I, Huiyan Wang, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Toxicology (Environmental Health).

It is entitled:
Toxicity and signaling mechanisms underlying interactions of Stachybotrys chartarum toxins with lung macrophages

Student’s name: Huiyan Wang

This work and its defense approved by:

Committee chair: Jagjit Yadav, PhD
Committee member: Gee Lau, PhD
Committee member: Michael Borchers, PhD
Committee member: Howard Shertzer, PhD
Committee member: Ying Xia, PhD
Toxicity and signaling mechanisms underlying interactions of *Stachybotrys chartarum* toxins with lung macrophages

A dissertation submitted to the

Division of Research and Advanced Studies
University of Cincinnati

in partial fulfillment of the
requirements for the degree of

DOCTORATE OF PHILOSOPHY (Ph.D.)

in the Department of Environmental Health
of the College of Medicine

2011

by

Huiyan Wang

M.S. Hebei Medical University, 1999
B.S. Hebei Medical University, 1996

Committee Chair: Jagjit. S. Yadav, Ph.D.
Professor
Department of Environmental Health
University of Cincinnati, College of medicine
Abstract

*Stachybotrys chartarum* (SC) is a mycotoxin-producing indoor mold, growing on humidified cellulose-based materials in water-damaged buildings. For the past two decades, SC has been linked to indoor mold-related health problems in residents and office personnel. SC produces highly toxic mycotoxins including several macrocyclic trichothecenes. Submicron sized trichothecene-containing air aerosols have been detected in moldy indoor environments. SC trichothecenes have been tested positive in the serum of workers exposed to moldy working environments. Experimentally, purified SC trichothecene or crude SC toxin extract and SC spores have been shown to induce extensive cytotoxicity in various cultured cell lines or lung tissue toxicity in animal models (mice and rats).

The inhaled toxin-containing aerosols pass nasal cavity and deposit in the deeper alveolar where resident macrophages could interact via phagocytosis and induction of host response.

To study the toxicity of SC trichothecenes to alveolar macrophages, we used the murine alveolar macrophage cell line MH-S and the methanol extracted toxin-mixture from the SC spores. SC toxin-treated MH-S cells showed apoptosis (including DNA ladder fragmentation, activation of Caspase 3/7, and sub-G₁ peak in flow cytometry analysis), DNA damage (comet assay and micronuclei formation assay), and activation of stress-inducible MAPK and DNA-damage responsive p53 signaling pathways (Chapter II).

To study the underlying mechanism of interaction of alveolar macrophage cells with SC toxin, genome-wide transcriptional changes were evaluated in SC toxin-treated MH-S cell line using gene expression microarray and qRT-PCR (Chapter III). The results implied that SC toxin regulated various genes involved in multiple cellular processes (cell growth, proliferation and
death, inflammatory/immune response, genotoxic stress and oxidative stress) and signal transduction pathways (MAPK-, NF-κB-, TNF-, and p53-mediated signaling), indicating that SC toxin activated cascades of cellular signaling events leading to apoptosis.

Several trichothecene mycotoxins including both simple (T-2 toxin and DON) and macrocyclic (saratotoxin G) types are known to bind to ribosome and inhibit protein synthesis. Since ribosomal proteins have also been reported to be involved in extra-ribosomal functions including cell cycle progression, cell proliferation, tumorigenesis, and apoptosis, we hypothesized that ribosomal component proteins might play a critical role in SC toxin (trichothecene)-induced toxicity in alveolar macrophage. In order to test this, we used yeast mat-a haploid yeast gene knockout library as a tool. The library was screened using both the SC toxin mixture and pure macrocyclic trichothecenes (Verrucarin A and Roridin A). The results revealed multiple ribosome-related genes and DNA damage/repair genes which could mediate SC toxin-induced toxicity, and could function as transducers in SC toxin-induced apoptosis signaling events (Chapter IV).
Acknowledgements

First and most, I thank my advisor, Dr. Jagjit Yadav, for giving me an opportunity to get into this doctorate research program and work in his lab. During the past years, I have learnt a lot from him. I really appreciate his supervision, advice, encouragement, and patience in my research work.

I want to thank my committee members, Dr. Ying Xia, Dr. Michael Borchers, Dr. Gee Lau, and Dr. Howard Shertzer, for their helpful suggestions and open-minded discussions. Dr. Ying Xia, being an expert in signaling transduction, gave me practical advices on how to perform MAPK activation assay and how to evaluate the microarray data; Dr. Michael Borchers, being an expert on lung toxicology, gave me valuable suggestions on detection of inflammatory response in alveolar macrophages; Dr. Gee Lau, generously provided me with a powerful research tool, the yeast deletion library, and taught me how to do the screening test; Dr. Howard Shertzer, encouraged me to finish my degree during all these hard years, taught me hands on how to do oxidative stress and glutathione assays. I truly thank each one of you, for spending time, energy, and sharing research resources with me, and for serving on my dissertation committee.

I would like to thank Dr. Mary Beth Genter for caring about my progress toward graduation. I thank Dr. Marian Miller for teaching me how to detect signals with fluorescent microscopy. Dr. Gleen Talaska and Brenda Shuman for helping me doing the DNA adduct assay; Dr. George Smulian and Dr. Francisco Gomez for teaching me how to perform Maldi-TOF and identify protein spots from the 2D gel as well as access instruments in their lab, and Dr. Rajiv Soman for teaching me how to do GC-MS to identify the components in SC toxin mixture and
letting me use the Mass spectrometry instrument in his laboratory. A Lot of thanks also goes to Dr. Puga, for generously letting me perform numerous microtiter plate reading on his instruments, and Yun Xia in Puga’s lab for helping me do flow cytometry for analysis of apoptotic cells. And I thank Dr. Ying Wai Lam for his help in the field of SC toxin chemical analysis and 2D gel image analysis. I also thank Dr. Zongqing Tan in Dr. Ying Xia’s Lab, for his helpful discussion in my proteomic experiments.

Thanks to Dr. Shuk-mei Ho, Dr. M.B. Rao, Dr. Howard Shertzer, and all my committee members to give me a chance to reactivate my student status and finish my degree; thank you all for your selfless support, which I really appreciate.

I also want to thank my lab members, for their day to day friendship; my thanks go to Venkataramanan Subramanian Evan, Suman, Syed, Eunice, Shalini, and my lab co-workers from the past, Izhar Khan, Harshavardhan Doddapaneni, Suresh Babu Selvaraju, Renuka Kapoor, Manish Gupta, Hansraj Bangar.

My special acknowledgements go to my classmates and friends, Dr. Xiaoqing Chang, Dr. Ying Chen, Dr. Hongbin Dong, Dr. Zhimin Peng, for their helps and encouragements.

Last but not the least, I want to thank my family, without your understanding and support, nothing is possible. I thank my Dad Zhilin Wang, my Mom HaiMei Liu, my husband Jianfeng Kong, and my daughter Amanda Kong, for their unconditional love and support.
# Table of contents

List of tables and figures........................................................................................................3

Abbreviations.........................................................................................................................5

## Chapter I: Background introduction.......................................................................................7

Fungi and molds.......................................................................................................................7
Mycotoxins and mycotoxicoses.................................................................................................8
Indoor molds and sick-building syndrome...............................................................................9
*Stachybotrys chartarum* and its toxicity potential.................................................................11
Why study alveolar macrophages...........................................................................................13
Trichothecenes and macrocyclic trichothecenes- structure, metabolism, and biological activity.................................................................................................................................15
Trichothecene binding to the ribosome ...............................................................................16
Trichothecene epoxide moiety and DNA damage .................................................................18
Yeast deletion library.............................................................................................................18

## Chapter II:
DNA damage, redox changes, and associated stress-inducible signaling events underlying the apoptosis and cytotoxicity in murine alveolar macrophage cell line MH-S by methanol-extracted *Stachybotrys chartarum* toxins..................................................................................20

Abstract.................................................................................................................................21
Introduction.............................................................................................................................22
Materials and methods........................................................................................................25
Results..................................................................................................................................33
Discussion.............................................................................................................................37
Figure legends.......................................................................................................................47
Tables and Figures.................................................................................................................50
References.............................................................................................................................61

## Chapter III:
Global gene expression changes underlying *Stachybotrys chartarum* toxin-induced apoptosis in murine alveolar macrophages: Evidence of multiple signal transduction pathways...........................................................................................................................65

Abstract.................................................................................................................................66
Introduction.............................................................................................................................67
Materials and methods........................................................................................................68
Results..................................................................................................................................68
Discussion.............................................................................................................................80
Chapter IV:
Ribosomal and DNA damage response pathway targets governing toxicity of the mold macrocyclic trichothecenes in eukaryotic cells

Abstract
Introduction
Materials and methods
Results
Discussion
Figure legends
Tables and figures
References

Chapter V:
Discussion and conclusions
References for Chapters I and V
List of tables and figures

Chapter II

Table 1 - Primers used for real-time quantitative RT-PCR in this study.........................50

Table 2 - Transcriptional changes in selected pro-inflammatory mediators in SC toxin-treated alveolar macrophages.................................................................51

Figure 1- Cytotoxicity of S. chartarum toxin mixture ("SC toxins") toward murine alveolar macrophages (MH-S cell line) in time-course treatments.................................................52

Figure 2 - Induction of apoptosis in murine alveolar macrophages by SC toxins............53

Figure 3 - Exacerbation of cytotoxicity caused by SC toxins in cultured alveolar macrophages pretreated (1 h) and co-incubated (24 h) with lipopolysaccharide..............................54

Figure 4 - Glutathione (GSH/GSSG) changes in SC toxin-treated alveolar macrophages.....55

Figure 5 - DNA damage in alveolar macrophages treated with SC toxins.......................56

Figure 6 - Micronuclei formation in alveolar macrophages treated with SC toxins at lower (non-apoptotic) concentration.................................................................57

Figure 7- Signaling changes in alveolar macrophages (MH-S) treated with SC toxins........58

Figure 8- Chemical inhibition of p38 and p53 reduced in part the extent of DNA fragmentation in alveolar macrophages treated with SC toxins.................................................59

Figure 9- Proposed signaling events during apoptosis of alveolar macrophages induced by S. chartarum toxins..............................................................................................60

Chapter III

Table 1- Primer sets used for real-time RT-PCR in this study........................................92

Figure 1 - Temporal analysis of SC toxins-induced apoptosis in murine alveolar macrophage cell line MH-S...............................................................................................93

Figure 2 - Up-regulation of stress-related genes as determined by microarray analysis......94

Figure 3 - Up-regulation of stress-inducible signaling as determined by microarray analysis
Figure 4 - Up-regulation of apoptosis-related genes as determined by microarray analysis

Figure 5 - Transcriptional changes in genes involved in inflammatory/immune response as determined by microarray analysis

Figure 6 - Transcriptional changes in genes involved in cell growth and cell cycle progression as determined by microarray analysis

Figure 7 - RT-PCR confirmation on selected genes, representing the impacted individual cellular processes and pathways, as determined by microarray analysis

Figure 8 - Proposed signaling pathways involved in SC toxin-macrophage interaction, as deduced on the basis of a global gene expression analysis

Chapter IV

Table 1 - siRNAs and targeted sequences

Table 2 - Primer sequences used for qRT-PCR

Table 3 - Primer sequences used for confirmation of the mutants’ genetic identity

Table 4 - Information on the yeast mutants showing common resistance to Verrucarin A (VA), Roridin A (RA), and SC macrocyclic trichothecenes mixture (SCMT)

Table 5 - Information on the yeast mutants showing common sensitivity to VA, RA, and SCMT

Figure 1 - Growth curve of S. cerevisiae parent strain BY4741 in 96-well plate

Figure 2 - Growth inhibition of the yeast parent strain BY4741 by VA, RA, and SCMT

Figure 3 - Yeast deletion mutants showing common resistance or sensitivity to VA, RA, and SCMT

Figure 4 - Confirmation of the genetic identity of resistant and sensitive mutants

Figure 5 - RPL8 gene silencing to verify its role in rescuing alveolar macrophages from cell death and p38 activation induced by SCMT treatment
Abbreviations

AMs- Alveolar macrophages
BAL- Bronchoalveolar lavage
DAPI- 4',6-Diamidino-2-phenylindole·2HCl
DON- Deoxynivelenol
FDR- False discovery rate
GSH- Reduced glutathione
GSSG- Oxidized glutathione
Hck- hematopoietic cell kinase
iNOS- Inducible nitric oxide synthase
IPH- Idopathic pulmonary hemorrhage
JNK- c-jun N-terminal kinase
LDH- Lactate dehydrogenase
LPS- Lipopolysaccharide
MAPK- Mitogen-activated protein kinase
MT- Macrocyclic trichothecene
NO- Nitric oxide
PFTH- Pifithrin-alpha, chemical inhibitor of p53
PKR- double-stranded RNA-activated protein kinase
RA-Roridin A
rRNA- Ribosomal RNA
SBS- Sick building syndrome
SB-SB202190, chemical inhibitor of p38

SC- Stachybotrys chartarum

SCMT- Stachybotrys chartarum macrocyclic trichothecene mixture

SGD- Saccharomyces genome database

SG- Satratoxin G

siRNA- Small interfering RNA

TNF- Tumor necrosis factor

tRNA- transfer RNA

VA- Verrucarin A
Chapter I

Background introduction

Fungi and molds

Fungi (plural for fungus) are lower eukaryotes which form a distinct biological kingdom, along with the biological kingdom of plants, animals, and bacteria. Fungi are unique in their cell wall composition which containing chitin and different from the cellulose of plant cell wall (Bowman and Free 2006). Fungi require preformed organic compounds as energy sources via decomposing organic matter in soil, plants, animals, and dead matter, and therefore play a fundamental role in nutrient cycling in the overall food chain. The fungal kingdom includes both microorganisms (yeasts and molds) and macroorganisms (mushrooms). Molds (or moulds) are one of the microorganism forms of fungi, featuring multicellular filaments (hyphae) and inter-connected network of hyphae (also known as mycelium) (Madigan and Martinko 2005).

Fungi have long been used as food source for human, including edible mushrooms and some molds which are key fermenting agents in making bread, wine, beer, and soy sauce. Fungi also have been used to produce antibiotics in medication and enzymes in industrial applications (Brakhage et al 2004, Abe et al 2006). However, some fungi grow on agricultural crops and cause diseases or destruction of the infected crops. Moreover, some fungi produce mycotoxins, which also contaminate food/feed, causing adverse health effect in both livestock and human beings, and incur extensive economic losses in agriculture annually worldwide (De Lucca 2007).
Mycotoxin and mycotoxicoses

Mycotoxins are toxic secondary metabolites of molds, which are produced naturally in certain environmental conditions. The production and toxicity of mycotoxins is regulated by environmental factors such as temperature, water, light, and growth substrate. One type of mycotoxin may be produced by more than one species of mold, and one mold species may produce more than one type of mycotoxin (Sweeney and Dobson 1999). Mycotoxins have been shown to be involved in multiple toxicological effects, such as antimicrobial, immunosuppressive, carcinogenic, mutagenic, cytotoxic, teratogenic, and neurotoxic, to name a few (Chu 1991). Generally, mycotoxins are chemically stable, showing resistance to digestion, cooking, freezing, and even autoclaving, which allow them to persist in the food chain. Most countries have set regulations for limiting mycotoxins in food products during international trading. Some of the mycotoxins, including trichotheccenes, fumonisins, zearalenone, aflatoxins, ochratoxins, citrinins, ergot alkaloids, and patulin, are of great concern to public health and being studied extensively in terms of their toxicity and underlying mechanism (Sweeney and Dobson 1998, Schlatter 1990, Richard 2007).

Mycotoxicosis (plural as mycotoxicoses) refers to adverse health effect or disease condition in animal and/or human, caused by exposure to toxic mycotoxin(s) via ingestion, skin contact, and inhalation. For example, aflatoxin B1, produced by Aspergillus in contaminated peanuts, cotton, spices, and maize, is a potent liver carcinogen in human and animal species (Choy 1993). Ochratoxin, a mycotoxin produced by Penicillium and Aspergillus, contaminating beers, wines, and juices, is a urinary tract carcinogen (Mally and Dekant 2009). Citrinin, another mycotoxin produced by Penicillium and Aspergillus, reported in wheat, rice, corn,
barley, oats, and rye, is associated with yellow rice disease in Japan (Flajs and Peraica 2009). Ergot alkaloids produced by *Claviceps*, contaminating flour and bread, caused ergotism in human in the forms of gangrenous ergotism and convulsive ergotism (Eadie 2003). Patulin, produced by the *Penicillium expansum* and *Aspergillus* species, contaminating fruits and vegetables, and has been shown to have adverse effect on the immune system in animal study (Roach et al 2002).

**Indoor molds and sick-building syndrome**

Molds are ubiquitous in both outdoor and indoor environments and grow on decomposable organic materials given appropriate temperature and humidity. In normal clean conditions, the number of mold spores in outdoor air is more than that of the indoor air; however, in water damaged indoor air environments, the spore number reached more than that of the outdoor air (Hsu et al 2011, Oliveira et al 2009). *Alternaria, Aspergillus, Penicillium,* and *Stachybotrys* are among the most frequently detected mold species in water-damaged indoor environments (Barbeau et al 2010). *Stachybotrys chartarum* produces several mycotoxins, usually more than what other molds produce when growing in the indoor environment (Jarvis et al 1998). Moreover, the profile of mold species in a contaminated indoor environment is different from the regular indoor molds profile and often comprises of more toxic species, such as *Stachybotrys chartarum* (Codina et al 2008, Baxter et al 2005, Shelton et al 2002). In water-damaged or high humidity indoor environment, molds usually co-exist with multiple bio-contaminants, including mycotoxins, bacteria, microbial particulates, glucans, endotoxins, and volatile organic compounds (Thrasher and Crawley 2009). Considering that most people spend more than 90% their health in long run.
Given the lack of direct evidence to establish causal relationship of indoor mold mycotoxins with sick building syndrome, more and more experimental data is being generated in support of the possible link (Pestka et al 2008, Hossain et al 2004). Mycotoxins have been detected in the spores and fragments, and the substrate that molds grow on (Vesper et al 2000, Gravesen et al 1999). Trichothecenes among other mycotoxins have been detected in bulk dust samples, indoor air aerosols, and ventilation systems of contaminated buildings (Bloom et al 2009, Gottschalk et al 2008). Macrocyclic trichothecenes have been detected on airborne particles of less than 2 micron size (Brasel et al 2005). Satratoxin and stachylysin (a protease produced by *Stachybotrys chartarum*) were detected in the sera of individuals who lived and/or worked in water-damaged moldy buildings (Vojdani et al 2003, Van Emon et al 2003, Brasel et al 2004). Routes of entry for molds and/or mycotoxins include ingestion, dermal exposure and inhalation. Symptoms of residents or workers who have been exposed to water-damaged moldy environments include allergic reactions, asthma episodes, irritations of the eye, nose and throat, sinus congestion, shortness of breath, dizziness, and memory problems (Hardin et al 2003, Straus, 2011; Al-Ahmad et al 2010).

Trichothecenes are produced by several species of fungi, including agricultural fungi, *Fusarium graminearum* (producing DON), *Fusarium sporotrichioides* and *Fusarium poae* (producing T-2 toxin); soil fungi, such as *Myrothecium roridum* and *Stachybotrys chartarum*, which produce macrocyclic trichothecenes (Desjardins et al 1993). Deoxynivalenol (DON) is frequently detected in wheat, rye, barley, oats (Trucksess et al 1995, Eskola et al 2001), and packaged food products such as beer, breakfast cereals, and bread (Wolf-Hall and Schwarz 2002, Schollenberger et al 1999). T-2 and HT-2 toxins are also detected in grains including...
wheat, corn, oats, rice, and rye, as well as in some commercial cereal-based foods (Schollenberger et al 1999). Macrocyclic trichothecenes (such as Satratoxins) have been detected in water-damaged indoors environments, contaminating building materials and indoor air (Gottschalk et al 2008, Straus and Wilson 2006).

