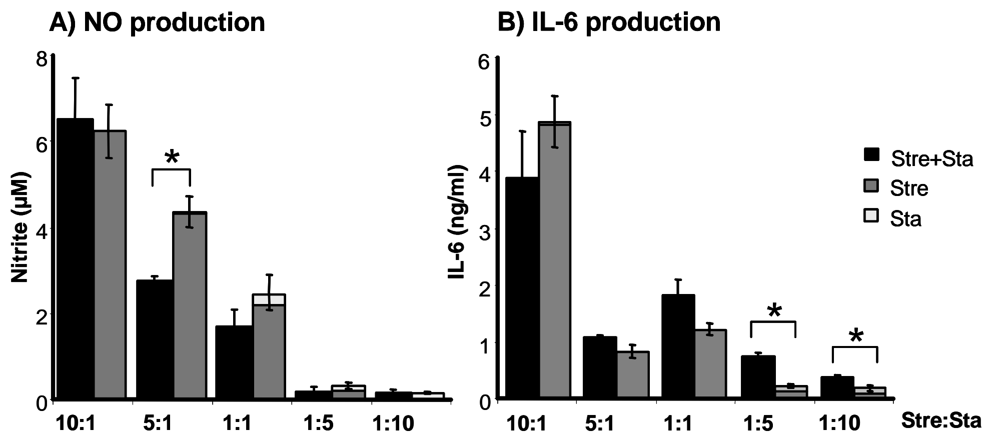

Figure 7. Effect of microbial spore ratio on induced A) NO and B) IL-6 production in mouse RAW264.7 macrophages after 24 hrs co-exposure (total dose 3×10^5 spores/ml) to the spores of *S. californicus* (Stre) and *S. chartarum* (Sta) or these microbes separately (study I). Each column represents mean ± SEM of four independent experiments. * indicates a statistically significant synergistic effect in co-exposure (Two-way ANOVA, $p<0.05$).

It was demonstrated in study II that the production of inflammatory mediators in RAW264.7 macrophages increased dose and time dependently after the exposure to the spores of co-cultivated *S. californicus* and *S. chartarum*, the spore-mixture of separately cultivated microbes and the spores of *S. californicus* alone (Figure 8A-B, see time course in paper II, Fig. 3B & Fig. 5B). Because microbial exposure caused extensive cytotoxicity in RAW264.7 macrophages, the interpretation of the measured inflammatory responses was not possible at the highest dose. Instead, the spores of *S. chartarum* alone were unable to cause inflammatory response at any tested dose or time point (Figure 8A-B).

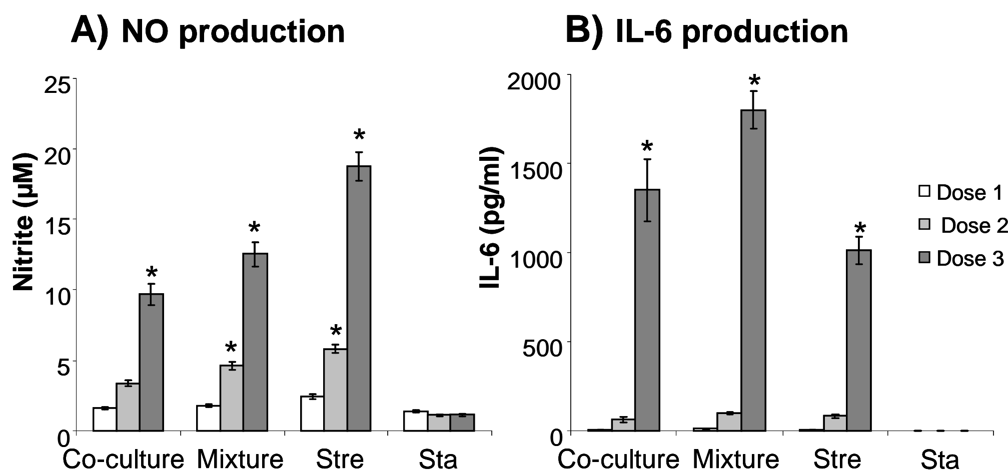

Figure 8. Dose dependent effect of induced A) NO and B) IL-6 production in mouse RAW264.7 macrophages after 24 hrs exposure to the spores of co-cultivated *S. californicus* (Stre) and *S. chartarum* (Sta), the spore-mixture of separately cultivated microbes or these microbes separately (study II). The used doses are presented in Table 2. Each column represents mean ± SEM of at least four independent experiments. Control levels were NO 1.7µM and IL-6 0.2 pg/ml. * indicates a statistically significant difference from control (Tukey, $p<0.05$).

5.6 Oxidative stress (V)

It was revealed in study V that all the evaluated microbial exposures i.e. the spores of co-cultivated *S. californicus* and *S. chartarum*, their separately cultivated spore-mixture and the spores of these microbes alone were capable of triggering oxidative stress by inducing intracellular peroxide production in RAW264.7 macrophages (see paper V, Fig. 1). ROS scavenger NAC prevented the co-culture induced apoptosis,

growth arrest, DNA damage and cytokine production by reducing the ROS production in macrophages (Figure 9). In contrast, NAC could not inhibit peroxide production triggered by the spores of *S. californicus* alone (see paper V, Fig. 2A). Although cell viability was enhanced and cytokine production declined (see paper V, Fig. 4A-B, Fig. 5A-C), *S. californicus* induced DNA damage and growth arrest remained unchanged by NAC (see paper V, Fig. 4D, Fig. 3A).