**Stachybotrys chartarum and its toxicity potential**

*Stachybotrys chartarum* (synonyms: *S. chartarum, S. atra, and S. alternans*), is an ascomycete fungus, reproducing by asexual spores (conidia). SC goes through its life cycle via spore germination, hypha/mycelium formation, colonization, and spore production (Florian 2000). The spores are ovate in form with smooth to irregularly walls. The size of spore ranges 3 to 4 by 7 to 10 microns. This mold grows on cellulose-based materials under room temperature with high humidity (Andersen et al 2002), and has been isolated from a variety of sources including contaminated grains, tobacco, indoor surfaces, insulator foams, and water-damaged buildings with high humidity (>93%) (Tucker et al 2007, Sorenson et al 1987). Multiple potent toxic macrocyclic trichothecenes have been identified from *S. chartarum* isolates, including satratoxins F, G, and H; roridin E; verrucarins B and J; and trichoverrols A and B. The toxin-containing spores and mold fragments were reported to be readily aerosolized under dry conditions with physical agitation caused by ventilation or vacuuming (Nielsen et al 2002, Barth et al 2002, Ayanbimpe et al 2010).

There have been several outbreaks of *Stachybotrys* mycotoxicosis in both livestock and farm workers. In1930s, horses fed with fodder contaminated by *Stachybotrys*, suffered stomatitis, rhinitis, and conjunctivitis, hemorrhaging and tissue necrosis, pancytopenia, and neurological disorders and subsequent death. Farm workers exposed to the moldy air by inhalation,
developed chest and upper airway symptoms, fever, leucopenia, dermatitis (Hintikka 1977). Another outbreak of *Stachybotrys* toxicosis was reported in sheep in the Republic of South Africa wherein *Stachybotrys chartarum* were isolated from the wheat and barley straw. The affected sheep had high body temperature, listlessness, epistaxis, hemorrhagic diarrhea, anemia, and leucocytopenia (Schneider et al 1979).

More recently, in 1990s, *Stachybotrys* was detected in the water-damaged homes of infants with pulmonary hemorrhage (Etzel et al 1998). In some moldy indoor environments, occupants complained of headache, sore throats, hair loss, flu symptoms, diarrhea, fatigue, dermatitis, and general malaise (Chapman 2003). Both the mold and aerosolized toxins have been detected in the flooded buildings (Gottschalk et al 2008, Brasel et al 2005). Moreover toxins were detected in the sera of individuals exposed to moldy indoor environments (Vojdani et al 2003).

In animal experiments, though relatively high doses of toxin or toxin-containing mold spores were used for relatively short duration, SC caused severe lung damage. Repeated exposure to mold extract induced asthma-like allergic reactions in a mouse model (Viana et al 2002). The live mold (*Stachybotrys*) was not found to be able to grow and cause infection within the lung of the exposed animals (Yike et al 2003). Methanol extraction of mycotoxins from the spores markedly reduced the lung toxicity in spore challenges (Rao et al 2000). In the lung of infant rats inhaled with SC spores, there was hemorrhage, hemosiderin-laden macrophages, and evidence of inflammation (increased number of alveolar macrophages, lymphocytes and neutrophils, and increased level of Hemoglobin, IL-1beta, TNF-alpha in bronchoalveolar lavage (BAL) fluid) (Yike et al 2002). The ultra-structural changes in alveolar type II cells
from juvenile mice exposed to *S. chartarum* spores and toxins include condensed mitochondria with separated cristae, scattered chromatin and poorly defined nucleolus, cytoplasmic rarefaction, and distended lamellar bodies with irregularly arranged lamellae (Rand et al 2002).

In other mouse model studies, inhalation of SC spores caused pulmonary hypertension and reversible pulmonary vascular remodeling (Nagayoshi et al 2011, Ochiai et al 2008). Intranasal exposure of mice to satratoxin G induced apoptosis in olfactory sensory neurons of the nose and brain in C57B16 mice (Islam et al 2009) and disrupted the synthesis and secretion of alveolar surfactant, which functions to adjusting the surface tension of the air-alveolar interface (Mason et al 2001).

The development of lung toxicity and localization/distribution of *S. chartarum* toxins were examined in a rodent model. Lung toxicity (in terms of cytotoxicity, hemorrhage, and inflammation) after a single dose of intratracheal SC spore instillation was detected as early as 6 h, peaked between 6 and 24 h, and subsided by 72 h (Rao et al 2000). Satratoxin G (SG) was mostly detectable in alveolar macrophages and to a much less extent in alveolar type II cells. SG was localized in the lysosomes, nuclear heterochromatin, and rough endoplasmic reticulum (RER). SG was not detected in neutrophils, fibroblasts, or other cells (Gregory et al 2004). In female C57B16 mice intranasally exposed to SG, the highest concentrations of SG toxin was detected in plasma in 5 min, and rapidly decreased and cleared from plasma following single-compartment kinetics within 60 min. SG toxin was shown to be distributed to nasal turbinate, kidney, lung, spleen, liver, thymus, heart, olfactory bulb, and brain, and finally excreted in feces and urine (Amuzie et al 2010).
Why study alveolar macrophages?

Alveolar macrophages (AMs) play an important role in host lung defense against inhalable pathogens (Fels and Cohn 1986). In humans, there are a series of lung defense mechanisms protecting the host from particulate pathogens. Upon inhalation of the particulate matter, particles with size over 10 µm will be cleared in the nasal cavity by nasal turbinate and mucus, whereas, smaller ones (5-10µm) escaping the nasal cavity will undergo clearance by the mucus and microvilli movement of the tracheal epithelial cells and pushed out via coughing. The finer particulates (around 1 to 2µm) will pass through the micro tracheal tubes and reach the alveolar region (Lohmann-Matthes et al 1994). There are three types of cells inside the alveolar sac. The Type I epithelial cell, accounting more than 90% of alveolar cells, forms the alveolar lining and therefore plays a direct role in air exchange of the lung. The Type II epithelial cell is in charge of surfactant (functions to adjusting the surface tension of alveolar sac) secretion and can transform into Type I cells in time of need. Alveolar macrophage cells moves around inside the alveolar sac, guarding the lung against exogenous inhaled particulates and pathogens, such as fungi, bacteria, parasites, virus, tumor cells, and other particulates. The AMs phagocytose the particulates and release a variety of biologically active mediators such as nitric oxide, reactive oxygen species, and cytokines/chemokines (IL-1, IL-6, and TNF-alpha) during the process of the phagocytosis. AMs are also involved in immune response to the antigenic particulates by antigen processing and antigen presentation to the lymphocytes (Dörger and krombach 2002).

In a mouse model intratracheally exposed to S. Chartarum spores, the toxin SG was detected mostly in alveolar macrophages (AM), and to a much less extent to alveolar type II cells, but
not in other cell types in the lung. These observations indicate that AMs play a critical role in clearance of and interacting with SC toxins (Gregory et al 2004). In different strains of SC exposed mouse, the relative sensitivity of AMs to SC toxins was related to the overall lung toxicity induced by SC toxins. The results showed the more sensitivity, the less lung toxicity, and conversely, the less sensitivity, the more lung toxicity (Lichtenstein et al 2010). This observation further emphasized the pivotal role of AMs in the SC toxin-induced lung toxicity. Based on the above background knowledge, a murine alveolar macrophage cell line, MH-S, was used in our study to investigate the toxicity and signaling mechanisms of SC toxins.

**Trichothecenes and macrocyclic trichothecenes-structure, metabolism, and biological activity**

The trichothecenes are a group of structurally related mycotoxins, characterized by a sesquiterpenoid ring structure. There are 4 types of trichothecene, including A, B, C, and D. All trichothecene types contain an epoxide at the C12-13 position. Type A trichothecenes (T-2 toxin, HT-2 toxin, and diacetoxyscirpenol (DAS)) have an isovalerate at C-8; Type B trichothecenes (deoxynivalenol (DON) and nivalenol) have a ketone at C-8; Type C trichothecenes (crotocin and baccharin) have an additional epoxide group at the C7, 8 or C9, 10 position. Type D refers to macrocyclic trichothecenes (satratoxin and roridin), containing a macrocyclic ring between the C4, 15 positions (Foroud and Eudes 2009, Sudakin 2003). Trichothecenes are non-volatile, chemically stable and resistant to UV/ionizing irradiation as well as autoclaving. The production of trichothecenes is encoded by specific genes which are regulated by the condition of temperature, humidity, and growth substrate (Mateo et al 2002, Nikulin et al 1994).
It has been shown that T-2 toxin is metabolized inside cells, and the extent of metabolism was related to the sensitivity of the cell (Carrasco et al 1973, Porcher et al 1988). T-2 toxin was shown to be metabolized by oxidation, de-epoxidation, hydrolysis, and glucuronide conjugation in liver, GI tract, and blood cells and excreted via feces and urine. Some cell lines have certain metabolic enzymes which capable of limited metabolism of T-2 mycotoxin. The half-life of T-2 toxin was short, and it varied in different cells (Yagen and Bialer 1993). In CHO and VERO cells, less than 50% T-2 toxin was metabolized in 5 hours, whereas, in cultured primary hepatocytes, about 87% of T-2 toxin was metabolized within 10 min (Pace 1986).

Macrocyclic trichothecenes (MTs) are highly toxic and have multiple biological activities, including antimicrobial (antibacterial, antifungal and antiviral), antimalarial, and anticancer. MTs are produced by *Fusarium* spp., *Myrothecium* spp., *Stachybotrys* spp., and *Trichothecium* spp. (Sy-Cordero et al 2010). The macrocyclic trichothecenes (such as Satratoxins) have been shown to cause lung toxicity in rodent model and induce apoptosis and protein synthesis inhibition in a variety of cell lines.

**Trichothecene binds to the ribosome**

One of the prominent mechanisms of trichothecene toxicity is *de novo* protein synthesis inhibition, resulting from binding of trichothecene with ribosomes. Radio-labeled T-2 toxin was found to specifically and reversibly bind to the cellular ribosomal components in both ribosomes-enriched cell lysate and Chinese hamster ovary cells. The binding of T-2 with ribosomes was shown to be in a time-, temperature-, concentration-dependent manner, with high affinity and a 1:1 ratio. The kinetics of this binding suggested that T-2 toxin easily and
immediately flows in and out of the cell membrane, no receptor-based transportation involved. The trichothecene-ribosome binding can reach saturation, but it is a dynamic equilibration of association and disassociation instead of a static status. The binding of T-2 toxin with the ribosomes was in accordance with its ability for protein synthesis inhibition (Middlebrook and Leatherman 1989).

Trichothecenes have been shown to disrupt one or more steps in protein translation, including initiation, elongation, termination, peptide bond formation, and activity of peptidyl-transferase. The toxicity of trichothecenes was shown to be related to chemical structure and concentration of the toxin, and the cell type. For example, T-2, Verrucarin A, and Roridin A have acetyl side groups on a hydrocarbon chain between carbons 4 and 13 of the basic ring structure. Loss of this side groups from either of these positions or loss of an isovaleryl group at carbon-8 position resulted in reduced protein synthesis inhibition (Thompson and Wannemacher 1986, Cundliffe and Davies 1977, Smith et al 1975, Cundliffe et al 1974).

Beside its well-known function in translating messenger RNA (mRNA) into polypeptides, ribosome was also reported to have additional extra-ribosomal functions, such as recruiting kinases involved in various signaling pathways. In rapidly growing yeast cells, the most transcription activity is committed to production of ribosomal RNA (rRNA) and ribosomal proteins (Warner 1999), which indicated the importance of ribosome function as well as fast turnover of ribosomes. The ribosomal small subunit (40S) of yeast is composed of an 18S rRNA and 32 ribosomal proteins, and the large subunit (60S) is comprised of three rRNAs (including 5S, 5.8S, and 25S rRNA) and 46 ribosomal proteins (Taylor et al 2009, Ben-Shem et al 2010); Small subunit mediates the correct interactions between the anticodons of the
tRNAs and the codons in the mRNA. The large subunit contains the peptidyl-transferase center (PTC), which catalyzes the formation of peptide bonds (Agirrezabala and Frank 2010).

Satratoxin G, one of the components of SC macrocyclic trichothecene mixture, was also shown to bind to 40S and 60S ribosomal subunits. This binding was detectable as early as 5 min in a concentration- and time-dependent manner, and preceded apoptosis induction (Bae et al 2009). Although the effects of trichothecene on ribosomal protein synthesis and the kinetics of trichothecene-ribosome binding have been extensively studied, little is known about the involvement and potential role of specific ribosomal proteins in the trichothecene-ribosome interaction. Given the observed trichothecene-ribosome binding and the reported extra-ribosomal function of some ribosomal proteins, we hypothesized that certain ribosomal proteins might be involved and play a role in SC toxin-induced cytotoxicity in alveolar macrophages.

**Trichothecene epoxide moiety and DNA damage**

The chemical structure of trichothecene has been shown to be important in its toxicity. Considering the presence of epoxide(s) (the 12, 13-epoxide moiety) in MTs, we hypothesized that macrocyclic trichothecenes might induce DNA damage. The mechanism could be similar to that of Aflatoxin B₁ (bio-activated form), in which the epoxide moiety binds to DNA, forming adduct and subsequently leads to DNA damage (Choy 1993).

**Yeast deletion library**

The yeast, *Sacchromyces cerevisiae*, has been used as a model for studying eukaryotes.
Certain critical and basic cellular functions are usually conserved in eukaryotes, including but not limited to protein translation and DNA-damage response. Importantly, all the ribosomal proteins in yeast have homologs in mammalian (Chen et al 1986). Moreover, trichothecene have been shown to inhibit growth of yeast (Engler et al 1999, Schappert and Khachatourians 1983).

A *S. cerevisiae* Mat-A haploid deletion library was generated from strain *BY4741* (MAT_his3_1 leu2_0 met15_0 ura3_0) by the Saccharomyces Genome Deletion (SGD) Project (http://sequence-www.stanford.edu/group/yeast_deletion_project/deletions3.html). The Mat-A haploid library contains 4,848 Mat-A haploid clones, with one specific gene deleted in each mutant. This knock-out library has been used in a variety of studies including chemical generic screening and searching for target genes that are important in mammalian pathogenesis (Ran et al 2003, Cheng et al 2007). This library provides a powerful tool to study the role of individual gene in the macrocyclic trichothecene-macrophage interaction (pure macrocyclic trichothecenes as well as the SC macrocyclic trichothecene mixture). In Chapter IV of the thesis, this yeast deletion library has been used in screening for macrocyclic trichothecene (MT)-resistant and sensitive mutant, in order to reveal the individual gene (possibly ribosomal-related and DNA damage/repair-related) which might play critical role in trichothecene-induced toxicity and signaling events.
Chapter II
DNA damage, redox changes, and associated stress-inducible signaling events underlying the apoptosis and cytotoxicity in murine alveolar macrophage cell line MH-S by methanol-extracted *Stachybotrys chartarum* toxins*

*This chapter has been published at: Toxicology and Applied Pharmacology 214 (2006): 297–308*
Abstract

Spore-extracted toxins of the indoor mold *Stachybotrys chartarum* (SC) caused cytotoxicity (release of lactate dehydrogenase), inhibition of cell proliferation, and cell death in murine alveolar macrophage cell line MH-S in a dose- and time-dependent manner. Apoptotic cell death, confirmed based on morphological changes, DNA ladder formation, and caspase 3/7 activation, was detectable as early as at 3 h during treatment with a toxin dose of 1 spore equivalent/macrophage and was preceded by DNA damage beginning at 15 min, as evidenced by DNA comet formation in single cell gel electrophoresis assay. The apoptotic dose of SC toxins did not induce detectable nitric oxide and pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) but showed exacerbated cytotoxicity in presence of a non-apoptotic dose of the known pro-inflammatory agent LPS (10 ng/ml). Intracellular reduced glutathione (GSH) level showed a significant decrease beginning at 9 h of the toxin treatment whereas oxidized glutathione (GSSG) showed a corresponding significant increase, indicating a delayed onset of oxidative stress in the apoptosis process. The toxin-treated macrophages accumulated p53, an indicator of DNA damage response, and showed activation of the stress inducible MAP kinases, JNK, and p38, in a time-dependent manner. Chemical blocking of either p38 or p53 inhibited in part the SC toxin-induced apoptosis whereas blocking of JNK did not show any such effect. This study constitutes the first report on induction of DNA damage and associated p53 activation by SC toxins, and demonstrates the involvement of p38- and p53-mediated signaling events in SC toxin-induced apoptosis of alveolar macrophages.

Keywords: *Stachybotrys chartarum*; Mycotoxin; Trichothecenes; Indoor mold; Apoptosis;
Introduction

Indoor exposure to toxic and allergic agents is a significant environmental and public health problem. Majority of the population spend more than 90% of their lifetime indoors and may frequently be exposed to indoor air pollutants, including molds, bacteria, and chemicals (Daisey et al., 2003). About 20–40% of the buildings have been estimated to encounter mold growth in Northern Europe and North America (Fog, 2003). *Stachybotrys chartarum* (SC) is a toxigenic mold reported to occur indoors in 6% of the American homes (Shelton et al., 2002). While growing on moisturized cellulose-based materials in indoor environments, SC produces a mixture of potent mycotoxins, including macrocyclic trichothecenes (Bata et al., 1985). Considering the nature and magnitude of the potential threat to public health on a daily basis via inhalation of toxic mold and mold products, SC has drawn increasing attention of public media, and emerged as a topic of intense research in Europe and North America.

SC was first noticed in 1930s for its association with several cases of stachybotryotoxicosis outbreaks in livestock consuming SC-infested straw. Coincidently, similar symptoms including skin/mucus irritation, and hemorrhage, were also reported in farm workers handling the SC-contaminated straw (Hintikka, 1977). More recently, SC was reported in the indoor air samples from homes associated with sick-building syndrome (SBS) and acute infant idiopathic pulmonary hemorrhage (IPH) (Dearborn et al., 1999; Hodgson et al., 1998). In the infant IPH outbreak during 1993–1998, 37 cases were reported in Cleveland, Ohio, of which 12 were fatal. Subsequently, additional 101 infant IPH cases were reported across North
America (Dearborn et al., 1999). In addition to the circumstantial epidemiological association of SC with SBS and IPH (Kuhn and Ghannoum, 2003), subsequent studies have provided additional support for the association of SC with respiratory disease. SC was isolated from the lung of a child diagnosed with IPH (Elidemir et al., 1999). In laboratory animal studies using young rodent models, toxin-containing SC spores induced severe inflammation and hemorrhage (Yike et al., 2002) and the presence of these SC components (spores and toxins) was detected in the alveolar macrophages based on immunocytochemical analysis (Gregory et al., 2003, 2004).

The inhaled toxin-containing SC spores first encounter the alveolar macrophages (AMs) in the exposed lung. AMs, the innate defense cells, are known to guard the lung against incoming particulates’ insult and ensuing deeper tissue damage, as a result of particulate phagocytosis, production of the potent inflammatory cytokines, and/or recruitment of other immune cells (Lohmann-Matthes et al., 1994). Nature of interaction between the inhaled SC toxins and the alveolar macrophages is crucial in determining whether these toxins will be partly or completely eliminated by the host and whether these will cause moderate or severe damage to the host lung tissue. Since the outcome is expected to depend on the overall effect of the mixture of toxins carried on or in the spores, it would be closer to the real-world exposure scenario to study the toxin mixture as a whole and not a particular isolated component. Considering the critical need to understand the specific interactions between the SC toxins and the host macrophages, and the importance of the mixed exposure, this study was designed using murine alveolar macrophage cell line (MH-S) and the whole toxin mixture extracted from SC spores.
Cytotoxicity of SC toxins toward macrophages was evaluated in terms of cell proliferation and cell death, and the mode of cell death was characterized. Cellular events underlying the cytotoxicity of SC toxins were investigated. Induction of inflammatory mediators, which is a typical response of macrophages to pathogens and various stimuli, was studied. Particularly, TNF-α and nitric oxide (NO), the common mediators reported in in vivo animal exposure studies (Yike et al., 2002), were assayed among other pro-inflammatory mediators, using RT-PCR. Since generation of reactive oxygen species and depletion of intracellular glutathione level are often involved in toxicant-induced apoptosis (Boulares et al., 2002), the intracellular glutathione level was monitored to study the possible effect of SC toxins on the cellular redox status and its contribution during the apoptosis of alveolar macrophages. Some mycotoxins such as aflatoxin B₁ act as genotoxic agents as a result of their metabolic activation that introduces DNA binding epoxide in their structure. Macrocyclic trichothecenes, the major components of the SC toxin mixture, have native epoxides in their chemical structure, similar to the DNA binding epoxide in the bioactivated aflatoxin B₁ (Choy, 1993), indicating their potential as genotoxic agents. Hence, SC toxin-treated macrophages were assayed for DNA damage using DNA comet assay and micronuclei formation. Considering that MAP kinases are frequently involved in toxicity and cell death, and that genotoxic stress involves the role of p53, we investigated the activation of the stress-responsive MAP kinases and accumulation of p53 in SC toxins-treated macrophages to explore the possible signaling events involved in the toxin–macrophage interaction. The role and relative contributions of these signaling events in cell death were evaluated by using respective specific inhibitors.
Materials and methods

Preparation of mold spores and the toxin mixture

The toxic indoor mold S. chartarum JS5817 (= ATCC 201211), originally isolated from a water-damaged home with an infant pulmonary hemorrhage patient (Jarvis et al., 1998) was used in this study. The mold (referred to as SC in this report) was grown on regular Whatman filter paper (number 1) layered on the modified malt extract agar (1% malt extract, 1% dextrose, 0.1% yeast extract, 0.1% peptone, and 2% agar) in Petri dishes (100 mm diameter) in a humidified incubator at 28 °C for 7 days. The spores were harvested and extracted with 100% methanol. Briefly, the spores from a uniform fungal growth in 10 Petri dishes were collected in 50 ml of 100% methanol using sterile cotton swabs, and subjected to repeated methanol extraction (2 × 50 ml) by shaking for 2 h each on an orbital shaker. The pooled methanol extract containing the toxins was dried under vacuum using rotary evaporator, concentrated under N₂, and suspended in 10% methanol to obtain the spore toxin mixture (referred to as “SC toxins” in this study). Meanwhile, the extracted spores were pelleted down by centrifugation (2000 × g for 15 min), and resuspended in distilled water (10 ml) by vortexing. Spore count was determined by making a 1:100 dilution (100 μl in 10 ml distilled water) of the above suspension and transferring a 10 μl aliquot of the diluted suspension onto a hemacytometer (Bausch and Lomb Optical Corp., Rochester, NY) for counting under a light microscope; the total number of spores was calculated as follows: Total spore number = [Spores counted in the designated hemacytometer area] × 10⁴ × 100 (dilution factor) × 10 (total volume of spore suspension). Based on the calculated initial total spore count taken, the spore toxin mixture was further diluted into an appropriate volume of 10% methanol to obtain
the desired final concentration (10⁸ spores equivalent/ml) of the SC toxins and was used as a stock solution. Since the spore toxin mixtures were prepared as needed in this study, the uniformity of these preparations in terms of toxicity was ensured by performing a cytotoxicity assay using MH-S cells (see the assay description in a following subsection). The acceptable preparations had a potency of ~50% lactate dehydrogenase (LDH) release in a 24 h treatment of MH-S cells at 10⁶ spores equivalent/10⁶ cells concentration and no detectable LDH release at 10⁵ spores equivalent/10⁶ cells concentration. The “SC toxins” preparations were filtered through a 0.2 μm cellulose acetate-based syringe filter (Nalgene company, Rochester, NY) before refrigeration for further use. The SC toxins were found to be stable under the storage conditions.

Murine alveolar macrophages—culturing, maintenance, and treatment

Murine alveolar macrophage cell line MH-S (ATCC CRL-2019) was used. The cells were cultured in 10-cm culture dish (Corning Inc., Corning, NY) using RPMI 1640 medium (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum and 1% streptomycin–penicillin–glutamate solution (Invitrogen, Carlsbad, CA). Active cells grown for 48 h at 37°C in a humidified 5% CO₂ incubator were collected, counted, and adjusted to a concentration of 5 × 10⁵ cells/ml for further use using standard protocols (Mbawuike and Herscowitz, 1989). The cell suspension was treated with the SC toxins using appropriate concentrations, as indicated. The concentration of SC toxins was expressed as spore equivalents.

Cytotoxicity assay

Cytotoxicity was measured in terms of release of lactate dehydrogenase (LDH) using the CytoTox96 non-radioactive cytotoxicity assay kit (Promega, Madison, WI). Varying
concentrations of the SC toxin mixture (10⁵ spores equivalent/10⁶ cells through 10⁷ spores equivalent/10⁶ cells) were tested for their cytotoxicity on MH-S cells in a treatment for varying periods of time (0, 1, 3, 6, 9, 12, and 24 h). In one of the treatments, the MH-S cells were pretreated with LPS (10 ng/ml) for 1 h before addition of a selected dose of SC toxin mixture (10⁶ spores equivalent/10⁶ cells) followed by incubation for varying periods of time as above. Cells treated with equal volume of vehicle solvent (10% methanol) were used as negative controls for estimating the spontaneous LDH release. The required 'maximum LDH release' sample was prepared by repeatedly freezing-thawing the cell suspension of the negative untreated control. The optical density (490 nm) was measured using a microtiter-plate reader. LDH release was calculated in terms of percent by dividing the OD₄₉₀ nm of samples with that of the maximum release control.

Cell proliferation assay

Freshly cultured MH-S cells (5 × 10⁵ cells/ml) were treated with varying levels of the SC toxins for different periods of time (0, 1, 3, 6, 9, 12, and 24 h) as discussed above in a 96-well microtiter plate (Fisher Scientific, Hanover Park, IL) with a total volume of 100 μl per culture well. Cell proliferation inhibition was measured using CellTiter 96 aqueous one solution cell proliferation assay kit (Promega, Madison, WI). This is essentially an MTT-like assay as it is based on measuring bioreduction of a tetrazolium compound into a stable color product (formazan) by actively metabolizing cells, as an index of proliferation. Briefly, 10 μl of the aqueous one reagent was added to each culture well. The reaction was allowed to proceed for 4 h at 37 °C in a humidified 5% CO₂ incubator and the absorbance was measured at 490 nm using a microtiter-plate reader.
Cell viability analysis by trypan blue exclusion

Aliquots (5 μl each) of the MH-S cell suspension (5 × 10^5 cells/ml) treated with varying levels of the SC toxins for different periods of time as given above were mixed with 45 μl of 0.4% trypan blue staining solution (Invitrogen, Carlsbad, CA). The number of viable cells (unstained) versus dead cells (blue stained) was determined by a hemacytometer. The percentage of viable cells was calculated by the following formula:

\[
\frac{(Viable \ cell \ number \ of \ the \ treated \ cells)}{(Viable \ cell \ number \ of \ the \ control \ cells)} \times 100%.
\]

Microscopic analysis

Phase-contrast microscopy

Freshly grown MH-S cells (10^6) were treated with the SC toxins using a selected concentration (10^6 spores equivalent) or with an equal volume of vehicle (10% methanol) for 24 h in a 24-well cluster. Morphological changes in the macrophage cells were examined and photographed directly using phase-contrast microscope.

Fluorescence microscopy

A sterile glass cover slip was placed at the bottom of a 10 cm-culture dish before addition of MH-S cell suspension for treatment with either SC toxin (10^6 spores equivalent/10^6 cells) or equal volume of vehicle (10% methanol). After the treatment, the cover slip was washed and fixed. The fixed cells were permeabilized using 0.5% NP-40 in PBS buffer and stained with 300 nM DAPI solution (Molecular Probes, Eugene, OR) followed by rinsing and drying of cover slips before mounting on the slides. The stained cells were examined under fluorescence microscope using an appropriate combination of excitation and emission wavelengths (Ex358/Em461nm).
DNA ladder analysis

The MH-S cells (SC toxin-treated and vehicle-treated) were lysed on ice for 30 min using lysis buffer (10 mM Tris–Cl pH 7.5, 50 mM EDTA pH 8.0, 0.5% NP-40), and the lysate was treated with RNase A (50 μg/ml) and proteinase K (100 μg/ml). DNA was precipitated and electrophoresed on 1% agarose gel (containing 10 μg/ml ethidium bromide) for analysis of fragmentation. The gel was photographed under a UV transilluminator. Caspase activity assay

Caspase activity was detected using the Apo-ONE Homogeneous Caspase-3/7 assay kit (Promega, Madison, WI). The caspase reagent was added to the macrophage cells after toxin treatment, and the contents of each well were gently mixed followed by incubation at room temperature for 12 h. The fluorescence was recorded at Ex485/Em530 nm using a microtiter-plate reader.

Glutathione (GSH/GSSG) assay

The toxin-treated and vehicle-treated control MH-S cells were tested for reduced (GSH) and oxidized (GSSG) form of glutathione using o-phthalaldehyde (OPA) assay (Senft et al., 2000). The levels of GSH and GSSG (pmol) were normalized based on cellular total protein level (μg), which was quantified using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

Alkaline DNA comet assay

The MH-S cells (toxin-treated and control) were collected and suspended in low-melting agarose gel (0.5% in PBS). A drop of the resulting soft agarose-cell suspension was added on a glass slide precoated with regular agarose gel (1% in PBS). The cells were lysed using the lysis solution (2.5M NaCl, 100 mM EDTA, 10 mM Tris base, pH 10, 1% NP-40) for 1 h at 4
The released DNA was allowed to denature under alkaline conditions (10 N NaOH, 200 mM EDTA pH 13) for 40 min at 4 °C. Following unwinding, the DNA was electrophoresed at 300 mA for 35 min. The DNA in the gel was stained with a fluorescent dye (DAPI) and observed under fluorescence microscope. The cells with no DNA damage showed intact nuclei, whereas those with damaged DNA showed varying degree of comet tail formation. The untreated and vehicle-treated cells were used as negative controls whereas the H2O2-treated cells served as positive control. The number of total comet-forming cells and that of each subtype (with variable comet tail length) were recorded (per 100 cells on each slide). The extent of DNA damage in the comet-forming cells was expressed using the standard DNA comet score, with scores assigned in terms of the tail length of the comet. Scores ranged from 0 to 4, corresponding to the following criterion: 0 = no tail or tail length of 0 nuclear diameter (ND), 1 = tail length of <1 × ND, 2 = tail length of <2 × ND, 3 = tail length of >2 × ND, and 4 = most of the DNA content becoming the tail, respectively.

### Micronuclei assay

Macrophage cells were cultured on sterile cover slip with and without SC toxin treatment. In this case, the cells were treated with lower concentration (10^5 spores equivalent/10^6 cells) of SC toxins for 72 h. After staining with DAPI, the cells were examined under fluorescence microscope, and the number of micronuclei (MN) was recorded in 10 randomly selected microscopic fields. A total of 100 cells were counted in each field. The MN numbers were averaged and analyzed statistically.

### Analysis of pro-inflammatory markers

Gene-specific RT-PCR technique was used to measure relative levels of different
pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and of inducible nitric oxide synthase (iNOS) as a measure of NO production, in the toxin-treated cells versus the negative control (vehicle-treated), and positive control (treated with bacterial lipopolysaccharide at 100 ng/ml) cells. The cells were subjected to total RNA extraction using TRI reagent kit (Molecular Research Center, Inc. Cincinnati, OH). The RNA preparation was further purified using the RNeasy mini kit (Qiagen, Valencia, CA). Gene-specific primers were designed for selected pro-inflammatory markers, namely TNF-α, IL-1β, IL-6, and iNOS using Generunner version 3.05 (Hastings software Inc., Hudson, NY) and were custom-synthesized by Integrated DNA Technologies (Coralville, IA). Sequences of the designed primers are listed in Table 1. Reverse transcription (RT) reaction and the real-time PCR reaction were performed using the GeneAmp Thermostable rTth Reverse Transcriptase RNA PCR Kit (Applied Biosystems, Foster City, CA). The RT reaction was performed at 60 °C on the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) for 10 min and the PCR reaction was performed on the Smart Cycler Real-time PCR system (Cepheid, Sunnyvale, CA), using the following reaction conditions: denaturation at 94 °C for 120 s, followed by 35 cycles of amplification (each involving denaturation at 94 °C for 30 s, annealing at 60 °C for 60 s, and extension at 68 °C for 120 s), and a final extension at 68 °C for 7 min. Ribosomal protein S18 (RPS18) was used as the internal control gene. The fold changes in gene expression were calculated as following using a published method (Chang et al., 2002). Fold change = 2^ΔΔCt, where Ct is the threshold cycle value, ΔCt is the difference between the Ct values for the target gene and internal control gene (RPS18), and ΔΔCt is the difference between the ΔCt values for the vehicle control cells and the treated cells.
The levels of nitric oxide were also measured directly in the culture supernatants using Griess reagent kit (Promega, Madison, WI).

**Western blot analysis to determine activation of p53 and MAP kinases**

Total protein was extracted from the treated and control (vehicle-treated) cells. Briefly, the cells were lysed with the M-PER mammalian protein extraction reagent (Pierce Biotechnology, Rockford, IL) supplemented with protease inhibitors (10 μg/ml Aprotinin, 10 μg/ml Leupeptin, 1 mM Phenylmethylsulfonyl fluoride) and phosphatase inhibitors (0.1 mM Sodium orthovanadate, 10 mM Tetrasodium pyrophosphate, 10 mM Sodium fluoride). Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). An aliquot (30 μg) of the total protein was resolved by 10% SDS-PAGE and the separated proteins were transferred onto a nitrocellulose membrane. Western blot analysis was performed using the Antiactive JNK/p38 polyclonal antibodies (Promega, Madison, WI) and full-length p53 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). HRP-conjugated goat-anti-rabbit-IgG was used as a common secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Protein visualization was accomplished by the SuperSignal west pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL).

**Role of activated MAP kinases (JNK, p38) and p53**

This was investigated using the specific chemical inhibitors and the effect was assessed using the CellTiter-Glo luminescent cell viability assay kit (Promega, Madison, WI) and DNA fragmentation assay. Briefly, the MH-S cells were treated with 10 μM p53 inhibitor Pifithrin-α (EMD Biosciences, San Diego, CA), 20 μM p38 inhibitor SB202190 (EMD Biosciences, San Diego, CA), or 20 μM JNK inhibitor SP600125 (EMD Biosciences, San Diego, CA), to
attain inhibition either before the onset of apoptosis or immediately after the onset of apoptosis. Two time points, 1 h before or 3 h after the SC toxin addition, were used. The treated cell suspension was mixed with 100 μl assay reagent, and the luminescence was recorded with a microtiter-plate reader, to assess the cell viability. DNA fragmentation was assayed as described above.

**Statistical analysis**

The results are presented as mean±standard deviation of three biological samples. Comparisons were made using Student's t test and P <0.05 as the level of significance.

**Results**

**Cytotoxicity of SC toxins on the alveolar macrophages**

In a time-course treatment (0–24 h) of the murine alveolar macrophages (MH-S cell line) with SC toxins (whole toxin mixture extracted from spores) at the concentrations 10^6 and 10^7 spores equivalent/10^6 cells, the toxins caused cytotoxicity (Fig. 1A) beginning at 12 h of treatment, as evidenced by release of lactate dehydrogenase (LDH). In an extended time-course treatment (24, 48, and 72 h) with these toxin concentrations (data not shown), the cytotoxicity peaked at 48 h (83.2% and 90.5%, respectively) and remained unchanged thereafter. However, the lower concentration of the toxin mixture (10^5 spores equivalent/10^6 cells) did not show any cytotoxicity in terms of a detectable difference in LDH release as compared to the vehicle control (10% methanol-treated cells) in either time course. Similarly, the cell proliferation assay (Fig. 1B) showed that only the upper range of concentration (10^6 and 10^7 spores equivalent/10^6 cells) of SC toxins caused marked inhibition of proliferation.
beginning 12 h of treatment and the effect peaked at 48 h (20.7% and 14.0%, respectively). The above trends were further confirmed by the more direct Trypan blue exclusion-based viability assay, which indicated a detectable increase in dead cells in the upper concentration-treated group as compared to the lower concentration-treated group (Fig. 1C).

**Apoptosis induction in alveolar macrophages**

The cultured macrophage cells (MH-S) treated with SC toxins, when examined directly under phase-contrast microscope, showed apparent changes in both cell morphology and cell viability in a time-course analysis. In contrast to the normally growing (round, large and confluent) control cells treated with the vehicle (10% methanol), the toxin-treated cells showed shrinkage and clustering, and floated in the medium with few small cells sparsely distributed on the bottom of the flask (Figs. 2A and B, top panel). Fluorescence microscopy using DAPI staining showed nuclear changes in the toxin-treated macrophages. As compared with the negative control cells, the toxin-treated cells showed chromatin condensation, shrinkage of the nuclei, typical apoptotic bodies, and micronuclei (Figs. 2C–F, top panel). Agarose gel electrophoresis demonstrated classic apoptosis feature, the “DNA ladder" formation (Fig. 2A, bottom panel), in the treated cells as compared to the intact DNA in the vehicle-treated control cells. Caspase 3/7 activity was increased (~16-fold at 9 h treatment) for the SC toxin-treated cells, as compared to the vehicle-treated control cells (Fig. 2B, bottom panel). In both of the above assays (morphological and DNA ladder formation), apoptosis became apparent beginning at 3 h of treatment and the intensity increased with time.

**Effect on inflammatory mediators**
In contrast with the bacterial lipopolysaccharide (LPS) treatment (positive control), which dramatically increased the mRNA levels of TNF-α, IL-1β, IL-6, and iNOS, the apoptotic dose of SC toxins (at 10^6 spores equivalent/10^6 cells) did not show considerable effect on the transcriptional expression of any of the above inflammatory mediators (Table 2). Similarly, SC toxins at 10^4 through 10^7 spores equivalent/10^6 cells showed no induction of nitric oxide (NO) in the treated macrophages at any of the incubation time points (from 24 h through 72 h) (data not shown). However, when macrophage cells were pretreated with LPS (10 ng/ml) for 1 h, before addition of SC toxins (10^6 spores equivalent/10^6 cells), the cytotoxicity as examined by LDH release assay at a series of time points (0, 1, 3, 6, 9, 12, 24 h) was profoundly exacerbated (38% vs. 47% at 12 h, and 58% vs. 98% at 24 h) (Fig. 3). Since LPS alone (10 ng/ml) did not induce cytotoxicity, this indicated a possible role of LPS-induced cytokines in exacerbating the cytotoxic effect of the SC toxins.

**Perturbations in intracellular glutathione (GSH and GSSG)**

In SC toxin-treated alveolar macrophages, reduced glutathione (GSH) level showed an initial transient elevation peaking at 3 h of treatment (~160% of the control), followed by a steady decrease to ~50% of the control in a 24-h treatment (Fig. 4A). The oxidized glutathione (GSSG) level did not show considerable change until 9 h, and showed significant increase to over 140% at 24 h (Fig. 4B).

**Toxin-induced DNA damage in macrophages**

MH-S cells treated with the apoptotic dose of SC toxins (10^6 spores equivalent/10^6 cells) showed DNA comet formation as early as 15 min, and the severity of DNA damage increased
with time (Fig. 5, top panel) as indicated by the comet score (Fig. 5, bottom panel). Since the caspase activation and “DNA ladder” formation were not detectable before 3 h period during the toxin treatment of the cells, the immediate onset (15–60 min) of DNA damage as indicated by comet assay is an independent effect and is not a secondary consequence of the SC toxin-induced apoptosis. The early DNA damage seen in SC toxin-treated MH-S cells pointed to a direct interaction of the SC toxins with nuclear DNA. Such effect of the SC toxins was further confirmed based on micronuclei formation using the non-apoptotic concentration of SC toxins. In micronuclei assay, MH-S cells treated with lower (non-apoptotic) concentration of SC toxins showed formation of micronuclei after 72 h incubation, when no morphological signs of apoptosis were detected (Fig. 6).