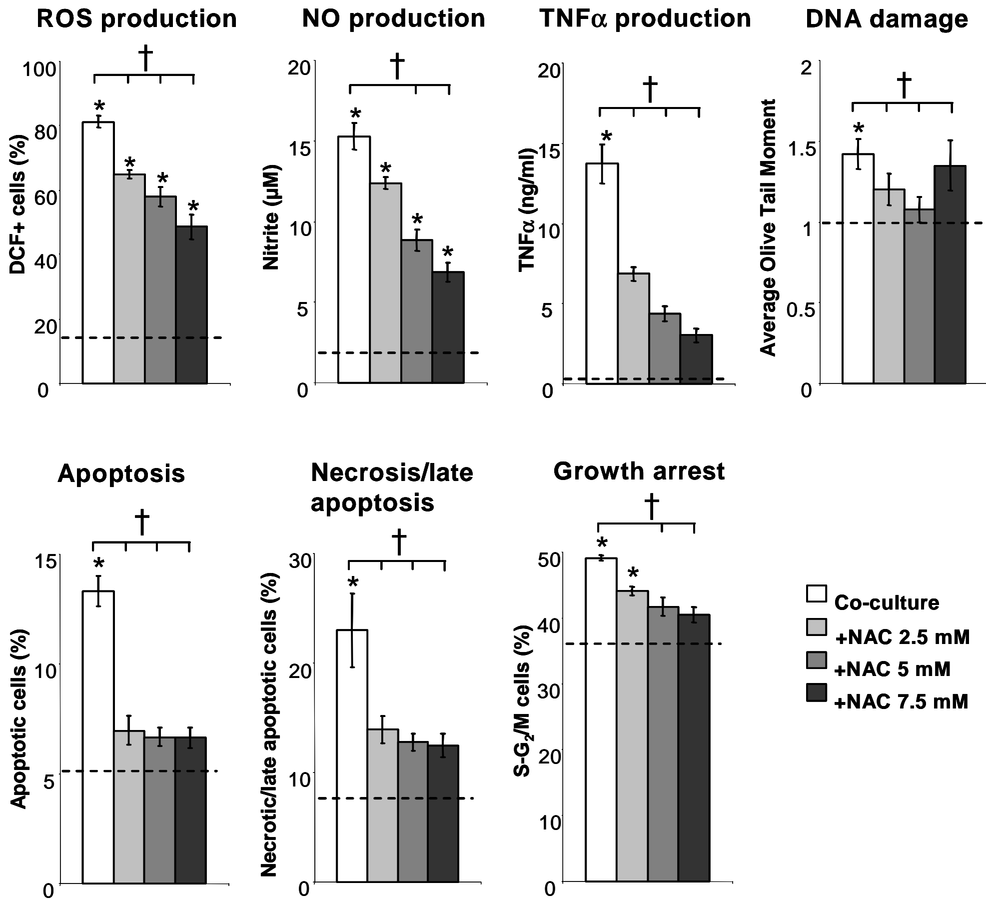

Figure 9. The RAW264.7 macrophages were exposed for 24 hrs to the spores of co-cultivated *S. californicus* and *S. chartarum* (dose 1×10^6 spores/ml) with ROS scavenger NAC (2.5 mM, 5 mM, 7.5 mM) or without NAC (study V). Each column represents mean ± SEM of at least three independent experiments and the dashed line expresses the control level. * indicates a statistically significant difference from control and † indicates significant changes caused by NAC (Tukey, $p<0.05$).

6 DISCUSSION

The occupants on moisture damaged buildings are exposed to a complex mixture of bioaerosols such as microbial spores, cells, structural components as well as biologically active metabolites produced by the micro-organisms (Górny, 2004; Hyvärinen et al., 2002). Previous studies have shown that some of the microbes isolated from moisture damaged buildings have strong inflammatory potential e.g. gram positive bacteria *Streptomyces californicus* and gram negative bacteria *Pseudomonas fluorescens*, whereas the others are extremely cytotoxic e.g. fungi *Stachybotrys chartarum* and *Aspergillus versicolor* (Huttunen et al., 2003). However, there is only a limited amount of toxicological data available on the possible cellular mechanisms accounting for mold-related health effects. In particular, little is known about the role of microbial interactions, although the complexity of the microbial ecosystem could be anticipated to produce alterations which would change the characteristics of any inhaled particles. In this thesis, the role of microbial interactions during co-cultivation and co-exposure and the cellular mechanisms underpinning the mold-related health effects were studied in detail.