**p53 accumulation and MAPK (JNK/p38) activation and their role in apoptosis**

As shown in Fig. 7 (top panel), treatment with SC toxins at the apoptotic concentration (10⁶ spores equivalent/10⁶ cells) caused accumulation of p53 in the macrophages during the treatment period (0.5 h through 3 h). The treatment also caused activation of the MAP kinases JNK and p38 as indicated by Western blot analysis using the specific antiphosphorylated JNK and p38 antibodies. The phosphorylation of both JNK and p38 that was evident in 0.5 h increased with time. Role of p53 accumulation and JNK/p38 activation in SC toxin-induced apoptosis was investigated based on the effect of their respective specific chemical inhibitors toward restoring the cell viability. Among the different treatment combinations tested, pretreatment (1 h before toxin addition) with 10 μM pifithrin-α (p53 inhibitor) or posttreatment (3 h after toxin addition) with 20 μM SB202190 (p38 inhibitor) partially rescued cell viability in the SC toxin-treated cells (Fig. 7, bottom panel). However, neither the
posttreatment with pifithrin-α or pretreatment with SB202191, nor the pretreatment or posttreatment with 20 μM SP600125 (JNK inhibitor) showed any impact on cell viability (data not shown). DNA ladder analysis further confirmed that chemical blocking of p53 or p38 decreased the extent of apoptosis, as indicated by reduced DNA fragmentation (Fig. 8).

**Discussion**

As a part of the innate defense mechanism of the host lung, alveolar macrophages (AMs) play a crucial role in clearance of various kinds of inhaled pathogens and toxicants (Lohmann-Matthes et al., 1994). In in vivo studies on SC exposure of rats using whole spores, AMs were found to phagocytose SC spores or fragments and one of the SC toxins (satratoxin G) was localized in the lysosome (Gregory et al., 2004). Severity of the pulmonary toxicity correlated with the number of toxin-containing SC spores instilled (Rao et al., 2000). These indirect studies using whole spores seemed to indicate that the SC toxins in large quantities overwhelm the capacity of AMs for phagocytosis and clearance, leading to a compromise of AM function in immune/inflammatory response and damage of lung tissues. In light of this background, we investigated the direct toxicological interaction of the SC toxins with the alveolar macrophages, using a murine lung cell line and a whole mixture of the toxins extracted from the SC spores. Cultured alveolar macrophage cell line instead of primary alveolar macrophage cells was used to facilitate the study as it involved the use of a range of variables and large number of macrophages. The MH-S cell line was chosen because this is a cell line originally derived from mouse primary alveolar macrophages and demonstrated to be morphologically and functionally similar to primary cells (Mbawuike and Herscowitz, 1989).
concentrations of SC toxins tested in vitro in this study were intended to coincide with the possible spore exposures in vivo. The tested concentrations (10⁵ through 10⁷ spores equivalent /10⁶ macrophages) are equivalent to 1:10, 1:1, and 10:1 spore:macrophage ratios, a likely scenario expected in in vivo spore exposures. So, if one macrophage is exposed to one SC spore-associated toxins in vivo, it represents the apoptotic dose (10⁶ spores equivalent SC toxins/ 10⁶ cells) used in this study. The SC toxin mixture caused induction of apoptosis as opposed to necrosis in the macrophages, as evidenced by periodic morphological examination of the treated MH-S cells. Increasing number of the treated cells shrank in size and condensed in both the cytoplasm and nucleus, which is typical for apoptotic cells, while cellular swelling, which is characteristic of necrotic cell death, was not detected. Furthermore, as is typical of the in vitro apoptosis, cells first showed apoptotic changes (3 h) which eventually led to increased porosity of the cell membrane and secondary necrosis as evidenced by the leakage of LDH (12 h). In contrast, apoptotic cells in vivo tend to undergo phagocytotic clearance by adjacent healthy cells and do not undergo secondary necrosis.

Apoptosis is a common mechanism used by a variety of pathogens and toxicants to evade host immune system (Navarre and Zychlinsky, 2000; Wang and Zhang, 2000). Macrocyclic trichothecenes have been shown to exert toxicity in other cell types (HL-60, RAW264.7, and U937) (Nagase et al., 2002; Nielsen et al., 2002; Yang et al., 2000) because of their common ability to cause protein synthesis inhibition via binding to the 18S rRNA of the ribosome large subunit (Cundliffe and Davies, 1977). This mode of action has been speculated to be a major mechanism underlying induction of cell apoptosis by this group of trichothecenes, such as in the SC toxin mixture. While some studies (Olmo et al., 2001) have demonstrated that protein
synthesis inhibition per se does not result in apoptosis but might induce quiescence (when the cells are still alive but metabolically less active), others showed that protein synthesis inhibitors cause either promotion or inhibition of apoptosis depending on cell-stimuli combination (Mattson and Furukawa, 1997). Considering the controversial status of the role of protein synthesis inhibition in cell apoptosis, other specific mechanism(s) underlying the SC toxin-induced apoptosis needed to be investigated.

**Induction and role of genotoxic stress**

Direct damage to the genomic DNA is a known trigger of the cytotoxicity by a number of environmental pollutants (Choy, 1993; Park and Schatz, 1999). For instance, in case of the known genotoxic pollutant chemicals aflatoxin and benzo (a) pyrene, the epoxide group in their bioactivated metabolites causes the interaction with DNA and DNA damage. Considering the presence of similar epoxide groups in the native chemical structure (Tuomi et al., 1998) of macrocyclic trichothecenes present in the SC toxin mixture, it appeared likely that these toxins cause a possible DNA damage in the treated macrophages. SC toxin-induced DNA damage was indeed observed in the present study. Furthermore, DNA damage has been known to trigger signaling events leading to cell apoptosis (Norbury and Zhivotovsky, 2004).

To extrapolate and test this possible mechanism in this study, we first ensured that the observed DNA damage in the SC toxin-treated macrophages is due to the direct toxin interaction and is not a secondary consequence of the ensuing apoptotic reactions such as caspase/endonuclease activity. In this context, the detectable apoptotic indicators, caspase activity and DNA fragmentation, did not show up in the first 3 h of toxin treatment at the apoptotic dose ($10^6$ spores equivalent/$10^6$ cells), unlike the onset of DNA damage which...
occurred prior to the appearance of any signs of apoptosis. Using the DNA comet assay, a sensitive method to detect DNA damage in single cells, the apparent DNA damage was detectable in as early as 15 min of toxin treatment, and the intensity of DNA damage increased with time based on comparison with the standard scores for comet formation. Additional line of evidence for the direct DNA damaging effect of SC toxins came from micronuclei assay, in which a non-apoptotic dose of SC toxin mixture was used for the treatment. Apparent elevation in the number of micronuclei in the treated cells revealed the potential of SC toxins (possibly of the major component toxins, the macrocyclic trichothecenes) to cause DNA damage in macrophages.

**Lack of inflammatory/immune response**

Toxin-containing SC spores instilled intratracheally in infant rat model were reported to cause severe inflammatory response, including granuloma formation, pulmonary hemorrhage/hemosiderosis, infiltration of lymphocytes/monocytes, and dramatic increase in IL-1β and TNF-α levels in bronchial alveolar lavage (BAL) fluid (Yike et al., 2002). It is not yet clear whether SC toxins are critical in inducing this response and whether these pro-inflammatory changes have any role in SC toxicity. Upon stimulation with a variety of pathogens and toxicants, macrophages are activated causing the release of pro-inflammatory cytokines and nitric oxide (NO) in order to amplify the immune defense response by recruiting neutrophils, lymphocytes, and other related cells, and to kill the pathogens (Lohmann-Matthes et al., 1994). Likewise, endotoxin, the lipopolysaccharide (LPS) component of the gram-negative bacterial cell wall, is a typical stimulant for macrophages and is known to cause dramatic induction of pro-inflammatory mediators as well as apoptosis in macrophages.
Macrophage apoptosis by LPS has been proposed to be associated with the induction of TNF-α and NO (Albina and Reichner, 1998; Xaus et al., 2000). Considering this causal relationship, and the fact that in vivo studies on exposure of infant rat model with toxic SC spores caused induction of inflammation (see above), the level of pro-inflammatory markers was examined in this study. However, SC toxins did not show any induction of NO (data not shown). This is consistent with our previous observations using the whole SC spores (Wang and Yadav, 2003). Since macrocyclic trichothecenes are protein synthesis inhibitors, a negative outcome in terms of induction at the protein level did not necessarily mean that the related gene transcription was not upregulated. Hence, we investigated transcriptional induction of the gene iNOS, encoding the inducible nitric oxide synthase (enzyme responsible for NO production), and of the genes encoding other cytokines (TNF-α, IL-β, and IL-6), using gene-specific quantitative real-time RTPCR. There were little changes, if any, in their levels in the SC toxin-treated AMs (Table 2). The results imply that SC toxins are not the considerable inducers of the pro-inflammatory response and the above mediators are less likely to be directly critical in the induction of apoptosis. Since this seems inconsistent with the in vivo animal model experiments, in which inflammation was found in SC spore-induced pulmonary toxicity (Yike et al., 2002), the observed inflammatory response might be elicited by some unknown spore wall structural components, or as a secondary consequence of the resulting lung tissue damage itself caused by the SC toxins. In vitro experiments with whole spores in other cell types have given some insights on the inflammatory response. Ruotsalainen et al. (1998) also reported a lack of induction of NO and iNOS by 21 strains of SC spores, and low induction of TNF-α and IL-6 was only associated with strains that showed lower cytotoxicity.
Further studies found that the Satratoxin-producing SC isolates were highly toxic, and the Atranone-producing SC isolates induced inflammatory mediators like TNF-α and IL-6 in RAW264.7 cells (Nielsen et al., 2002). In this study, as the SC toxins were extracted from a potent satratoxin-producer strain by a relatively polar solvent (methanol), the majority of the SC toxins are expected to be the trichothecenes (satratoxins), which are not a significant inducer of inflammatory response. If a non-polar solvent was used, it is likely that other extracted components of spores could have shown some inflammatory response and altered cytotoxicity profile. In this context, cotreatment of MH-S cells with a known pro-inflammatory reagent LPS (at a non-toxic dose) led to exacerbation of cytotoxicity caused by SC toxins. This observation extends our understanding of the toxicity of SC spores in real-world in vivo exposures, wherein pro-inflammatory changes occur and coinhalation of SC spores and bacteria (or endotoxin) is expected.

**Induction and role of oxidative stress**

Generation of reactive oxygen species and subsequent depletion of the major intracellular antioxidant glutathione (GSH) by its oxidation (conversion to GSSG) have been shown to induce apoptosis in other cell type-stimuli combinations (Boulares et al., 2002; Swamy and Huat, 2003). In our study, the time line of the decrease in GSH level coincided with that of the increase in GSSG level, with both happening during late phase of apoptosis (at 9 h treatment). This indicated that the observed depletion of GSH was the result of oxidative stress. However, the possibility that GSH was depleted in part by complexing with SC toxins and/or its metabolites cannot be excluded. In the present study, depletion of GSH started after the onset of apoptosis (3 h), as detected by both DNA fragmentation and caspase activity. This suggests
that oxidative stress as a result of depletion of GSH is not responsible for triggering the apoptosis at the early time point, but may be involved in its progression or exacerbation at a later time point. Pretreatment (4 h) and coincubation (24 h) with N-acetyl-L-cystein (NAC), a GSH precursor, at selected protective concentrations (5 mM and 10 mM) could not rescue MH-S cells from SC toxin-induced apoptosis (data not shown). This is contrary to the observations on the SC toxin mixture component satratoxin H in other studies (Nusuetrong et al., 2005), wherein supplementation with GSH, NAC, and glutathione reductase inhibited toxin-induced apoptosis of PC12 cells (derived from rat adrenal gland pheochromocytoma). This difference in observations could be cell type-specific, resulting from the differential responses of the alveolar macrophages versus the PC12 cells. Furthermore, the transient elevation of GSH level before and/or during the onset of apoptosis may actually promote apoptosis in macrophages. For instance, according to Boggs et al. (1998), RES cell line, which is an apoptosis-resistant cell line-derived from RAW264.7 macrophages, had lower levels of GSH and higher oxidative state than RAW264.7 cells, and depletion of GSH rescued RAW264.7 macrophages from spermine NONOate (a nitric oxide donor)-induced apoptosis.

**Stress-inducible signaling events**

It has recently been proposed that ribosome-binding protein synthesis inhibitors, including some trichothecenes, cause “ribotoxic stress”, leading to activation of stress-related MAP Kinases and induction of apoptosis (Shifrin and Anderson, 1999). We, therefore, examined the activation of JNK/p38 in the SC toxin mixture-treated macrophages using the specific antiphosphorylated JNK/p38 antibodies. Both JNK and p38 were found to show phosphorylation in 0.5 through 3 h treatment time range and the intensity increased with time.
Activation of JNK/p38 has been shown to be involved in either proliferation or apoptosis depending on the duration of activation, which may vary from minutes to hours. The prolonged activation, as in this study, has been found to be mostly associated with or believed to be responsible for apoptosis (Lin and Dibling, 2002). The role of JNK/p38 activation in “ribotoxic stress”-mediated apoptosis has been investigated for different protein synthesis inhibitors, albeit with differing conclusions. For instance, Watanabe et al. (2002) reported that chemical inhibition of JNK by its specific inhibitor had little impact on caspase activation and cell death induced by anisomycin. Smith et al. (2003) showed that blocking of p38 with specific inhibitor SB202190 successfully prevented cell death and caspase3 activation by Shiga toxin I. Yang et al. (2000), however, showed that blocking of p38 with the p38-specific inhibitor SB203580 had no effect on apoptosis induction by the highly toxic trichothecene, satratoxin G (a component of SC toxin mixture), but moderately inhibited apoptosis induction by the less toxic trichothecene vomitoxin. Our data showed that blocking JNK at either time point (1 h before or 3 h after the toxin addition) failed to affect apoptosis. However, inhibition of p38 at 3 h after SC toxin addition, but not 1 h before toxin addition, partially rescued cells from apoptosis induced by SC toxins. This seems consistent with earlier studies on other cell–stimuli combinations, wherein it has been demonstrated that the MAP kinase p38 plays an antiapoptotic role at early time point, but plays a pro-apoptotic role in prolonged activation (Mackay and Mochly-Rosen, 2000). It appears that our pretreatment with p38 inhibitor (1 h before toxin addition) blocked both the early antiapoptosis function as well as the subsequent pro-apoptotic function, with the combination resulting in the little effect observed. When treated with p38 inhibitor after the early onset of apoptosis (3 h posttreatment), only the
pro-apoptotic function was blocked, resulting in a net protection against apoptosis. In DNA damage-triggered apoptosis by different genotoxic stimuli such as UV, p53 has been shown to be an important mediator in apoptosis. Considering that SC toxins caused DNA damage, we examined the accumulation and role of p53 in SC toxin–macrophage interaction (apoptosis). Chemical inhibition of p53 with pifithrin-α at 1 h before (but not 3 h after) addition of SC toxins moderately reduced apoptosis. These results suggested that p53 functioned at early time point as an upstream signaling event promoting apoptosis, which is consistent with its demonstrated role in apoptosis by other genotoxic stresses (Komarov et al., 1999). Inhibition of either p38 or p53 with their specific inhibitors at the used concentrations caused only partial reversal of apoptosis and failed to completely abolish AM apoptosis induction by the SC toxins. The possible reason for the observed phenomenon could be that the use of lower concentrations of the chemical inhibitors due to toxicity reasons may have resulted in incomplete blocking of their target signal (and its function); a higher concentration of these chemical inhibitors led to independent cytotoxicity even without SC toxins (data not shown). Activated p53 and p38, which are known to mediate apoptosis via the intrinsic (mitochondrial) pathway for other stimuli (Asakura and Ohkawa, 2004), may likely have a similar role in SC toxin-induced apoptosis. Nevertheless, the observed accumulation of p53 indicates that the alternate extrinsic (deathreceptor mediated) pathway of apoptosis (Chi et al., 2004) may also be involved. This is consistent with the fact that both capase 8 (specific initiator caspase for death-receptor-mediated apoptosis pathway) and capase 9 (specific initiator for mitochondrial apoptosis pathway) were activated in satratoxin-treated HL-60 cells (Nagase et al., 2002). Taken together, the above signaling changes indicate that there are multiple
signaling pathways involved in SC toxin-induced apoptosis of alveolar macrophages. A proposed scheme of signaling events associated with SC toxin-induced apoptosis is presented in Fig. 9. In conclusion, the toxins associated with spores of the indoor mold S. chartarum are responsible for apoptosis and cytotoxicity in alveolar macrophages in a dose- and time-dependent manner. The SC toxins, though unable to induce proinflammatory markers, cause early DNA damage, GSH perturbation, and associated activation of stress-inducible signals such as p53 and MAP kinase (p38), that contribute in part to the apoptotic process in the SC toxin-treated alveolar macrophages. Co-treatment with non-apoptotic concentration of LPS exacerbated the cytotoxicity effect of the SC toxins.

**Acknowledgments**

The study was supported in part by the University of Cincinnati Research Investigator Award (JSY) and the University Graduate Scholarship Award (HW). We thank Marian Miller, PhD for her help in the microscopic work and Howard Shertzer, PhD for help in GSH analysis.
Figure legends

Fig. 1. Cytotoxicity of *S. chartarum* toxin mixture (“SC toxins”) toward murine alveolar macrophages (MH-S cell line) in time-course treatments. Freshly grown MH-S cells (1×10⁶) were treated with SC toxins at varying concentrations (in terms of spores equivalent/10⁶ cells) as described in Materials and methods. (A) Induction of lactate dehydrogenase (LDH) release. (B) Inhibition of cell proliferation. (C) Loss of cell viability. *Statistically significant based on Student's t test (P < 0.05).

Fig. 2. Induction of apoptosis in murine alveolar macrophages by SC toxins. Freshly grown MH-S cells (1×10⁶) were treated with SC toxin mixture (10⁶ spores equivalent) at 37°C for various periods of time, as described in Materials and methods. Top panel: Morphological examination of the apoptotic changes in SC toxin-treated cells based on phase contrast (A and B) and DAPI-based fluorescence (C through F) microscopy. (A) Vehicle control cells. (B) SC toxin-treated cells (cell shrinkage and reduced cell number). (C) Vehicle control cells. (D) SC toxin-treated cells (apoptotic bodies). (E) SC toxin-treated cells (chromatin condensation). (F) SC toxin-treated cells (micronuclei formation). Bottom panel: Biochemical analysis of the SC toxin-induced apoptosis in alveolar macrophages. (A) DNA fragmentation. (B) Caspase3/7 activation. Abbreviations: M (DNA marker); RFLU (Relative Fluorescence Unit). *Statistically significant based on Student's t test (P< 0.05).

Fig. 3. Exacerbation of cytotoxicity caused by SC toxins in cultured alveolar macrophages pretreated (1 h) and co-incubated (24 h) with lipopolysaccharide. MH-S cells were treated with the following: vehicle (10% methanol), LPS (10 ng/ml), SC toxins (10⁶ spores equivalent/10⁶ cells), and LPS (10 ng/ml)+SC toxins (10⁶ spores equivalent/10⁶ cells). The cytotoxicity was measured in terms of LDH release at the indicated periods of time.
*Statistically significant based on Student's t test (P < 0.05).

**Fig. 4. Glutathione (GSH/GSSG) changes in SC toxin-treated alveolar macrophages.**
Freshly grown MH-S cells (1×10^6) were treated with SC toxins at an apoptotic concentration (10^6 spores equivalent/10^6 cells) and compared with the vehicle-treated control, as described in Materials and methods. (A) GSH level changes. (B) GSSG level changes. *Statistically significant based on Student's t test (P < 0.05).

**Fig. 5. DNA damage in alveolar macrophages treated with SC toxins.** Freshly grown MH-S cells (1×10^6) were treated with SC toxins (10^6 spores equivalent/10^6 cells) or vehicle (10% methanol) as described in Materials and methods. Top panel: (A) Vehicle control. (B) Positive control (H_2O_2 treatment). (C) SC toxins for 15 min. (D) SC toxins for 30 min. (E) SC toxins for 60 min. (F) Score of DNA comet formation deduced based on the standard score chart. Bottom panel: Standard score chart for the DNA comet assay based on the relative tail-length. *Statistically significant based on Student's t test (P < 0.05).

**Fig. 6. Micronuclei formation in alveolar macrophages treated with SC toxins at lower (non-apoptotic) concentration.** Freshly grown MH-S cells were treated with SC toxins (10^5 spores equivalent/10^6 cells) and the vehicle (10% methanol) for 72 h, as described in Materials and methods. Fluorescence-based microscopic examination of micronuclei formation: (A) Vehicle control. (B) SC toxins. (C) Quantification of micronuclei per 1000 cells in vehicle control and treated cells. *Statistically significant based on Student's t test (P < 0.05).

**Fig. 7. Signaling changes in alveolar macrophages (MH-S) treated with SC toxins.** An
apoptotic dose (10^6 spores equivalent/10^6 cells) of the SC toxin mixture was used for the treatment. Top panel: SC toxin-induced accumulation of p53 and activation of JNK and p38. Equal volume of total protein (30 μg) was loaded in each lane. Bottom panel: (A) Treatment with p38 inhibitor SB202190 (20 μM). (B) Treatment with p53 inhibitor Pifithrin-α (10 μM). The increased cell viability values in SB202190 posttreatment (3 h after toxin addition) and Pifithrin-α pretreatment (1 h before toxin addition) were statistically significant (P < 0.05). Abbreviations: SB (SB202190); PFTH (Pifithrin-α). *Statistically significant based on Student's t test (P < 0.05).

**Fig. 8. Chemical inhibition of p38 and p53 reduced in part the extent of DNA fragmentation in alveolar macrophages treated with SC toxins.** An apoptotic dose (10^6 spores equivalent/10^6 cells) of the SC toxin mixture was used in a 24-h toxin treatment. (A) Posttreatment with p38 inhibitor (20 μM SB202190) 3 h after toxin addition. (B) Pretreatment with p53 inhibitor (10 μM pifithrin-α) 1 h before toxin addition.

**Fig. 9. Proposed signaling events during apoptosis of alveolar macrophages induced by S. chartarum toxins.** Solid line: event evidenced in this study; dotted line: hypothesized event.
**Tables and figures**

**Table 1. Primers used for real-time quantitative RT-PCR in this study**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>Forward</td>
<td>5'-CCT GTA GCC CAC GTC GTA GC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-TTG ACC TCA GCG CTG AGT TG-3'</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Forward</td>
<td>5'-AGT GTG GAT CCC AAGCAA TAC C-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-ATG GTT TCT TGT GAC CCT GAG C-3'</td>
</tr>
<tr>
<td>IL-6</td>
<td>Forward</td>
<td>5'-CGG AGA GGA GAC TTC ACA GAG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CTC TGA AGG ACT CTG GCT TTG-3'</td>
</tr>
<tr>
<td>iNOS</td>
<td>Forward</td>
<td>5'-CAG CCT TGC ATC CTC ATT GGG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GCA CGC TGA CCT CAT TGG C-3'</td>
</tr>
<tr>
<td>RPS18</td>
<td>Forward</td>
<td>5'-GTT GTA GAG CTT GGG TAC TG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GTT GGT CAG CTG GCA ATC ACC TC-3'</td>
</tr>
</tbody>
</table>
Table 2. Transcriptional changes in selected pro-inflammatory mediators in SC toxin-treated\(^a\) alveolar macrophages

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Fold change in transcripts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SC toxins</td>
</tr>
<tr>
<td><em>TNF-α</em></td>
<td>0.99 ± 0.35</td>
</tr>
<tr>
<td><em>IL-1β</em></td>
<td>1.58 ± 0.39</td>
</tr>
<tr>
<td><em>IL-6</em></td>
<td>1.15 ± 0.38</td>
</tr>
<tr>
<td><em>iNOS</em></td>
<td>0.90 ± 0.27</td>
</tr>
</tbody>
</table>

\(^a\) MH-S cells were treated with SC toxin mixture at 10\(^6\) spores equivalent/10\(^6\) cells in experimental or with lipopolysaccharide (LPS) at 100 ng/ml in positive control wells. The values represent means of triplicates with standard deviation.
Fig. 1.

A

LDH release %

0 1 3 6 9 12 24

Treatment Time (h)

B

Proliferation %

0 1 3 6 9 12 24

Treatment Time (h)

C

Viable cells %

0 1 3 6 9 12 24

Treatment Time (h)

- Vehicle Control (10% Methanol)
- SC toxins (10^5 spores equivalent/10^6 cells)
- SC toxins (10^6 spores equivalent/10^6 cells)
- SC toxins (10^7 spores equivalent/10^6 cells)
Fig. 2.
Fig. 4.
Fig. 6.
Fig. 7.

![Graph A: Treatment with Veh, 5B, Sc Iboins, Sc Iboins + SB (3h), Sc Idoins + SB (3h) showing luminescence levels.](image)

![Graph B: Treatment with PTH, Sc Idoins, PTH + Sc Idoins, Sc Idoins + PTH + Sc Idoins showing luminescence levels.](image)
Fig. 8.
Fig. 9.

![Diagram showing the relationship between SC toxins, DNA damage, Ribotoxic stress, p53, p38, and Apoptosis.](image-url)
References


*Stachybotrys chartarum* spores and spore-impacted mouse lung using immunocytochemistry. Toxicol. Pathol. 32, 26–34.


Chapter III

Global gene expression changes underlying *Stachybotrys chartarum* toxin-induced apoptosis in murine alveolar macrophages: Evidence of multiple signal transduction pathways*

*This chapter has been published in: Apoptosis (2007) 12:535–548.*
Abstract

The overall mechanism(s) underlying macrophage apoptosis caused by the toxins of the indoor mold *Stachybotrys chartarum* (SC) are not yet understood. In this direction, we report a microarray-based global gene expression profiling on the murine alveolar macrophage cell line (MH-S) treated with SC toxins for short (2 h) and long (24 h) periods, coinciding with the pre-apoptotic (<3 h) and progressed apoptotic stages of the treated cells, respectively. Microarray results on differential expression were validated by real-time RT-PCR analysis using representative gene targets. The toxin-regulated genes corresponded to multiple cellular processes, including cell growth, proliferation and death, inflammatory/immune response, genotoxic stress and oxidative stress, and to the underlying multiple signal transduction pathways involving MAPK-, NF-kB-, TNF-, and p53-mediated signaling. Transcription factor NF-kB showed dynamic temporal changes, characterized by an initial activation and a subsequent inhibition. Up-regulation of a battery of DNA damage-responsive and DNA repair genes in the early stage of the treatment suggested a possible role of genotoxic stress in the initiation of apoptosis. Simultaneous expression changes in both pro-survival genes and pro-apoptotic genes indicated the role of a critical balance between the two processes in SC toxin-induced apoptosis. Taken together, the results imply that multiple signaling pathways underlie the SC toxin-induced apoptosis in alveolar macrophages.

**Keywords**  Microarray. *Stachybotrys*. Trichothecene. Apoptosis. Genotoxic stress. Alveolar Macrophages
Introduction

_Stachybotrys chartarum_ (SC) is a toxigenic mold reported to occur in indoor environments in about 6% of the American homes and in more than 50% of the flooded buildings [1, 2]. This toxic mold has been associated with idiopathic pulmonary hemorrhage and sick building syndrome [3, 4]. It produces a mixture of mycotoxins, with macrocyclic trichothecenes being the most toxic components of the toxin mixture. Inhalation of toxin-containing SC spores or free toxin-contaminated dust particulates is the major route of human exposure in indoor environments [5]. The inhaled toxic particulates containing trichothecene mycotoxins are subjected to phagocytosis and clearance by the host alveolar macrophages (AMs), which act as a critical first line of innate defense in the host lung against inhaled particulates [6]. Our studies have shown that the toxins associated with SC spores cause cell death and cytotoxicity in the exposed alveolar macrophages [7]. Hence, it is important to understand the mechanisms underlying the toxicity of SC toxin toward alveolar macrophages. Such investigation could help understand the molecular basis of macrophage activation/fate and ensuing tissue injury by the SC toxins in the host lung.

Limited recent _in vitro_ and _in vivo_ studies have attempted to understand the toxicity mechanism(s) of SC toxins. Trichothecenes, in general, have been shown to inhibit _de novo_ protein synthesis in cultured macrophages (RAW264.7), and cause ribotoxic stress by binding to 28S rRNA, accompanied by activation of MAP Kinases [8, 9]. Other studies have shown an increase of inflammatory mediators (TNFα and IL-1) in bronchoalveolar lavage (BAL) fluid from the SC spores-treated mouse lung [10]. Macrocyclic trichothecene satratoxin showed activation of caspases 3, 8, and 9 in the treated cultured HL-60 cells [11]. Our recent studies
revealed the involvement of DNA damage and oxidative stress in SC toxins-treated murine alveolar macrophages, and demonstrated the role of apoptotic signals, mediated in part via p53 and stress-inducible MAP kinase p38 [7]. However, the specific mechanisms and pathways underlying the apoptosis and toxicity of SC toxins in lung cells are not yet completely understood.

With an aim to obtain a comprehensive understanding of the underlying mechanisms and signaling events of SC toxin-induced macrophage apoptosis and cytotoxicity, we established the pre-apoptotic and advanced-apoptotic stages in the toxin-treated macrophages based on flow cytometry and DNA ladder formation, and investigated a temporal global gene expression profiling using oligonucleotides-based microarray analysis. The SC toxin-treated alveolar macrophages from a pre-apoptotic stage (2 h-treatment), and from the progressed apoptotic stage (24 h-treatment) when extensive cytotoxicity and apoptosis are apparent, were used for the analysis in order to understand the early and late events in the macrophage-toxin interaction, and apoptosis.

**Materials and methods**

**Fungal culturing and toxin extraction**

*Stachybotrys chartarum* strain JS58-17 (ATCC 201211), originally isolated from a water-damaged home associated with infant pulmonary hemorrhage [12], was used. The fungus was grown on agar plates containing modified malt extract agar (1% malt extract, 1% dextrose, 0.1% yeast extract, 0.1% peptone, and 2% agar) overlaid with Whatman filter paper No. 1, by incubation at 28°C for 7 days in a humidified incubator. The spores were harvested
by collecting the black fungal mass from the filter surface using sterile swabs followed by low vacuum-filtration using glass wool filter to remove mycelia. Considering that methanol extraction of SC spores dramatically reduces their lung toxicity [13], this could be the preferred solvent to isolate all SC toxins (the components responsible for toxicity) from the harvested SC spores. Preparation of the whole toxin mixture involved extraction (3x) of SC spore suspension by methanol, drying under nitrogen gas followed by resuspension of the nitrogen-dried toxin extract in 10% methanol using our optimized protocol described previously [7]. The prepared SC toxin mixture (referred to as “SC toxins” in this study) was clarified and sterilized by filtration using 0.2 μm syringe filters (Nalge Company, Rochester, NY).

Macrophage culturing

Murine alveolar macrophage cell line MH-S (ATCC CRL-2019), originally derived from alveolar macrophages of a 7 week-old male BALB/c mouse, was used. The cells were cultured in 75 cm² tissue culture Flask (Cat. No. 430641, Corning Costar Corporation, Cambridge, MA) using RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), and other supplements (Invitrogen, Carlsbad, CA), namely L-glutamine (0.29 mg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml), and incubation in a 5% CO₂ incubator at 37°C.

Flow cytometric analysis

Using a 12-well cell culture plate, MH-S cells (5 × 10⁵ cells/ml; 2 ml/well) were treated with SC toxins for 0, 2, 3, and 24 h at the apoptotic concentration of 1 spore/macrophage (using
1×10^6 spores equivalent toxin/1 × 10^6 macrophage cells in each well) as determined in our previous studies [7]. The treated cells were collected by gentle scraping with a cell scraper (Corning Incorporated, Corning, NY), spun down by centrifugation (800×g for 3 min), washed with phosphate-buffered saline (PBS), followed by fixing of the pelleted cells in 75% cold ethanol. After fixation, the cells were washed twice with PBS, using centrifugation at 800×g for 3 min, followed by RNase A treatment (50 μg/ml; 37°C for 15 min) and propidium iodide (PI) staining (3 μM; 37°C for 15 min). Cellular DNA content was examined for apoptosis by flow cytometric analysis on the FACSCalibur™ system (BD Bioscience. San Jose, CA) using CellQuest ProTM (BD Bioscience. San Jose, CA) for data acquisition and data analysis.

**DNA ladder formation assay**

After treatment with SC toxins as above, the MH-S cells were resuspended in lysis buffer (10 mM Tris-Cl pH 7.5, 25 mM EDTA pH 8.0, 0.5% NP-40) and vortexed for a few seconds, before incubating on ice for 15 min. Supernatant obtained after centrifugation at 1,000 × g for 5 min, were transferred to new tubes and subsequently treated with RNase A(50 μg/ml; 37°C for 30 min) and proteinase K (100 μg/ml; 56°C for 60 min). The fragmented DNA was then precipitated with ice-cold ethanol (100%) and subjected to 1% agarose gel electrophoresis. A typical DNA ladder pattern on the gel indicated apoptosis of the cells.

**Total RNA preparation**

MH-S cells for gene expression analysis were cultured in 100 mm culture dishes (1.0 × 10^7 cells/20 ml medium) and treated with the SC toxin mixture for 2 h or 24 h using the predetermined apoptotic concentration (1 spore equivalent toxin/macrophage). Each treatment
was performed in triplicate. Total RNA was isolated from the pooled toxin-treated cells and the vehicle-treated (control) cells using TRI reagent kit (Molecular Research Center, Inc. Cincinnati, OH) according to the manufacturer’s instructions. The RNA preparations were further purified using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. The total RNA preparations meant for microarray work were subjected to a quality check, using Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA) analysis.

**Microarray analysis**

Microarray analysis was performed at the Genomics and Microarray Laboratory (GML) of the University of Cincinnati. In-house prepared microarray slides used in the study were spotted with 70-mer oligonucleotide probes derived from the mouse oligo library (Operon Biotechnologies, Huntsville, AL). The oligo lists are available at the GML website (http://microarray.uc.edu). Cy3/Cy5 labeling, hybridization and scanning were performed using the GML optimized protocols available at http:// microarray.uc.edu/ Resources/protocols.htm. Mouse 70-mer oligo library (V3.0) and its prior version were used for the 2 h and 24 h treatments. At either time point (2 h and 24 h), the pooled RNA samples obtained from biological triplicates for vehicle-treated versus toxin-treated cells were hybridized using three microarray slides (chips), two chips using Cy3 versus Cy5, and the third chip using a dye flip (Cy5 versus Cy3) for hybridization. Hybridization data were collected using a GenePix 4000A Scanner (Axon Instruments, Inc. Sunnyvale, CA) at 635 nm (Cy5) and 532 nm (Cy3) channels. Being dual-labeled (Cy5 and Cy3), the global gene-expression hybridizations required that the ratio for the whole scan area for the two signals be adjusted to 1.0, and,
therefore, no reference RNA was required. The results were analyzed using GeneSpring 6.0 (Agilent Technologies, Inc. Palo Alto, CA), for identifying up-regulated or down-regulated genes, and sorting the affected genes into various functional categories. An arbitrary fold-change criterion was used to understand differential expression; the genes showing more than 2-fold increase in at least 2 of the 3 slides were considered up-regulated. Similarly, genes with more than 2-fold decrease in at least 2 of the 3 slides were considered down-regulated. The fold-change values presented represent averages of the triplicates.

**Real-time RT-PCR**

Forward and reverse PCR primers for the genes of interest were designed using Gene runner 3.05 (Hastings software Inc, Hudson, NY) and custom synthesized by Integrated DNA Technologies (Coralville, IA). Different primer sets used in this study are listed in Table 1. Reverse transcription (RT) reaction and the real-time PCR reaction were performed using GeneAmp Thermostable rTth Reverse Transcriptase RNA PCR kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. The RT reaction was performed at 60°C for 10 min using GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The PCR reaction was performed on the Smart Cycler system (Cepheid, Sunnyvale, CA) using the following conditions: denaturing at 94°C for 120 sec, followed by 35 cycles of amplification, each involving denaturing at 94°C for 30 sec, annealing at 60°C for 60 sec and extension at 68°C for 120 sec, and a final extension at 68°C for 7min. Ribosomal protein S18 (RPS18) was used as the internal control. The gene expression fold changes were calculated as described previously [7]. Fold change = $2^{\Delta\Delta Ct}$, where $Ct$ is the threshold cycle, $\Delta Ct$ is the difference between the $Ct$ values of the target gene and the internal control gene (RPS18), $\Delta\Delta$
Ct represents the difference between the ΔCt value for the vehicle control cells and the treated cells.

**Statistical analysis**

For microarray analysis, the raw data generated by GenePix® Pro version 5.0 software were input into R statistical software using the *limma* package of Bioconductor and analyzed to identify differentially expressed genes [14]. Briefly, data normalization was performed by log-transformation of background adjusted intensities and array-specific local regression. The statistical analysis (p-value) was performed for each time point comparison and for each gene separately by fitting ANOVA model. The False Discover Rate (FDR) was calculated using the Benjamini and Hochberg method. For flow cytometry, the data were statistically analyzed using student *t*-test, and presented as average±standard deviation representing three independent experiments.

**Results**

**Induction of apoptosis in MH-S cells by SC toxins**

In order to establish pro-apoptotic and advanced-apoptotic stages in SC toxin-treated macrophages, a predetermined [7] physiologically relevant apoptotic dose of the SC toxins (1 spore equivalent toxin/macrophage) was applied. The original rationale for selecting this dose is that in the spore-exposed macrophage population, a given impacted macrophage encounters at least one spore and the toxins associated with that spore. Temporal flow cytometric analysis on SC toxin-treated MH-S cells for apoptosis induction revealed that there were no detectable apoptotic cells in 0 h and 2 h treatments. At 3 h, there were moderate number (~7%) of
apoptotic cells, and the number increased significantly at 24 h (~60%) (Fig. 1(A)) as evidenced by the percentage of sub-G\(_1\) cells (cells with less DNA content than \(G_0/G_1\) cells).

Similarly, in the DNA ladder formation assay for detection of apoptosis, there were no detectable fragmented DNA bands at the 0 h and 2 h treatments, while at the 3 h time-point, there was visible apoptotic fragmented DNA, which increased dramatically at 24 h (Fig. 1(B)). Taken together, these results allowed us to select pre-apoptotic (2 h) and progressed-apoptotic (24 h) stages of the toxin treatment, for use in subsequent gene expression profiling.

**Microarray-based gene expression changes in SC toxin-treated MH-S cells**

According to the conventional 2-fold change filtering criterion, a total of 304 genes were up-regulated and 13 genes down-regulated in the 2 h treatment, whereas a much larger number of genes were altered (1002 genes up-regulated and 1206 genes down-regulated) in the 24 h treatment. In terms of the percentage of genes impacted, the 2 h and 24 h-treatments caused 0.998% and 16.430% changes, respectively. Statistical significance of the differential expressed genes was based on \(p\)-value \((p < 0.05)\) and false discovery rate (FDR\(< 0.05)\) for multiple hypothesis testing adjustment. It turned out that most of those genes that passed the 2-fold change criterion are also statistically significant, although some genes which did not pass the 2-fold change criterion also showed statistical significance.