6.1 Comparison of responses induced by the spores of *S. californicus* and *S. chartarum* alone (II, IV, V)

The studied microbes, *Streptomyces californicus* and *Stachybotrys chartarum*, caused significant cytotoxic responses in RAW264.7 macrophages, but compared to the spores of *S. chartarum*, the spores of *S. californicus* induced cytotoxicity at lower doses. On the other hand, the spores of *S. californicus* could not trigger apoptotic cell death in the manner of the spores of *S. chartarum*. Thus, *S. californicus* induced cell death was most likely necrotic, which is associated with the extensive production of inflammatory mediators. Consistent with earlier findings, the spores of *S. californicus* induced significant inflammatory responses in RAW264.7 macrophages, whereas the spores of *S. chartarum* did not trigger those responses at any tested dose (Huttunen et al., 2003). These results support the view that the cytotoxicity caused by the spores of *S. chartarum* may represent a model for one type of apoptotic cell death occurring without any major inflammation (Rathmell & Thompson, 1999). It is most likely that the induced apoptosis proceeds via oxidative stress, since the ROS scavenger NAC was capable of decreasing the amount of apoptotic cells when this compound was provided to the RAW264.7 macrophages exposed to the spores of *S. chartarum*. This is supported by previous

studies demonstrating that cellular oxidative stress plays an important role also in mycotoxin induced toxicity (Hassen et al., 2007; Nusuetrong et al., 2005).

Although the spores of *S. chartarum* were capable of inducing apoptosis, they did not induce DNA damage or evoke cell cycle arrest at any tested dose. In contrast, the spores of *S. californicus* caused significant DNA damage, cell cycle arrest and p53 accumulation in both the nucleus and the cytoplasm. Several metabolites produced by streptomycetes have similar immunosuppressive and genotoxic properties (Islaih et al., 2005; Robles et al., 1999). The present study also demonstrated that chemotherapeutic drugs DOX, AMD, MMC and PHLEO were able to induce cell cycle arrest at G_2/M, which has been considered as a typical reaction of normal mammalian cells when exposed to a range of compounds capable of causing DNA damage (Lin et al., 1999; Robles et al., 1999). Interestingly, the ROS scavenger, NAC, could not inhibit the intracellular peroxide production induced by the spores of *S. californicus* alone, since the DNA damage and growth arrest in the RAW264.7 macrophages remained unchanged by treatment with NAC. Previously it has been proposed that some of the metabolites produced by streptomycetes have potent abilities to trigger oxidative damage, but there are also compounds inducing DNA damage via different mechanisms of actions e.g. topoisomerase inhibition and DNA cross-linkage (Chabner et al., 2001; Mizutani et al., 2005). On the other hand, NAC was capable of inhibiting the inflammatory responses triggered by *S. californicus* i.e. NO and cytokine production. The present findings suggest that NO production is associated with cytokine production, and since NAC was able to prevent *S. californicus* -induced NO production, the cytokine production in macrophages also declined. This reduction in the production of inflammatory mediators might also affect the cell survival by enhancing viability of macrophages after NAC treatment.

In many cases, the fungus *Stachybotrys chartarum* has been considered as the most toxic and harmful indoor air microbe in moisture damaged buildings (Hossain et al., 2004; Kuhn & Ghannoum, 2003), but these results clearly demonstrated that the actinobacterium *Streptomyces californicus* can cause significant cytotoxicity at an even lower dose than *S. chartarum*. In addition, the spores of *S. californicus* have significant immunosuppressive and genotoxic properties and thus they might be capable of acting as cancer-provoking agents.

6.2 Microbial interactions during co-cultivation (II-V)

The spores of co-cultivated *S. californicus* and *S. chartarum* were more cytotoxic than the spore-mixture of separately cultivated microbes. Both of these spore suspensions induced apoptotic and necrotic cell death, but significant responses were seen already at a lower dose when they were grown in the co-culture. In addition, the co-culture induced apoptosis was detected already at a lower dose than that seen after exposure to the spores of *S. chartarum* alone. Apoptotic cell death triggered by the spores of co-cultivated microbes proceeded via mitochondria membrane depolarization, leading to caspase-3 enzyme activation and DNA fragmentation. A significant collapse of $\Delta\psi_m$ was also detected after exposure to the spores of separately cultivated microbes, but they did not induce caspase-3 activation or DNA fragmentation at the same dose. The results of the MTT-test supported the findings pointing to mitochondrial dysfunction because the number of functional mitochondria (i.e. viable cells) was significantly decreased after exposure to both the co-culture and the mixture. This indicates that both of these spore suspensions were able to irritate the mitochondria of RAW264.7 cells, but the spores of co-cultivated microbes were more potent at triggering apoptosis than the spores of separately cultivated microbes. Increased apoptosis can impair the ability of macrophages to protect the host against bioaerosols including micro-organisms present in the indoor air. This may lead to immunosuppression, which was seen in a previous *in vivo* study which described a decreased number of splenocytes after exposure to the spores of *S. californicus* (Jussila et al., 2003). In addition, the significant inflammatory responses triggered by the spores of both the co-cultivated and the separately cultivated microbes may be associated with the observed necrotic cell death or oxidative stress.