Functional classification of the genes showing expression changes (up-regulation or down-regulation) allowed us to identify several individual categories of genes including those related to cell apoptosis, as described below.

**Up-regulation of stress-related genes**
Genes involved in two types of cellular stress namely, genotoxic stress and oxidative stress, were up-regulated in the SC toxin-treated macrophages.

Genotoxic stress-related genes

At 2 h, 9 genes related to DNA damage and repair response were significantly ($p<0.05$) up-regulated (Fig. 2), including $H2AFX$ (NM 010436), $RAD52$ (NM 011236), $GTF2H4$ (NM 010364), $GTF2H1$ (NM 008186), $POLI$ (NM 011972), $REV1L$ (NM 019570), $RAD9$ (NM 011237), $TLK2$ (NM 011903), and $RAD18$ (NM 021385). At 24 h, the above genes (except for $H2AFX$ and $GTF2H4$ that were down-regulated), showed even greater up-regulation, together with 10 additional DNA damage and repair response genes, namely $MHS3$ (NM 010829), $MLH1$ (NM 026810), $XPA$ (NM 011728), $REV3L$ (NM 011264), $ERCC2$ (NM 007949), $PARP2$ (NM 009632), $RAD50$ (NM 009012), $HUSI$ (NM 008316), $PRKDC$ (NM 011159), and $MSH2$ (NM 088628).

Oxidative stress-related genes

Two genes related to oxidative stress, namely $PTGS2$ (NM 011198) and $TXNIP$ (NM 023719), were up-regulated in the SC toxin-treated cells (Fig. 2) at both 2 h and 24 h.

Regulation of genes involved in stress-inducible signaling

Genes involved in MAPK- and p53-signaling and some stress-related transcription factors showed expression changes in SC toxin-treated alveolar macrophages.

MAPK-related genes

Both MAP phosphatases and MAP kinases showed transcriptional changes after SC toxin-treatment. As for MAP phosphatases, the 2 h treatment led to an up-regulation of
DUSP16 (NM 130447) and DUSP10 (NM 022029), the inactivators for JNK and p38 (Fig. 3(A)), and the 24 h treatment caused an up-regulation of another inactivator for ERK and p38, the dual-specificity MAP phosphatase DHCR24 (NM 053272), in addition to DUSP16. As for MAP kinases (activators), the 24 h SC toxin treatment led to an up-regulation of the p38 activators, MAP2K3 (NM 008928) and MAP3K7IP1 (NM 025609), and an up-regulation of the JNK activators, MAPK8IP3 (NM 013931), MAPKBP1 (NM 011941) and MAP4K5 (NM 024275), and a down-regulation of the ERK activator MEK1 (NM 008927).

**p53-pathway genes**

Four genes that are known to contribute to p53 functions in cell cycle arrest and apoptosis were up-regulated (Fig. 3(B)). Except for JMY (NM 021312), which showed upregulation at only 24 h, NFKBIA (NM 010907), GADD45g (NM 011817), and MTBP (NM 134092) were significantly ($p < 0.05$) up-regulated at both 2 h and 24 h.

**Stress-inducible transcription factors**

Several stress-inducible transcription factors were up-regulated in SC toxin-treated cells (Fig. 3(C)). Components of transcription factor AP-1, including c-JUN (NM 010591), JUNB (NM 008416), JUND (NM 010592), and FOS (NM 010234), were up-regulated. Stage-specific up-regulation was observed for the following factors: FOS (2 h only), JUNB (24 h only), c-JUN and JUND (both time points). Other stress-inducible transcription factors that were also up-regulated at both 2 h and 24 h included small MAF genes [MAFF (NM 010755), MAFK (NM 010757), and MAFG (NM 010756)], cyclin genes [CCNL1 (NM 019937), CCNL2 (NM 018856), and CCNT1 (NM 009833)], and ATF/cAMP response element binding protein (ATF/CREB) family genes [ATF3 (NM 007498) and ATF4 (NM 009716)].
Regulation of apoptosis-related genes

Genes involved in death receptor-mediated (extrinsic) apoptosis pathway and mitochondria-mediated (intrinsic) apoptosis pathway were transcriptionally changed, in the toxin-treated macrophages.

Death-receptor pathway-related genes

At 2 h, two death-receptor pathway components, TNF-α (NM 013693) and CASP8AP2 (NM 011997) were statistically significantly up-regulated (Fig. 4(A)). The prolonged (24 h) treatment led to an up-regulation of pro-apoptotic TNF superfamily members, namely TNFRSF5 (CD40) (NM 011611), TNFRSF7 (CD27) (L24495), and FAF1 (NM 007983), down-regulation of pro-survival TNF superfamily members, namely FAIM (NM 011810), TNFRSF9 (NM 011612) and TNFSF13 (NM 033622), and upregulation of one of the pro-survival TNF members, TNFRSF11B (OPG) (NM 008764).

BCL-2 family-related genes (involved in mitochondria apoptosis pathway)

In the 2 h toxin treatment, BH3-only pro-apoptotic proteins BIM (NM 009754), NOXA (NM 021451), and PUMA (NM 133234) were up-regulated (Fig. 4(B)). In the 24 h toxin treatment, in addition to NOXA that continued to be upregulated, the expression levels of BAX (NM 007527) and BAX activator gene MOAP1 (NM 022323) also increased, whereas those of the pro-apoptotic gene BAK (NM 007523) and anti-apoptotic gene TEGT (NM 026669) were decreased (Fig. 4(B)). In addition, the 2 h treatment led to statistically significant (though not passing the 2-fold filtering criterion) down-regulation of TEGT and up-regulation of MOAP.

Caspase-related genes

CASP2 (NM 007610) showed significant ($p < 0.05$) upregulation at the 2 h treatment (Fig.
4(C)). At 24 h, different caspase-related genes were regulated either way: pro-apoptotic genes
_CASP2_ and _CASP4_ (NM 007609) showed up-regulation, whereas the other pro-apoptotic
caspase genes [_CASP1_ (NM 009807), and _CASP6_ (NM 009811)] showed down-regulation.
Likewise, the pro-apoptotic _BCAP31_ (caspase 8 substrate) and _HIP1_ (complex partner for
Caspase 8) were down-regulated, whereas the anti-apoptotic gene _CEBPB_ (NM 009883) that
acts as caspase-antagonist showed up-regulation, (Fig. 4(C)).

**Regulation of genes involved in inflammatory/immune response**

NF-κB target genes and Toll-like receptor genes were found either up-regulated or
down-regulated in both 2 h and 24 h treatments.

**NF-κB signaling pathway genes**

The 2 h-treatment led to an up-regulation of several NF-κB target genes (Fig. 5(A)), which
also act as positive feedback regulators for NF-κB, namely _TNF-α_ (NM 013693), _PTGS2_ (NM
011198), _CXCL2_ (NM 009140), _CCL3_ (NM 011337), and _BIRC3_ (NM 007464). In contrast, at
24 h, some of these genes, namely _TNF-α_, _CXCL2_, and _CCL3_, were less upregulated,
non-regulated, or dramatically down-regulated, respectively. Other positive feedback
regulators that showed no change at 2 h, showed either up-regulation, as in case of _C3_ (NM
009778) and _CARD14_ (NM 130886) or down-regulation, as in case of _C4_ (NM 009780),
_CCL4_ (NM 013652), _IL2_ (NM 008366), and _IL2RA_ (NM 008367). NF-κB complex
components _RELA_ (NM 009045) and _RELB_ (NM 009046) were also up-regulated and the
fold-changes were more dramatic at 24 h. Other NF-κB target genes, which act as negative
feedback to inhibit the activation of NF-κB signaling, namely _TRAF1_ (NM 009421), _TNFAIP3_
(NM 009397), and _CFLAR_ (NM 009805), showed up-regulation at 2 h treatment. At 24 h,
CFLAR level was sustained whereas TNFAIP3 was further up-regulated. Additionally, another negative feedback regulator, TRAF3 (NM 011632) was up-regulated.

Toll-like receptors

TLR2 (NM 011905) was significantly ($p < 0.05$) upregulated both at 2 h and 24 h time points (Fig. 5(B)). At 24 h, TLR3 (NM 126166) showed up-regulation (though statistically non-significant), whereas multiple Toll-like receptors, including TLR4 (NM 021297), TLR6 (NM 011604) and TOLLIP (NM 023764), were down-regulated (Fig. 5(B)).

Regulation of cell growth- and cell proliferation-related genes

Some oncogenes and other genes involved in cell growth and cell cycle progression were transcriptionally changed during the SC toxin treatment.

Oncogenes

Pro-apoptotic oncogenes L-MYC (NM 008506) and c-MYC (NM 010849) showed up-regulation at both 2 h and 24 h (Fig. 6(A)). At 24 h, 8 more oncogenes were regulated, of which, 3 genes were up-regulated and 5 down-regulated. Among the pro-apoptotic oncogenes, FES (NM 010194) was down-regulated, whereas PIM1 (NM 008842) and RRAS (NM 009101) were up-regulated. Among the pro-survival oncogenes, DEK (NM 025900) was up-regulated, whereas LYN (NM 010747), ETS1 (NM 011808), MERTK (NM 008587), and NRAS (NM 010937) were downregulated.

Other genes involved in cell growth and cell cycle

At 2 h, only PLK2 (NM 152804) showed up-regulation (Fig. 6(B)). At 24 h, among the positive cell growth regulators, PLK2, CDK6 (NM 009873), CCND1 (NM 007631), CDC2A (NM 007659), PDGFC (NM 019971), and CATNB (NM 007614) were down-regulated,
whereas *MCM6* (NM 008567), *CDC2L1* (NM 007661), and *RHOU* (NM 133955) were up-regulated. Among the negative cell growth regulators, *SMAD3* (NM 016769) and *IGFBP3* (NM 008343) were up-regulated, whereas *TGFB3* (NM 009368) was down-regulated.

**RT-PCR confirmation of the microarray-based expression patterns for selected genes involved in cell apoptosis**

At least one representative gene for each of the individual processes and pathways underlying the apoptosis process that showed up-regulation of expression in the 2 h- and/or 24 h-treatment based on microarray was confirmed using quantitative real-time RT-PCR. The RT-PCR confirmation of a total of 15 selected representative genes was performed for both the 2 h (Fig. 7(A)) and 24 h (Fig. 7B) time-points. The RT-PCR analysis did confirm the microarray changes in transcriptional patterns of these genes, albeit with variable absolute fold-change values for some genes, as normally expected in such comparisons.

**Discussion**

**Understanding overall transcription changes in SC toxin-treated MH-S cells**

It is known that trichothecenes in general act as protein synthesis inhibitors [8, 9]. Inhibition of *de novo* protein synthesis was reported to happen as early as 20 min of treatment with SC trichothecenes and inhibition up to 80% using high doses of these toxins [9]. Conventionally, it has been assumed that cell death induced by these protein synthesis inhibitors is due to a decreased synthesis and eventual lack of macromolecules such as proteins, RNA, and DNA, which are essential for cell survival. If this is the sole reason, such inhibition will be expected
to involve down-regulation of most if not all genes. However, in this study, instead of seeing an overall down-regulation of genes either at 2 h or at 24 h treatment, the microarray data (as confirmed by RT-PCR) showed both down-regulation and up-regulation of genes in SC toxin-treated MH-S cells at both the time points. The total number of changed genes was much higher at 24 h than that at 2 h treatment. The quantitative difference in terms of number of genes showing altered expression in the two time points suggests a progressive increase in transcriptional changes with the time of treatment. Nevertheless, a secondary response to the early changes in gene expression could, in part, have led to the proportionately larger numbers of regulated genes in the prolonged treatment.

**Identifying signaling events in SC toxin-induced apoptosis of MH-S cells**

The global and temporal transcriptional changes detected by microarray analysis, coupled with RT-PCR confirmation on selected representative genes, provided useful information for identifying the possible signaling events involved in SC toxin-induced apoptosis in MH-S cells. Transcriptional changes in functionally related genes indicated involvement of certain cellular processes and/or signaling pathways, and the timing of the transcriptional changes in a given gene suggested its involvement in initiation and/or progression of apoptosis.

**DNA damage-mediated apoptosis pathway**

Up-regulation of DNA damage- and DNA repair-related genes indicated that SC toxins cause DNA damage response in the exposed macrophages. There were less number of total up-regulated genes in 2 h than in 24 h, and for the statistically significant up-regulated genes (both at 2 h and 24 h), the expression levels at 2 h were less than those at 24 h, indicating a
milder genotoxic-stress response at 2 h, and a more extensive DNA damage and repair activity in the later stages (24 h). These microarray data are consistent with the DNA damage data (based on comet assay and micronuclei) in our earlier study [7] that demonstrated a milder DNA damage (evidenced by DNA comet assay) before the onset of apoptosis (as early as in 15 min treatment), and a temporal progression of DNA damage as evidenced by increased comet tail length. Considering the timeline of both DNA damage (DNA comet assay) and DNA damage response (microarray analysis), it appears that DNA damage is involved, partly if not solely, in the initiation of apoptosis in SC toxin-treated MH-S cells. While the alkaline comet assay implied either single or double strand breaks, the impacted genes with known biological function in the microarray analysis could help predict the specific mode of DNA damage and repair. For instance, up-regulation of certain DNA repair genes implies that double-strand DNA break [15, 16] (repair genes such as RAD52, H2AFX, TLK2, RAD50, and PRKDC) and DNA adduct formation [17] (repair genes such as HUS1) may be the major forms of DNA damage involved in this SC toxin-alveolar macrophage interaction. Likewise, the expression changes in the relevant DNA repair genes indicated that the mechanisms of DNA repair may include, homologous recombination repair (such as RAD52), nucleotide/base excision and mismatch repair [18, 19] (such as GTF2H4, MSH3, MLH1, MSH2, XPA, ERCC2, and PARP2), and translesion repair [20, 21] (such as POLI, REV1L, and REV3L).

Oxidative-stress-mediated apoptosis pathway

Up-regulation of PTGS2 and TXNIP at the 2 h and 24 h time points of the toxin treatment, suggests that oxidative stress may be involved in both early and late phases of the SC toxicity
to alveolar macrophages. This is partly consistent with our previous report [7] that was based on measurement of reduced glutathione (GSH) level and oxidized glutathione (GSSG) levels. These biochemical measurements had implied that oxidative stress (as evidenced by decrease of GSH and increase of GSSG) occurred at the late stage of apoptosis, and not at the onset of apoptosis (3 h treatment). This ambiguity on the involvement of oxidative stress in early stages of apoptosis, or in terms of whether or not oxidative stress contributes to the initiation of SC toxin-induced apoptosis in MH-S cells, could be explained by the following two arguments. First, although up-regulation of PTGS2 can cause depletion of glutathione, it may need quite some time to really deplete it. Alternatively, there may be alternate mechanism (not revealed by microarray analysis in this study) by which cells could boost glutathione level in response to the SC toxin-induced stress, thereby counterbalancing the PTGS2-based reduction of glutathione level at the early stage of apoptosis. Second, besides changes in glutathione level, oxidative stress might still be involved in both early and late stage of SC toxin-induced apoptosis in MHS cells in the form of lipid peroxidation and inhibition of thioredoxin mediated by up-regulation of PTGS2 [22] and TXNIP [23].

MAPK-mediated apoptosis pathway

Up-regulation of MAPK-pathway related kinases and/or phosphatases in the two treatments, together with several stress-inducible transcription factors such as c-JUN and FOS, indicated the involvement of MAPK-mediated apoptosis pathway in SC toxin-induced apoptosis of MH-S cells. MAPK pathway has been shown to be involved in apoptosis induced by different forms of stress in various cell types. Studies by us and others have shown that both isolated trichothecenes (Satratoxins) and whole toxin mixture from S. chartarum induce prolonged
activation of MAPK [7, 9]. Unlike previous assumption on the role of JNK in trichothecene-mediated apoptosis [9], our studies showed the role of p38 instead as chemical inhibition of p38 (3 h-post treatment) partially rescued SC toxin-induced apoptosis in MH-S cells [7]. Activation of MAP kinases by trichothecenes has been ascribed to ribotoxic stress response as a result of binding of these toxins to the ribosomes in the treated cells [8, 9]. However, ribotoxic stress is poorly understood at the molecular level in terms of its critical components. Consequently, the transcriptional changes, if any, due to ribotoxic stress could not be ascribed. It is known that transient activation of MAPK is prosurvival whereas prolonged activation leads to apoptosis. Duration and magnitude of MAPK activation are crucial factors in deciding the cell fate, which depends on a balance between the activities of the upstream activators (MAP kinases) and the specific inactivators (MAP phosphatases). In our recent study, post-translational activation (phosphorylation) of JNK/p38 by SC toxins was detected as early as in 15 min and it continued through 3 h [7]. Since we did not detect a simultaneous up-regulation of JNK/p38 genes or their upstream kinases in 2 h treatment in this study, the prompt activation of MAPK does not seem to require de novo transcription. Nevertheless, the observed upregulation of phosphatases genes (DUSP16 and DUSP10) [24, 25] in 2 h analysis might be a partial negative feedback for the MAPK regulation, which functions to limit the magnitude and duration of MAPK activation. On the other hand, in the prolonged (24 h) exposure, several of the JNK/p38 activators [26, 27] (such as MAP2K3, MAP4K5, MAPKBP1, MAPK8IP3, and MAP3K7IP1) were transcriptionally up-regulated, and could be responsible for the sustained activation of JNK/p38. In support of the apoptosis outcome, ERK1/2 MAPK (pro-survival) seems to be negatively regulated as indicated by
down-regulation of its activator (*MEKI*) [28] and up-regulation of its inhibitor (*DHCR24*) [29]. Such opposing changes in MAP kinases (JNK/p38 versus ERK) are known to favor cell death over cell survival.

*P53-mediated apoptosis pathway*

p53 is often involved in stress-induced apoptosis, due either to genotoxic stress or to non-genotoxic stress. p53-mediated apoptosis occurs via the transcription-dependent pathway and/or transcription-independent pathway, both of which can occur in SC toxin-induced apoptosis in macrophages. This is corroborated by our previous studies [7], wherein p53 protein was shown to be activated based on Western blot analysis and inhibition of p53 by chemical inhibitors partly rescued cells from apoptosis. Based on the temporal changes, this activation appeared to be at least partly if not solely due the observed DNA damage. The microarray data in this study support this observation that p53 is involved and further indicates that the p53 activation occurs likely via regulation of the related pathway genes, *NFKBI A* [30], *MTBP* [31], *JMY* [32], and *GADD45g* [33].

*Death receptor-mediated apoptosis pathway*

Up-regulation of the death-receptor pathway members, such as *TNF-α* and *CASP8AP2* [34], at 2 h and of additional members, *TNFRSF5 (CD40)* [35], *TNFRSF7 (CD27)* [36], and *FAFI*, subsequently at 24 h could be responsible for the SC toxin-induced apoptosis in MH-S cells via death receptor-mediated apoptosis pathway. This pro-apoptotic effect is perhaps further enhanced by the observed simultaneous down-regulation of several pro-survival TNF superfamily members. This is consistent with the reported activation of Caspase 8 in
Satratoxin-treated HL-60 cells, as caspase 8 is an initiator caspase activated through each receptor pathway [11]. The observed up-regulation of caspase 2 in this study also corroborated the involvement of death-receptor pathway, as this is another initiator caspase activated through death receptor pathway and connected with mitochondria-mediated apoptosis pathway [37].

**Mitochondria-mediated apoptosis pathway**

Members of the apoptotic protein family BCL-2 are critical in controlling the transition in the permeability of mitochondria outer membrane, during mitochondria-mediated apoptosis pathway [38]. The observed early or sustained up-regulation of these BCL-2 family pro-apoptotic genes implies that the mitochondria-mediated pathway is involved in the SC toxin-induced apoptosis of alveolar macrophages. Microarray data not only imply the involvement of specific BCL-2 family members in SC toxin-induced MH-S apoptosis, but also provide clues on the order of their involvement. For instance, up-regulation of BAX indicated its involvement in SC toxin-induced apoptosis. Further, its late up-regulation as compared to the up-regulation of NOXA, PUMA and BIM, is consistent with the fact that BAX acts as a downstream signal in the mitochondria apoptosis pathway, and is activated by NOXA, PUMA, or BIM [39, 40].

**NF-κB signaling pathway**

NF-κB signaling plays an important role in inflammatory/immune response and stress response. NF-κB activation has been shown to prevent apoptosis and promote cell survival [41]. At both time points (2 h and 24 h), we observed counterbalancing factors, favoring
activation or inactivation of NF-kB, and the balancing seemed to change with time. In early phase, the activating factors (such as TNF-α, PTGS2, CXCL2 [42], CCL3, BIRC3, RELA, and RELB) balanced with the inactivating factors (such as TRAF1 [43], TNFAIP3 [41], CFLAR [41]), and in the later phase, as the treatment prolonged, the activating factors seem to be getting weaker and the inactivating factors getting stronger. The net inactivation of NF-kB might be responsible for favoring cell death over survival. This is consistent with our previous macrophage toxicity studies in which real-time RT-PCR failed to detect any up-regulation of some classic NF-kB target genes (TNF-α, iNOS, IL-1β, and IL-6) in a 24 h treatment [7]. Nevertheless, TNFα was moderately up-regulated at 2 h, as evidenced by microarray analysis and RT-PCR confirmation in this study.

Overall, the genome-wide microarray analysis, coupled with quantitative real-time RT-PCR analysis on selected upregulated apoptosis genes, revealed a complex set of transcriptional changes in gene expression during the interaction between alveolar macrophages and SC toxins. Collectively, these expression patterns implied that multiple signaling pathways are involved in this SC toxin-macrophage interaction and that the outcome (apoptosis) is a net result of the counteracting pro-apoptotic and anti-apoptotic changes in gene expression. In other words, the cell death outcome is a result of the fact that pro-apoptotic and anti-proliferation factors prevailed over the anti-apoptotic and pro-proliferation factors. Differences in the activity of these factors between the early and late phases of the toxin-treatment indicated a dynamic nature of a given signaling pathway, or a dynamic balance among its regulators. Particularly this seemed true for the predicted NF-kB and MAPK pathways. For a given signaling pathway, upstream and downstream signaling
molecules could be differentially modified, such as in the WNT signaling pathway [44] (wherein an upstream activator RHOU was up-regulated while a downstream molecule CATNB was down-regulated) and in TGF pathway [45] (wherein an upstream activator TGFB3 was down-regulated while a downstream molecule SMAD3 was up-regulated). This could be the result of a combination of effects due to individual toxin components or due to the cross-talk between multiple signaling pathways.

**Conclusion**

Based on overall changes in gene expression, it is hypothesized that the SC toxin mixture induces multiple stress responses via ribotoxic, oxidative, and genotoxic stresses and causes apoptosis in alveolar macrophages via multiple signaling pathways, including MAPK pathways (induced by ribotoxic stress, oxidative stress, and/or genotoxic stress), Death receptor-mediated pathway (induced by DNA damage response via TNF-α and via p53) and their cross-talk. Macrocyclic trichothecenes, being major component of the whole SC toxin mixture, may have some commonality in their mechanism of apoptosis induction with other simple trichothecenes, but their unique structure and multiple congeners in addition to the presence of other unknown toxic components in the whole toxin mixture could be responsible for the observed complexity of the signaling mechanisms (Fig. 8) in the SC toxin-treated alveolar macrophages. It is expected that the deduced mechanisms in this study will form the basis for future detailed studies to investigate the specific role of individual pathways using protein-based and/or gene knockout/knockdown approaches.

**Acknowledgments** The study was supported in part by the University of Cincinnati Research
Incentive Award (JSY) and the University Graduate Scholarship Award (HW). We thank Dr. Mario Medvedovic of the Department of Environmental Health for the statistical help.
Figure legends

Fig. 1. Temporal analysis of SC toxins-induced apoptosis in murine alveolar macrophage cell line MH-S. The MH-S cells were treated with SC toxins at an apoptotic dose (1 × 10^6 spores equivalent toxin/1 × 10^6 cells) for 0, 2, 3, and 24 h as described in Materials and methods. (A) Flow cytometric DNA content analysis. (B) Apoptotic DNA ladder assay. *p < 0.05 by Student t-test

Fig. 2. Up-regulation of stress-related genes as determined by microarray analysis. *p < 0.05 and FDR< 0.05

Fig. 3. Up-regulation of stress-inducible signaling as determined by microarray analysis. (A) MAPK-related genes. (B) p53 pathway-related genes. (C) Stress-inducible transcription factors. *p < 0.05 and FDR< 0.05

Fig. 4. Up-regulation of apoptosis-related genes as determined by microarray analysis. (A) Death-receptor pathway-related genes. (B) BCL-2 family-related genes. (C) Caspase-related genes. *p < 0.05 and FDR< 0.05

Fig. 5. Transcriptional changes in genes involved in inflammatory/immune response as determined by microarray analysis. (A) NF-kB-related genes. (B) Toll-like receptor-related genes. *p < 0.05 and FDR< 0.05

Fig. 6. Transcriptional changes in genes involved in cell growth and cell cycle progression as determined by microarray analysis. (A) Oncogenes. (B) Other genes
involved in cell growth and cell cycle progression. *$p < 0.05$ and FDR < 0.05

Fig. 7. RT-PCR confirmation on selected genes, representing the impacted individual cellular processes and pathways, as determined by microarray analysis. (A) Correlation plot for the 2 h-treatment data. (B) Correlation plot for the 24 h-treatment data

Fig. 8. Proposed signaling pathways involved in SC toxin-macrophage interaction, as deduced on the basis of a global gene expression analysis.
### Table 1. Primer sets used for real-time RT-PCR in this study

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer</th>
<th>Primer sequence</th>
</tr>
</thead>
</table>
| TNF-α     | Forward 5-CCTGTAGCCCACGTCGTAGC-3  
Reverse 5-TTGACCTCAGCGTCGTAGTGTG-3 |
| GADD45g   | Forward 5-GTCCGCCAACAGTCCTGAATGTG-3  
Reverse 5-TGGGCAACCCAGTCGTAGTGTG-3 |
| BAX       | Forward 5-AGGGTCTATCCAGGATCGACG-3  
Reverse 5-GTCAGTCTCCAGCCCATGATG-3 |
| JMY       | Forward 5-TCCGAGACATCGAGAACTGGG-3  
Reverse 5-CTGCTGCCCACGATGCTTTG-3 |
| RAD52     | Forward 5-GCAGCTCCTGCACTCGTG-3  
Reverse 5-AGGTCCCACATTGCAAGTCTTC-3 |
| TNFRSF5   | Forward 5-GTGGTTTTAAAGTGCCCGATGC-3  
Reverse 5-TCAAGGCTATGTCTGTTGTAC-3 |
| c-JUN     | Forward 5-TGCCAGCATGCTGCTGCTTC-3  
Reverse 5-CAGACCAGTCTCCCAGCAGA-3 |
| c-MYC     | Forward 5-CAGCAGCAGACTCTGAAAGAG-3  
Reverse 5-GACCCCTGCCCCTGAC-3 |
| TNFAIP3   | Forward 5-GCCTGAGATCAAGCCAGCATG-3  
Reverse 5-TCCGACTCTGAGCACTAG-3 |
| MAP2K3    | Forward 5-AGCTACCTGGAGCTGATGGCAAC-3  
Reverse 5-AGGTCTCTTTCAGTTGCACT-3 |
| PTGS2     | Forward 5-CAGCTAGGTTGTGCACGTAG-3  
Reverse 5-CGCCATGGTTGCACT-3 |
| TLR4      | Forward 5-AGGTGTGCACACTCTACTCTC-3  
Reverse 5-TTGCTGAGTTTCTGATCCAG-3 |
| NFKBIA    | Forward 5-GAGGATGAGCTGCCCTATGATG-3  
Reverse 5-AACAGAAACAGCGAGATAC-3 |
| DUSP16    | Forward 5-CCTCAAGTTGTGCGAGAGAG-3  
Reverse 5-CTGTTGCTGAGGAAACAGAGC-3 |
| PLK2      | Forward 5-CGAAATGCTGCTGATAGATG-3  
Reverse 5-CTGTTGCTGAGGAAACAGAGC-3 |
| RPS18     | Forward 5-CTGTTGCTGAGGAAACAGAGC-3  
Reverse 5-CTGTTGCTGAGGAAACAGAGC-3 |
Fig. 1.

A.  

B.  

<table>
<thead>
<tr>
<th></th>
<th>0h</th>
<th>2h</th>
<th>3h</th>
<th>24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0h</td>
<td>1.86±0.57</td>
<td>1.27±0.46</td>
<td>7.42±1.77</td>
<td>60.62±3.35</td>
</tr>
</tbody>
</table>

*
Fig. 2.
Fig. 3.

A.

B.

C.
Fig. 4.

A.

B.

C.
Fig. 5.

A.

B.
Fig. 6.
Fig. 7.

A.

Fold Change

\( \Delta \) Microarray

\( \bullet \) RT-PCR

B.

Fold Change

\( \Delta \) Microarray

\( \bullet \) RT-PCR
Fig. 8.

[Diagram showing the interaction between SC toxins, DNA, ribosomes, and various growth factors and proteins involved in early and late phases of cell response.]
References


43. Carpentier I, Beyaert R (1999) TRAF1 is a TNF inducible egulator of NF-kappa B

Chapter IV

Ribosomal and DNA damage response pathway targets governing toxicity of the mold macrocyclic trichothecenes in eukaryotic cells*

*This chapter is a manuscript in preparation.
Abstract

Macrocyclic trichothecenes (MTs) produced by the indoor mold *Stachybotrys chartarum* (SC) cause cytotoxicity and apoptotic cell death in alveolar macrophages. While role of ribotoxic and genotoxic stress response pathways in macrophage apoptosis has been shown, little is known on the specific molecular targets mediating signals in these pathways. In this study, we investigated MT interaction with ribosomal proteins and DNA damage response targets using chemical genomic approach (based on screening of the haploid yeast gene knock out library) coupled with siRNA-based validation in alveolar macrophages. A complete genome-wide gene knock out library as well as a selected sub-library representing a collection of only the ribosomal protein genes and DNA damage/repair pathway genes, were screened against two purified MTs Verrucarin A (VA) and Roridin A (RA) and the SC macrocyclic trichothecene mixture (SCMT). The deletion mutants showing resistance or sensitivity to VA were validated using the other two MTs (RA and SCMT). In all, 15 mutants showing resistance (10) or sensitivity (5) to all three MTs (VA, RA, and SCMT) were identified. Among the 10 resistant mutants, 6 were deleted in ribosomal protein genes, 3 were in genes involved in DNA damage/repair response and 1 in a gene involved in vacuole function. Among the 5 sensitive mutants, 3 were impacted in ribosomal components, and 2 in DNA damage/repair response targets. In order to validate the relevance of the identified yeast gene targets in macrophage cell death due to MTs, selected candidate gene encoding ribosomal protein L8 was knocked down using siRNA in SCMT- treated alveolar macrophage cell line MH-S. Silencing this ribosomal protein partly rescued the MH-S cells from MT–induced cell death.

**Key words:** macrocyclic trichothecene; *Stachybotrys*; yeast deletion library screening;
Introduction

Trichothecene mycotoxins produced by certain species of molds/fungi are prevalent in the contaminated food and feed sources (Edwards 2004, Kraska et al 2001) as well as the indoor/outdoor environments (LeBouf et al 2008). Among the fungal trichothecenes, two of the simple trichothecenes viz. deoxynivalenol (DON) and T-2 toxin have been the most intensely studied and have served as prototypes for research on this group of toxins (Pestka 2010, Kruber et al 2011, Wu et al 2011, Chen et al 2011). However, these simple forms do not always represent the macrocyclic trichothecenes, which differ in their toxicity potential due to the presence of a macrocyclic ring in addition to their basic trichothecene skeleton (Sy-Cordero et al 2010).

*Stachybotrys chartarum* is an indoor mold most often encountered in water-damaged or flooded homes and buildings (Ayanbimpe et al 2010, Bellanger et al 2009). Several outbreaks of stachybotryotoxicosis have been reported in animals and farmers in the old literature (Hintikka 1977). In the recent literature, this toxic mold has been associated with indoor mold-related morbidity and mortality, including sick building syndrome and idiopathic pulmonary hemorrhage in infants (Al-Ahmad et al 2010, Wilson and Straus 2002). Among the many toxins that *Stachybotrys* produces, macrocyclic trichothecenes have proven to be the most toxic components causing cytotoxicity in vivo and in vitro (Black et al 2006, Flemming et al 2004). Our recent studies exploring the mechanism of apoptosis caused by the SC macrocyclic trichothecenes (SCMT) mixture in murine alveolar macrophage cell line MHS

Trichothecenes including the macrocyclic forms are known to have the ability to inhibit protein synthesis via interaction with ribosome (Middlebrook and Letherman 1989) and cause ribotoxic stress (Yang et al 2000). However, little is known on the role of specific ribosomal components in causing the toxicity phenotype by these toxins. On the other hand, trichothecenes may interfere with other cellular macromolecules (DNA and RNA). Given the epoxide moiety in the structure of the macrocyclic trichothecenes, one may speculate their native ability to interact with DNA as happens in case of the bioactivated aflatoxins (Bedard and Massey 2006). While no DNA adducts could be reliably detected unlike with aflatoxins, we demonstrated DNA damage in SCMT-treated MHS cells, based on comet tail and micronuclei formation (Wang and Yadav 2006) as well as dysregulation of genes involved in DNA damage response and repair pathways based on genome-wide gene expression analysis (Wang and Yadav 2007).

Yeast gene deletion library is a useful screening tool to identify gene targets that play a pivotal role in cellular response to external chemical stimuli (Ran et al 2003, McLaughlin et al 2009). The prerequisite for such screening applications is that the external stimulus being studied should trigger a measurable response in yeast. In this context, the available literature indicated that trichothecenes such as T-2 toxin cause inhibition of growth in yeasts including Saccharomyces (Schappert and Khachatourians 1983). Moreover, since yeast is easier to grow and manipulate, it could provide a rapid approach to screen for targets of trichothecene toxicity to macrophages. Our working hypothesis is that the yeast and mammalian cells, being
eukaryotes, share a common mechanism of trichothecene interaction with conserved targets such as ribosomes and DNA damage effectors that are implicated in macrocyclic trichothecene-induced toxicity. To explore these concepts, we screened both an entire yeast deletion library (all 4,848 genes) and a targeted sub-library (genes representing the two specific pathways viz., ribotoxic stress response and genotoxicity pathways) for growth inhibition by macrocyclic trichothecenes using purified toxins (VA and RA) and the SC macrocyclic trichothecene mixture (SCMT). Fifteen common gene targets conferring either resistance or sensitivity to all three macrocyclic trichothecenes (VA, RA, and SCMT) were identified. From the pool of identified targets, a selected ribosomal target (RPL8) was knocked down using siRNA to verify its role in MT-induced apoptosis in alveolar macrophages. This is the first report on demonstration of the involvement of a ribosomal protein in mediating the MT-induced apoptosis in alveolar macrophages.

**Materials and methods**

**Fungal culturing and toxin extraction**

*Stachybotrys chartarum* (SC) strain JS58-17 (ATCC 201211) was used as a source of macrocyclic trichothecenes. The fungus was grown on Whatman filter paper-overlaid modified malt extract agar plates. The spores were harvested after 7 days of incubation at 28°C in a humidified incubator and the SC toxins was extracted from the harvested spores using methanol, as described in our previous report (Wang and Yadav 2006).

**Macrophage culturing**

Murine alveolar macrophage cell line MH-S (ATCC CRL- 2019) was cultured at 37°C in a 5%
CO₂ incubator using RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and other supplements (Invitrogen, Carlsbad, CA), namely L-glutamine (0.29 mg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml).

**Assay for Yeast growth inhibition by macrocyclic trichothecenes**

*Saccharomyces cerevisiae* BY4741 MAT a (his3_1 leu2_0 met15_0 ura3_0), the parent strain for the yeast deletion library, kindly provided by Dr. Gee Lau, was used to set up the growth inhibition assay for *S. chartarum* macrocyclic trichothecenes (SCMT), as described earlier (Engler, 1999). Commercially available purified macrocyclic trichothecenes Verrucarin A and Roridin A (Sigma-Aldrich Co. St. Louis, MO) were used for comparison. Briefly, the yeast frozen stocks were revived by incubation in YPD medium (yeast extract 1%, peptone 1%, and dextrose 2%) at 30°C for 24h. The fully-revived yeast suspension was then diluted 20-fold with the fresh YPD medium supplemented with the membrane permeability modulator polymyxin B sulfate (PMBS) at 30μg/ml (EMD BioSciences, Inc. San Diego, CA). The assay was set up in a sterile 96-well microtiter plate. Diluted yeast suspension (100μl per well) was treated with the given trichothecene toxin at variable concentrations viz. Verrucarin A (12.5-100ng/ml), RA (31.25-250ng/ml) or SCMT (1.25x10⁶ - 2x10⁷ spores equivalent toxin/ml). The vehicle (10% methanol in water) was used for the negative control. The plates were incubated in a shaker (150 rpm) at 30°C overnight. The cell density was measured at 630nm using microtiter plate reader (Wallac Victor 1420 Multilabel Counter, Perkin Elmer, Shelton, CT).

**Screening of the yeast deletion libraries (whole and partial)**

A whole library of deletion mutants of *S. cerevisae* Mat-A haploid (originally obtained from
Invitrogen Corporation. Carlsbad, CA), was kindly provided by Dr. Gee Lau (Ran et al 2003). The library consisted of 4,848 Mat-A haploid clones generated by genome-wide deletion of the individual genes in the yeast genome (Winzeler et al 1999). For the targeted library (sub-library), a list of all ribosome- and DNA repair/DNA damage-related ORFs was prepared by searching the Saccharomyces genome database (http://www.yeastgenome.org/) and the corresponding individual yeast gene deletion mutants were retrieved from the whole library according to the MAT-A plate ID information available on the Invitrogen website (http://clones.invitrogen.com/deletions/). These selected mutants were rearranged into a new set of three plates for use as sub-library. The frozen master stocks of the whole library or the sub-library were revived by re-arraying (using steel-pronged replicator) into new sets of 96-well microtiter plates containing 100μl/well of the YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) supplemented with 200μg/ml of geneticin (Invitrogen Corporation. Carlsbad, CA) in a shaker (150 rpm) at 30ºC for 72h. Subsequently, the final set of microtiter plates meant for use in toxin screening was prepared by another round of arraying using a 12-channel pipet to transfer 5 µl of yeast cell suspension from each of the revived clones into 95 µl of PMBS-supplemented YPD medium in each well. The 53-plate whole library and the 3-plate sub-library were first screened using VA (50ng/ml) for resistant and sensitive mutants, followed by further confirmation of their resistance using a higher concentration of VA (100ng/ml), RA (250ng/ml), and SCMT (5x10⁶ spore equivalent toxin), and sensitivity using a lower concentration of VA (12.5ng/ml), RA (31.25ng/ml), and SCMT (1.25x10⁶ spore equivalent toxin).

The resistance or sensitivity of a given mutant was measured in terms of Relative Growth as
compared to the parent strain, per the following formula:

\[
\text{Relative Growth} = \frac{(F-B)/(E-A)}{(H-D)/(G-C)}
\]

The individual values (abbreviated letters) in the above equation represented the \( \text{OD}_{630\text{nm}} \) values listed in the table below.