The spores of co-cultivated microbes were also capable of inducing DNA damage, causing p53 accumulation and triggering significant cell cycle arrest at G_2/M already at the relatively low dose. Similar genotoxic effects (DNA damage, p53 accumulation, cell cycle arrest) were also detected after exposure to the spores of *S. californicus* alone, but the responses were not significant until the higher doses of spores were used. Thus, co-cultivation with *S. chartarum* clearly increased the ability of the spores of *S. californicus* to evoke genotoxic and cytostatic effects already at lower doses. Since the spores of *S. chartarum* alone did not induce DNA damage or trigger cell cycle arrest at any tested dose, the results suggest that when these micro-organisms are growing in the same habitat, *S. chartarum* potentiates or stimulates *S. californicus* to synthesize some highly toxic components, which caused these cytostatic and genotoxic effects. These toxic components are still unidentified, but we have already demonstrated that they have similar properties than cancer

chemotherapeutic agents such as doxorubicin and actinomycin D. Interestingly, the genotoxic activity of these spores was strongly associated with interactions occurring during co-cultivation, since no similar responses were detected when the RAW264.7 macrophages were exposed to the corresponding spore-mixture of the same microbes which were grown separately from each other. Interestingly, the spore-mixture of separately cultivated microbes could not induce DNA damage in the same way as the spores of *S. californicus* alone, which means that the genotoxic properties of *S. californicus* was inhibited by the interaction during co-exposure with *S. chartarum*.

In the search for a plausible mechanism to explain the cellular damages induced by the spores of co-cultivated *S. californicus* and *S. chartarum*, the ROS scavenger, NAC, was used to evaluate the role of oxidative stress. By treating with NAC simultaneously during exposure to the co-culture, we were able to demonstrate that oxidative stress is involved in all these aspects of cellular damage. NAC prevented the co-culture induced apoptosis, growth arrest, DNA damage and cytokine production by reducing the intracellular ROS production in macrophages. Previous studies have demonstrated that the major targets of oxidative stress are nuclei and mitochondria, and that this results in damage to membrane lipids, protein enzymes, and deletion or modification of DNA (Sauer et al., 2001). Oxidative stress can also lead to apoptosis by inducing the mitochondria permeability transition (Halliwell & Gutteridge, 2007). Previously it has been shown that oxidative stress triggers RAW264.7 macrophages to undergo activation of growth arrest and either apoptosis or cell survival by regulating the genes which encode distinct protein families and signaling pathways (Zhang et al., 2005). The p53 protein is one of the factors operating as a sensor of ROS, but it also directly regulates ROS levels and further mediates the cell cycle arrest and apoptotic cell death (Sharpless & DePinho, 2002). These findings further confirm our hypothesis that oxidative stress is the factor triggering the cellular damage evoked by the production of these compound(s) and this process was stimulated by microbial interactions during co-cultivation. Figure 10 illustrates the activated cellular mechanisms induced by the spores of co-cultivated microbes.

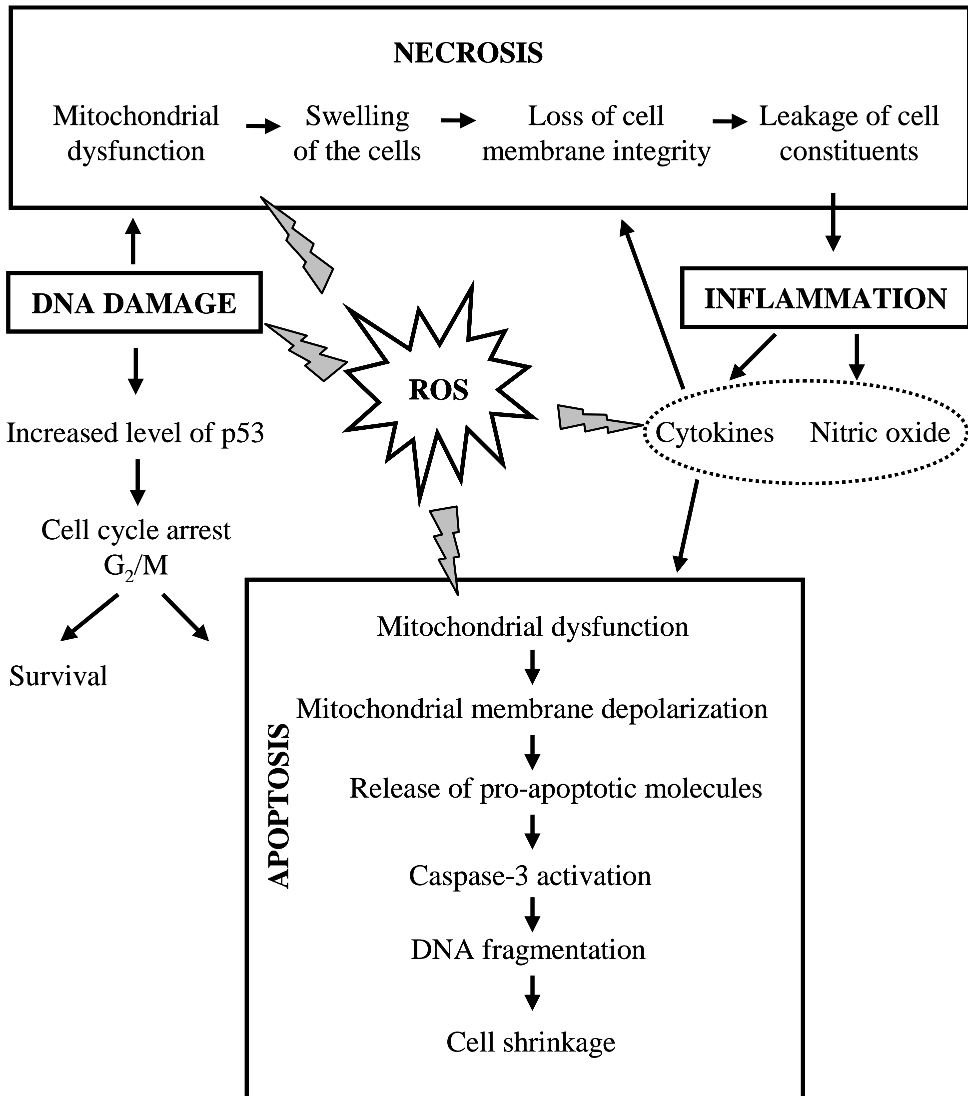

Figure 10. Summary of the main findings and cellular immunotoxic mechanisms triggered by the spores of co-cultivated *Streptomyces californicus* and *Stachybotrys chartarum* in mouse RAW264.7 macrophages. Co-culturing of these microbes increased the ability of the spores to trigger the production of reactive oxygen species (ROS) followed by DNA damage. Subsequently cells may die through apoptosis or necrosis, which can proceed via the mitochondria. Necrosis is

associated with inflammation detected by increased amount of inflammatory mediators e.g. cytokines and nitric oxide. Furthermore, increased amounts of cytokines and nitric oxide are able to induce apoptotic and necrotic cell death. ROS could also modify on cytokine production and mitochondria functions directly. This process is self-amplifying and can provoke also other cellular responses.

Interactions between the microbial agents demonstrated in these studies were induced by the co-culture of microbial spores containing five times more *S. californicus* spores than *S. chartarum* spores. The final proportion of the microbial spores reached during the co-cultivation is not predictable, and in real life there might be an infinite number of different kinds of co-cultures, depending on their growth conditions and microbial species present in moistured building material. Though the presence of different micro-organisms in a damp environment is ubiquitous the actual interactions between the micro-organisms may be condition-dependent, resulting in the production of unique combinations of biologically active compounds with unexpected cellular effects. Although these studies as such cannot be directly extrapolated to the real life situations, in conjunction with previous studies they demonstrate the importance of microbial interactions especially during co-cultivation (Meyer & Stahl, 2003; Murtoniemi et al., 2005; Yli-Pirilä et al., 2007).

6.3 Microbial interactions during co-exposure (I)

Indoor air of moisture damaged building includes various micro-organisms to which residents are exposed simultaneously. Thus, the other aspect of exposure is the interactions occurring during co-exposure situation. Previously it has been shown that in simultaneous exposure situations, the spores of *S. chartarum* are able to potentiate the inflammatory responses of *S. californicus* (Huttunen et al., 2004), but it is not known how the proportions of microbes affect their interactions and their induced inflammatory responses. This study demonstrated that mutual proportions of fungal and bacterial spores in simultaneous exposure affect the nature of cellular responses leading to increased or suppressed production of inflammatory mediators in mouse RAW264.7 macrophage cell line.

The synergistic interaction in cytokine production (MIP2, TNFα and IL-6) of RAW264.7 macrophages was most clearly apparent when co-exposure contained more of the fungal spores of *S. chartarum* than the bacterial spores of *S. californicus*. These results are in line with previous studies performed with microbial spores or toxins (Chung et al., 2003; Huttunen et al., 2004; Sugita-Konishi & Pestka, 2001; Zhou et al., 1999). In all these studies, the most potent synergistic

interaction occurred when the co-exposure contained the lowest amount of the bacterial component, which evoked relatively low levels of cytokine production. Previously has been demonstrated that the spores of *S. californicus* are able to induce the nuclear binding activity of transcription factor NF-κβ in RAW264.7 cells, but the synergistic interaction between *S. californicus* and mycotoxin trichodermin can not be explaned by amount of NF-κβ in the nucleus (Huttunen et al., 2004).

In contrast to the increased cytokine production, a suppression of NO production was detected after co-exposure to the spores. A significant antagonistic response was detected when the co-exposure contained more bacterial *S. californicus* than fungal *S. chartarum* spores compared to the response induced by the bacterial spores alone. Although, a low number of fungal spores alone did not trigger the NO production in RAW264.7 macrophages, they were able to significantly suppress the response evoked by the bacterial spores in the co-exposure. Both potentiating and suppressive inflammatory responses were also seen in human macrophages after co-exposure to fungal DON and bacterial LPS (Sugita-Konishi & Pestka, 2001). While TNFα production was synergistically increased in macrophages, the IL-6 response was bidirectional, i.e. significantly suppressed IL-6 production when DON concentration was higher than LPS, and significantly increased IL-6 production in cases where bacterial LPS was the prevalent compound (Sugita-Konishi & Pestka, 2001). These results demonstrate clearly that the mutual proportions of spores present in the exposures can affect the nature of interactions leading to changes in the biological responses.

6.4 Methodological considerations

6.4.1 Relevance of *in vitro* assays

In vitro assays are a useful way to obtain a detailed understanding of biological effects induced by potential causative agents present in the indoor environment. Moreover, *in vitro* experiments are ethically and economically preferable and they are invaluable in producing essential data on activated cellular mechanisms in different cell types in host defence system. Although *in vitro* studies play an important role in proper risk assessment, there are issues that need to be considered while evaluating the relevance of *in vitro* data. The *in vitro* systems lack the feed-back mechanisms of tissues and whole organisms, since these experiments are conducted using single types of cells, either human or animal cell lines or primary cells. The cell lines grow well with a minimal loss of viability. Their responses are highly reproducible and well characterized, but the responses (e.g. cytokine production) may partly differ from those activated in ‘normal’ cells. Instead, the primary cells which are representatives of normal cell systems suffer a loss of

viability, especially in prolonged experiments. In addition, the previous exposure history of the donor may affect the induced responses in the experimental settings. Thus, the reproducibility of the primary cell systems is lower than those which can be activated with cell lines.

As is always the case, direct extrapolation from the experimental data to human risk assessment is not possible. Although *in vitro* experiments produce crucial data for risk assessment, it could describe only part of the highly complex local systemic responses seen in tissues and whole organs. Therefore, in the search for causative agents and pathophysiological mechanisms behind adverse health effects associated with damp buildings, *in vitro* data needs to be combined with experimental animal data *in vivo*, human clinical data and epidemiological findings.

6.4.2 Cell line and exposure agents

The primary route of the exposure to the complex mixture of indoor air microbes and their constituents is through inhalation. Therefore, immunological cells of the host defence are the most relevant for *in vitro* studies. Macrophages are present not only in alveoli-region of the lungs but also in the upper airways (Abbas et al., 2007). They are the primary defence cells against inhaled pathogenic and other insoluble material, and they can produce large amounts of proinflammatory mediators and chemotactic substances, which recruit other inflammatory cell types into airways. Previous studies have shown that the mouse RAW264.7 macrophage cell line, which was selected for use in the studies of the present thesis, is very sensitive and a widely used model for evaluating murine responses to a variety of stimuli (Hirvonen et al., 1997; Huttunen et al., 2003; Ji et al., 1998; Wong et al., 1998; Yang et al., 2000, Zhou et al., 2005). For example, the same dose of microbial spores induced at least one hundred fold greater cytokine response in RAW264.7 cells than in human 28SC macrophages or A549 lung epithelial cells (Huttunen et al., 2003).

It was recently reported that out of the six studied microbial strains, only *Stachybotrys chartarum* was able to potentiate the inflammatory response of *Streptomyces californicus* (Huttunen et al., 2004). Based on these findings, the fungus *S. chartarum* and the actinobacteria *S. californicus* were selected for the present more detailed experiments focussing on the immunotoxic responses triggered by microbial interactions.

6.4.3 Valid dose level and time point

While exploring *in vitro* responses, the measurements must be done at their most feasible time-point and dose. Studies on multiple endpoints relating inflammation,

cytotoxicity, genotoxicity and oxidative stress need to be done by using several dose levels and exposure times. However, there is no *ONE* time-point at which one can obtain the highest responses to all members of a large scale of parameters. The studies on dose response are crucial since a too low dose may be totally ineffective and too high might lead to a plateau –effect or overload.

The initiation of immunotoxic responses can proceed via different membrane receptors activated by either soluble or particulate substances. Several *in vitro* studies are done by using soluble agents e.g. mycotoxins, which are commercially available pure compounds (e.g. Yu et al., 2006; Zhou et al., 2005). These studies are essential to clarify the cellular mechanisms for exact compounds, but as mentioned previously in moisture damaged buildings, the occupants are not exposed to a single agent. In addition, toxins can be separated from spores by solvent extraction e.g. methanol (e.g. Wang & Yadav, 2006). However, it is almost impossible to extract all the toxins and bioactive metabolites from the spores, and this process itself may affect the spores and released compounds. Since the principal route of the microbial exposure in moisture damaged buildings is thought to be the inhalation of particles, the doses used in this study are based on equivalent numbers of spores. It was hypothesized that the effects of microbial spores would mimic the real life exposure to these microbes, because the inhalable spores may as such affect the induced responses in lungs. The spore-dose also needs special attention, since the spores of the studied microbes are different in their size (aerodynamic diameter of *S. chartarum* 4.6 µm and *S. californicus* <1 µm).

It is also well known that even inert particles can cause an oxidative burst, which may induce cellular damages in macrophages. In these experiments, inert particles were not included as a negative control or as a filler material. A thorough consideration of this aspect led us to conclude that in this case the biological metabolites carried by the microbial spores are most probably more important than the particle effect. This view is supported by the results of previous experiments on the inflammatory and cytotoxic responses by both of the studied microbes. It has been shown that the same spore concentrations of several *Streptomyces* strains (i.e. the very same particle size) can cause highly strain specific responses (Hirvonen et al., 1997). The very specific effects were also induced by different *Stachybotrys* strains (Nielsen et al., 2001; Ruotsalainen et al., 1998). Moreover, there are many other properties that affect the particulate induced responses such as particle solubility, particle shape, surface area and the bioavailability of the causative components in the spores.

To estimate the macrophage : spore ratio, it is important to bear in mind that at the beginning of these exposures the cell culture contained about 2×10^6 macrophages and the total volume was 2 ml. The spore doses of the co-culture or the mixture used

in this study were between 1×10^4 - 3×10^6 spores/ml, meaning that macrophage : spore ratio ranged from 100:1 to 1:3. Most of the cellular effects were observed already at a dose of 3×10^5 spores/ml corresponding to a ratio of 3:1 between macrophages and microbial spores. Hence, the detected responses cannot be explained by overloading effects even at the highest dose used.

It is difficult to compare the spore doses used in *in vitro* studies and the factual indoor air concentration in moisture damaged buildings. To complicate comparison even further, there are remarkable spatial and temporal variations in the concentrations measured from the indoor air of moisture damaged buildings (Hyvärinen et al., 2001b). In addition, a recent study has shown that indoor air microbial concentrations as measured by new techniques, quantitative PCR (qPCR), do not necessarily correlate well with cultured based methods (Pietarinen et al., 2008). However, there is no conclusive evidence that increased concentrations of fungi in the indoor air are the direct cause for reported health effects, which suggest that other issues (e.g. microbial interactions) may have a crucial role in evoking these adverse heath outcomes.

6.4.4 Comparison of cytotoxicity assays

Dying cells undergo a complex network of metabolic events resulting in either apoptotic or necrotic cell death. One important aspect of this study was to apply several new methods for the evaluation of cellular events underlying the cytotoxicity evoked by damp building related microbes more precisely. Overall, in this study seven different kinds of assays detecting cell viability or cell death were used: the live gate analysis, the MTT test, the trypan blue staining, the PI exclusion test, the DNA content analysis, the assay for mitochondria membrane depolarization and the caspase-3 activity assay. The results were highly repeatable and they correlated very well with each other.

Since the used 'overall cytotoxicity' methods (the live gate analysis, the MTT test and the trypan blue staining) measure different parameters (see Table 3, page 38), the absolute values could not to be expected to be identical. However, the dose response curves were surprisingly similar (e.g. see paper IV, Fig. 1, Fig. 2). For example, all the used methods indicated that the two highest doses of the co-culture were the most toxic. In addition, only the highest dose of the spores of *S. chartatum* was capable of inducing severe cytotoxicity in RAW264.7 cells.

The DNA content analysis (Sub G_1 peak) was mainly used in these experiments to determine the proportion of apoptotic cells. This method is widely used and it correlates well to other generally used apoptosis assays (Darzynkiewics et al., 1992).

We also confirmed the results detected by PI staining by using caspase-3 assay, which is a more specific assay for apoptosis (see paper II, Fig. 2 and paper III, Fig. 5). Our results of caspase-3 enzyme activity assay supported the findings detected by DNA content analysis. In addition, detected mitochondrial depolarisation by flow cytometric analysis correlates well with the present findings (see paper III, Fig.3). The present results demonstrate that a variety of different assays are needed for in-depth understanding of the pathways of cell death caused by microbial agents, and for the proper evaluation of their toxicity.

6.5 Clinical implications

Even though the measured microbiological concentrations are not necessarily high in the indoor air, dampness, moisture damage and mold growth have been linked with several detrimental health problems such as asthma, frequent respiratory infections and clusters of autoimmune diseases (Bornehag et al., 2001, 2004; Husman, 1996; Kilpeläinen et al., 2001; Jaakkola et al., 2005; Luosujärvi et al., 2003; Pekkanen et al. 2007; Zock et al., 2002). The current *in vitro* data showed that significant cytotoxic, genotoxic and inflammogenic effects can be observed in experimental settings *in vitro* already at a relatively low spore dose (3×10^5 spores/ml), meaning that in the cell culture, the ratio between macrophages and microbial spores was 3:1. However, the causal relationships between observed health effects, microbial agent and cellular mechanisms are still largely unknown.

Adverse health effects in moisture damaged buildings are usually associated with an inflammatory response (Nielsen et al., 1995). The present data suggest that the actinobacterium *Streptomyces californicus* is one relevant microbe which could cause inflammatory related symptoms in the residents of damp buildings. The strong inflammatory potency of *S. californicus* and its capability to induce oxidative stress at relatively low concentrations have been already previously described (Hirvonen et al., 1997; Huttunen et al., 2003; Jussila et al., 1999), but the important finding here was that these responses triggered by *S. californicus* were synergistically potentiated by interactions after growing with other micro-organisms. The present data on immunosuppressive and DNA damaging properties of *S. californicus* link this microbe also to possibility of activation of the complex cascade leading to the autoimmune diseases and cancer, although there is still no epidemiological data available on this possibility. This finding is supported by a previous *in vivo* study, which indicated that the spores of *S. californicus* were capable of decreasing the number of splenocytes after repeated intratracheal instillation in mice (Jussila et al., 2003). In addition, it is well known that *Streptomyces* have the capacity to produce several bioactive secondary metabolites,

such as antibiotics, immunosuppressive agents and antitumor substances, which are capable of damaging DNA (Behal, 2000). However, *S. californicus* originated from moisture damaged buildings has not been studied as thoroughly as *S. chartarum*, which has been considered as the most toxic indoor air microbe.

The fungus, *Stachybotrys chartarum*, can produce a diverse spectrum of mycotoxins (Hossain et al., 2004; Kuhn & Ghannoum, 2003; Nielsen, 2003). These agents have been characterized and are known to suppress immune function by inducing apoptosis (Nasage et al., 2002; Yang et al., 2000). Our studies demonstrated that only a rather high concentration of *S. chartarum* could cause a significant cytotoxic response. Previously it has been suggested that neurotoxicity could be one potential health effect of exposure to *Stachybotrys* or its toxins in the indoor air of moisture damaged buildings (Islam et al., 2006). It has been also shown that trichothecenes produced by *Stachybotrys* have adverse effects on dendritic cells, which are the most potent antigen-presenting cells of the immune system (Hymery et al., 2006). Due to the high concentration of *S. chartarum* required for toxic effects, it is obvious that it is difficult to establish an unequivocal link between *Stachybotrys* and some specific health effect reported by the residents of moisture damaged building.

The present findings suggest that microbial interactions could be one explanation for the adverse health effects observed already at relatively low microbial concentrations. Although *S. chartarum* alone did not induce a significant immunotoxic response until present at relatively high doses, even a low dose was capable of synergistically potentiating the responses triggered by *S. californicus*. In addition, interactions during co-cultivation stimulated the production of highly toxic compounds, which significantly inceased the cytotoxic and genotoxic properties of the spores causing the responses at lower doses than the microbes alone. Interactions between microbial exposures and other indoor exposures can be complex and therefore they need to be carefully considered when evaluating the health effects experienced by the residents of moisture-damaged buildings.

6.6 Future direction

The present findings clearly demonstrated that there is a need for further studies concerning microbial interactions. Firstly, these *in vitro* results needs to be confirmed by experimental animal studies to gain more detailed information on the effects induced at the tissue level and in the whole animal. Secondly, it is crucial to isolate and identify the biologically active compound(s), the production of which was stimulated during microbial co-cultivation. Thirdly, it is important to study also other mechanisms (e.g. neurotoxicity, reproductive toxicity), which can be activated after non-infectious microbial exposures, and which can contribute to the adverse health effects associated with indoor air dampness and microbes. Fourthly, it is noteworthy that the responses seen in the present study were induced by the combination of only two microbes. Because a wide variety of microbes are present in the moisture damaged buildings, it may be worthwhile to investigating the interactions between other microbes.

In a more practical setting, there is a need to develop and improve toxicological methods, which can be used for evaluating the harmfulness of indoor air in moisture damaged buildings. Microbiological analyses can reveal the diversity of microbes present in indoor air, but these analyses are not able to estimate the toxic properties of the microbes, which can be intensified while micro-organisms grow together in the same moistured building material. Furthermore, indoor air includes other harmful components in addition to those originating from microbes, and thus it is important to study this kinds of interactions and synergistic effects e.g. caused by moldy house microbes and particles from outdoor sources.

In summary, a combination of toxicological studies with comprehensive microbiological and chemical analysis of indoor samples is crucial if one wishes to identify the causal relationship between exposure and adverse health effects experienced by the inhabitants of moisture damaged buildings.

CONCLUSIONS

The spores of actinobacterium *Streptomyces californicus* isolated from a moisture damaged building have cytotoxic, cytostatic, genotoxic and inflammogenic properties. The spores of fungus *Stachybotrys chartarum* induced significant cytotoxicity at relatively high concentrations, but no cytostatic, genotoxic or inflammogenic activity was observed.

During simultaneous exposure, the mutual proportion of these microbes affects the nature of cellular responses, leading to either an increased or suppressed inflammatory response in RAW264.7 macrophages.

Microbial interactions during co-cultivation of *Streptomyces californicus* and *Stachybotrys chartarum* can stimulate or potentiate the production of highly toxic compound(s), leading to stronger immunotoxic and genotoxic responses in RAW264.7 macrophages than those induced by an equal spore-mixture of separately cultivated microbes.

Compound(s) produced during co-cultivation of *Streptomyces. californicus* and *Stachybotrys chartarum* has/have potent cytotoxic, cytostatic and genotoxic properties, and the mechanism of cell death resembles the apoptotic pathway evoked by the chemotherapeutic drugs, doxorubicin and actinomycin D which themselves originate from streptomycetes.

Oxidative stress is involved in all the detected cellular damages caused by the spores of co-cultivated *Streptomyces californicus* and *Stachybotrys chartarum*. This was demonstrated by administering the ROS scavenger, NAC, at the same time as the RAW264.7 macrophages were exposed to the spores of co-cultivated microbes.

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