<table>
<thead>
<tr>
<th>OD(_{630\text{nm}}) (0 h)</th>
<th>Mutant vehicle control</th>
<th>Mutant Toxin treatment</th>
<th>Parent strain Vehicle control</th>
<th>Parent strain Toxin treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>OD(_{630\text{nm}}) (20h)</td>
<td>E</td>
<td>F</td>
<td>G</td>
<td>H</td>
</tr>
</tbody>
</table>

**Silencing of the identified target gene homologs in alveolar macrophages**

For each identified mutant (resistant or sensitive), the corresponding yeast ORF was retrieved from the “Saccharomyces genome database” (SGD). The corresponding mammalian gene homolog and its mRNA sequence for the selected yeast gene targets were then obtained from the NCBI Genbank database. The siRNA for each target mRNA was pre-designed and purchased from Qiagen Sciences, Inc. (Germantown, MD). Transfection of siRNA into MHS cells was performed using the CodeBreaker\textsuperscript{TM} siRNA transfection reagent (Promega Corporation, Madison, WI) according to the manufacturer’s instructions. The sequences of siRNAs used in this study are presented in Table. 1. The efficiency of transfection was confirmed using a fluorescent dye labeled siRNA, the siGLO cyclophilin B siRNA (Dharmacon, Inc., Chicago, IL), before proceeding further in our siRNA-based silencing experiments.
qRT-PCR verification of the gene silencing

The siRNA-based silencing of the selected target gene was verified by analyzing the corresponding mRNA levels in the gene-specific siRNA-transfected cells and the scramble siRNA-transfected cells (control) using real-time qRT-PCR. Total RNA was isolated using Tri-reagent (Molecular Research Center, Inc. Cincinnati, OH) and purified using RNeasy mini RNA purification kit (Qiagen Sciences, Inc. Germantown, MD) according to manufacturer’s protocols. The qRT-PCR reaction was performed using the Brilliant SYBR green QRT-PCR master mix kit (Agilent Technologies, Santa Clara, CA) on the Smart Cycler system (Cepheid, Sunnyvale, CA). The sequences of the primers used are listed in Table 2. The qRT-PCR was performed by using the following reaction conditions: RT reaction at 50°C for 30 min, initial denaturation at 94°C for 10 min, followed by 40 cycles of amplification, each involving denaturing at 94°C for 10 sec, annealing and extension at 60°C for 60 sec. Beta-actin gene was used as the internal control. The fold change in the transcript level was calculated as $2^{\Delta\Delta C_t}$ based on the cycle threshold (Ct) measurements, where

$\Delta\Delta C_t = \Delta C_t$ of vehicle control - $\Delta C_t$ of toxin-treated

$\Delta C_t = C_t$ of the target gene - $C_t$ of beta actin (Chang et al 2002).

Cell viability assay

Considering that the gene targets for silencing in macrophages correspond to those associated with trichothecene-mediated growth inhibition phenotype in yeast, the effect of siRNA-mediated silencing on these genes was measured in terms of viability changes in the alveolar macrophage (MH-S) cells treated with SCMT. The cell viability changes were measured using CellTiter-Glo Luminescent cell viability assay kit (Promega, Madison, WI).
according to the manufacturer’s instructions. Briefly, 100μl of CellTiter-Glo® Reagent was added to each well containing the cell culture and the contents mixed for 2 min on an orbital shaker to induce cell lysis. The plate was allowed to incubate at room temperature for 10 min to stabilize the luminescence signal.

**Genetic identity confirmation of yeast deletion mutants**

Genetic identity of the identified resistant/sensitive yeast mutants was confirmed by PCR using primers specific for the deleted gene target. The sequence information on the target genes was retrieved from the website (http://www-sequence.stanford.edu/group/yeast_deletion_project/strain_a_mating_type.txt.) provided by the yeast deletion project. Briefly, genomic DNA was extracted from the individual mutants and the parent yeast, and a pair of PCR reactions was setup using the DNA template from the mutant and the parent strain. The PCR was performed on GeneAmp PCR system 2700 (Applied Biosystem) with ExTaq PCR kit (Takara, Shiga, Japan) in a 50μl total reaction volume, using 0.5μl Ex-Taq DNA polymerase, 5μl 10x Ex-Taq buffer, 4μl dNTP mix, 1μl genomic DNA (500ng), 5μl A primer (10μM), 5μl KanB primer (10μM), and 29.5μl nuclease-free water. The reaction conditions included denaturation at 95°C for 3min, 40 amplification cycles each at 95°C for 15sec, 57°C for 30 sec, and 72°C for 30sec, followed by a final extension step at 72°C for 3min. An aliquot (5μl) of each PCR product was loaded onto 1% agarose gel (supplemented with 25ng/ml of ethidium bromide) for electrophoresis (90v for 40min). The primer sequences used for the genetic identity confirmation are listed in Table.3.

**Monitoring changes in p38 activation in RPL8 silenced cells by Western blot analysis**
MH-S cells silenced with RPL8 siRNA were treated with SCMT toxin at 24 hours post-transfection. At 0min, 10min, 30min, and 3 hours intervals post-toxin treatment, the cells were extracted for total protein followed by Western blot analysis for p38 activation as described in our previous report (Wang and Yadav 2006). Transfection reagent-treated cells and scramble siRNA-treated cells were used as vehicle control and negative control, respectively. The same blot was stripped and probed for beta-actin as the loading control.

Statistical analysis

The results are presented as mean+standard deviation of three biological samples. Comparisons were made using Student’s t test and p<0.05 as the level of significance.

Results

Condition optimization and screening for yeast growth inhibition by macrocyclic trichothecenes

Our initial experiments focused on optimization of yeast growth in microtiter plate culture format and generation of growth curves using the parent yeast *S. cerevisae* BY4741, MAT-A (his3_1 leu2_0 met15_0 ura3_0). Relationship between the cell number and the corresponding OD is shown in Fig.1A. The two sets of data were regressed to a linear curve, represented by $Y = 5 \times 10^8 X + 0.0471$, where $X$ is the number of yeast cells and $Y$ is the corresponding OD value. The growth curve showed that the yeast started growing exponentially after 6 hours and the growth continued through 20 hours, followed by a slow transition to the stationary phase (Fig. 1B).

Using the optimized microtiter-based growth assay, we compared the growth inhibition
pattern by the individual test macrocyclic trichothecenes (VA, RA, and SCM). Verrucarin A (VA) showed a concentration-dependent inhibition of yeast growth viz. slight growth inhibition at lower concentration (12.5ng/ml), 50% growth inhibition at moderate concentration (50ng/ml VA), and about 80% growth inhibition at high concentration (100ng/ml VA) as compared to the vehicle control (Fig. 2A). Roridin A (RA) also showed concentration-dependent growth inhibition pattern with slight inhibition at 31.25ng/ml and 50% inhibition at 250ng/ml (Fig. 2B). Likewise, the SCMT toxin mixture showed a concentration-dependent inhibition in the initial test range of the toxin concentration. An SCMT concentration of 5x10^6 spores equivalent toxin/ml inhibited about 50% of the yeast growth, which was equivalent to that caused by 50ng/ml of VA. However, at the higher test range (1x10^7 and 2x10^7 spores equivalent toxin/ml), the growth inhibition by SCMT was lowered and showed a reverse correlation with the toxin concentration (Fig. 2C). The biphasic model of SCMT-induced growth inhibition in the yeast parent strain (BY4741) might have been caused due to the presence of unknown growth protective/stimulatory ingredient(s) in the toxin mixture.

**MT gene targets identified based on the library screenings**

In the whole library screening, 6 mutants showed common resistance (none showing common sensitivity) to the three toxin types, confirming their specific responsiveness to a macrocyclic trichothecene. Among the 6 resistant mutants, 3 were disrupted in genes encoding ribosomal proteins, namely RPL2A (YFR031C-A), RPL16A (YIL133C), and RPL20A (YMR242C). 2 were disrupted in genes encoding DNA damage/repair pathway components, namely MMS
(YGL087C) and SIR4 (YDR227W), and one was deleted in PFA3 (YNL326C) which is involved in vacuole fusion (Fig. 3A).

The sub-library consisting of 155 ribosome-related mutants and 61 DNA damage/repair-related mutants was maintained in three 96 well-plates, with plates #1 and #2 arrayed with ribosomal mutants and plate #3 arrayed with DNA damage/repair-related mutants. Screening of the sub-library for resistant mutants using high concentrations of VA, RA, and SCMT toxins yielded 4 resistant mutants that showed resistance to all three toxins, two from plate #1 [RPL6B (YLR448W) and RPL7A (YGL076C)], one each from plate #2 [RPL16B (YNL069C)], and plate #3 [UBC13 (YDR092W)] (Fig. 3B).

The sensitivity phenotype of the yeast deletion mutants in the sub-library was tested using lower concentrations of the toxins. This yielded 5 commonly sensitive mutants, including 2 mutants from plate #1 [RPS10B (YMR230W) and RPS28A (YOR167C)], 1 mutants from plate #2 [RPS29B (YDL061C)], and 2 mutants from plate #3 [RAD14 (YMR201C) and MAD1 (YGL086W)] (Fig.3C).

Gene-specific PCR analysis (Fig.4) confirmed the genetic identity of the above identified 15 mutants (10 resistant and 5 sensitive).

**Gene silencing-based verification of the role of the identified gene targets in macrophage cell death**

RPL8 was selected for siRNA-based verification of its functional role in cell death in alveolar macrophage cell line. The effect of silencing of the gene target RPL8, measured based on the mRNA levels, reached as high as 70% (Fig. 5A.). RPL8-knock down cells showed slight
resistance (around 10% increase in viability) to SCMT as compared to the scramble siRNA-treated cells (Fig. 5B). However, the activation of p38 in terms of timing and intensity did not show any obvious difference between the RPL8-knock down cells and the controls viz. vehicle control and negative control (Fig. 5C).

Discussion

Trichothecenes including macrocyclic trichothecenes of the mold *S. chartarum* are known to induce apoptosis in various cell lines (Huang et al 2007, Wang and Yadav 2006) and in exposed animal models (Mikami et al 2010, Sehata et al 2005). Ribotoxic stress has been proposed to be one of the underlying mechanisms (Shifrin and Anderson 1999). In this mode of action, trichothecenes (such as T-2 and Satratoxin G) are hypothesized to bind to ribosomes, and activate p38/JNK2, which in turn transduces the death signal through mitochondria and/or death receptor pathway (Yang et al 2000, Wang and Yadav 2006). Between ribosomal binding and MAPK activation, two kinases, namely Hck (hematopoietic cell kinase) and PKR (double-stranded RNA-activated protein kinase) have been suggested to act as transducers (Bae et al 2010, Gray et al 2008, Zhou et al 2005). However, the specific mechanism of binding or interaction of trichothecene with the ribosome leading to transducing of the signal is not known.

In eukaryotic cells, including lower eukaryotes like yeast, ribosomes are highly conserved sub-cellular organelles responsible of protein synthesis. Each yeast ribosome contains one copy of the four ribosomal RNAs (5S, 5.8S, 8S, and 25S) and 78 different ribosomal proteins (Venema and Tollervey 1999). Ribosome is comprised of one large subunit (60S) and one
small subunit (40S). The large subunit contains 46 proteins and three RNA molecules (25S RNA, 5.8S RNA, and 5S RNA) and the small subunit contains 18S rRNA and 32 proteins (Verschoor et al 1998). Each yeast ribosomal protein has its mammalian homolog (Mager et al 1997).

Besides their conserved function in ribosomal protein synthesis, ribosomal proteins have been shown to perform extra-ribosomal functions, such as in regulating gene transcription, translation, cell cycle progression, cell proliferation, and apoptosis to name a few (Lee et al 2010, Wool 1996). Considering this, we took advantage of the available yeast gene knock out library (Ran et al 2003, McLaughlin et al 2009) in order to identify the specific ribosomal proteins for their role in the ribotoxic stress response pathway governing macrocyclic trichothecene-induced apoptosis mechanisms in macrophages. In addition, we investigated conserved gene targets of DNA damage response pathway; this pathway was targeted considering the outcome of our initial studies (Wang and Yadav 2006, Wang and Yadav 2007) on genotoxicity potential of *Stachybotrys* trichothecenes (SCMT). Those studies provided the first evidence of DNA damage in the treated macrophages (Wang and Yadav 2006) and upregulation of DNA damage-responsive genes and pathways (Wang and Yadav 2007) in response to *Stachybotrys* trichothecenes mixture. Subsequently, other studies had confirmed these genotoxic phenomena using purified trichothecenes T-2 and Satratoxins G, H (Chaudhari et al 2009, Rakkestad et al 2010). In light of these studies, here we investigated both purified (VA and RA) and mixed (SCMT) macrocyclic trichothecenes to identify the specific gene targets involved in macrocyclic trichothecene-induced DNA damage/repair pathway.
Based on the overall screening data from the sub-library and whole library screenings, a total of 6 ribosomal protein genes were implicated in resistance as against 3 ribosomal genes implicated in conferring sensitivity to the three tested macrocyclic trichothecenes (SCMT, VA, and RA). Information on these and other identified gene targets is listed in Tables 4 and 5.

Deletions in ribosomal genes or DNA damage/repair-related genes were identified in the resistant and sensitive yeast strains. Most notably, all 6 ribosomal genes associated with increased resistance to MT, namely RPL2A (YFR031C-A), RPL6B (YLR448W), RPL7A (YGL076C), RPL16A (YIL133C), RPL16B (YNL069C), RPL20A (YMR242C), are the structural components of the ribosomal large subunit (60S). This is in accordance with the existing notion that trichothecenes (such as T-2 toxin) bind to the large subunit of the ribosome (Smith et al 1975) and with the recent observation on the binding of the macrocyclic trichothecene Satratoxin G to both 60S and 40S subunits (Bae et al 2009).

Yeast RPL2A contacts almost every domain of the large subunit rRNA and participates in an inter-subunit bridge with the small subunit rRNA (Meskauskas et al 2008). Binding studies show that L7 (mammalian homolog of yeast RPL7A) binds preferentially to 28S rRNA (von Mikecz, 1999). L6 (mammalian homolog of yeast RPL6B) and L7 were shown to bind to 5S rRNA in rat liver (Ulbrich et al 1982). Yeast RPL20A was shown to bind to the double-stranded region of rRNA in yeast (Reyes et al 1978). Later studies showed yeast RPL20A binding to 25S rRNA (Yeh and Lee 1998).

Ribosomal RNA (rRNA) is important in medicine considering that it acts as the target for a number of antibiotics such as chloramphenicol (Marconi et al 1990), erythromycin (Lee et al
rRNA has also been reported to be a target of biological toxins such as ricin (Uchiumi et al 1999) and sarcin (García-Ortega et al 2010). Among the rRNA species, 5.8S rRNA plays an important role in elongation and translocation; its mutation caused cell growth inhibition and disruption of translation (Abou Elela and Nazar 1997). p53 has been shown to covalently bind to 5.8S rRNA, implying its role in signaling transduction (Fontoura et al 1992). 5S rRNA links all of the functional centers of the ribosome, and thus may play a critical role in transmission and coordination of the functional centers of the ribosome (Dinman 2005). Recently, it was proposed that MAPK docks in the ribosome and the two kinases PKR and Hck may transduce the trichothecene-ribosome binding and ribotoxic stress signaling to p38 (Pestka 2010). However, it is not clear how the transducer(s) function between the trichothecene and the ribosome. Based on the results in the current study, we propose that MT might specifically bind to rRNA and the identified proteins are either dislodged by MT or the functional /spatial changes are sensed by the transmitters inside the ribosome, which in turn function to recruit or activate subsequent signaling events, such as activation of stress-inducible MAPK (p38) or p53, among others. Subsequent work would determine the binding site of MT on rRNA, and the effect of rRNA binding on the association of ribosomal protein(s), and subsequent signaling.

The identified DNA damage/repair pathway target genes associated with MT resistance (MMS2, SIR4, and UBC13) or sensitivity (RAD14 and MAD1) in this study may be involved in multiple cellular processes such as nucleotide excision repair (RAD14/ YMR201C) (Guzder et al, 2006 ), post-replicative DNA repair (MMS2/ YGL087C, UBC13/ YDR092W)
(McKenna et al 2001), cell cycle checkpoint (MAD1/ YGL086W) (Hardwick and Murray 1995), and telomere maintenance (SIR4/ YDR227W) (Hsu et al 2004). These targets imply that multiple types of DNA damage and responses might be induced by MT, probably including the one leading to DNA-adduct formation.

In conclusion, this is the first report revealing the involvement of multiple ribosomal proteins and DNA damage/repair pathway proteins in the MT-induced cytotoxicity in a eukaryotic cell system. Since the observations are common to multiple MTs and are based on single gene-knock out mutant screening, the identified candidates especially those conserved in eukaryotes (with homologs in mammalian cells) could be the real signaling target molecules; these targets may interact closely with MT, working either directly as upstream targets and/or as transducers for subsequent activation of the already proven downstream targets p53 and p38.
Figure legends

Fig. 1. Growth Curve of yeast parent strain *BY4741* in 96-well plate format. A. Linear regression of yeast cell number with OD$_{630nm}$; B. Growth curve.

Fig. 2. Growth inhibition of the yeast parent strain *BY4741* by verrucarin A (VA), Roridin A (RA), and *S. chartarum* macrocyclic trochothecene mixture (SCMT). A. Dose-dependent growth inhibition by VA; B. Dose-dependent growth inhibition by RA; C. Bi-phasic growth inhibition by SCMT.

Fig. 3. Yeast gene deletion mutants showing common resistance or sensitivity to VA, RA, and SCMT. A. Resistant mutants identified from the whole library screening; B. Resistant mutants identified from the sub-library screening; C. Sensitive mutants from the sub-library screening. *: statistically significant based on Student’s t test (p<0.05).

Note: resistance or sensitivity of a given mutant as compared to the parent strain was measured in terms of the relative growth (Y-axis). This was calculated as given in the Materials and methods section.

Fig. 4. Confirmation of genetic identity of the resistant and sensitive mutants. A. Panel A, lanes A1 and A17 correspond to the 1kb DNA ladder, lanes A2 through A16 show PCR amplicons (amplified using mutant genomic DNA template and gene-specific primers, one from the mutant insert and the other from the flanking genomic region) from mutants knocked out in genes encoding RPL2A, RPL6B, RPL16A, RPL16B, RPL7A, RPL20A, MMS2, UBC13, SIR4, PFA3, RPS10B, RPS28A, RPS29B, RAD14, and MAD1, respectively. B. Panel B, lanes B1 and B17 represent 1kb DNA ladder, lanes B2 through B16 show separation
of PCR reaction products from the parent strain genomic DNA using the mutant-specific primers (in the same order as listed for panel A) to rule out non-specific amplifications in panel A.

**Fig.5. RPL8 gene silencing to verify its role in rescuing alveolar macrophages from cell death and p38 activation induced by SCMT treatment.** A. qRT-PCR based quantification of relative transcript levels in RPL8-siRNA transfected cells versus scramble-siRNA transfected cells; B. RPL8 gene knock down conferred partial resistance to SCMT in murine alveolar macrophage cells. C. The timing and intensity of p38 activation in RPL8 knock down cells after treatment with SCMT for 0min, 10min, 30min, and 180min, in comparison to the control cells (vehicle-treated and scramble siRNA-treated).*: statistically significant based on Student’s t test (p<0.05).
**Table. 1. Various siRNAs and their targeted sequences used in this study**

<table>
<thead>
<tr>
<th>siRNA Name</th>
<th>Target gene (Accession No.)</th>
<th>Target Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mm_RPL8_1</td>
<td>RPL8</td>
<td>TAGAGTTCAGGAGCTAATAAA</td>
</tr>
<tr>
<td>Mm_RPL8_2</td>
<td>(NM_012053)</td>
<td>CAAGTACAAGGCAAAGAGGAA</td>
</tr>
<tr>
<td>Mm_RPL8_3</td>
<td></td>
<td>AACCACCAGCAGCATTTGGCAAAA</td>
</tr>
<tr>
<td>Mm_RPL8_4</td>
<td></td>
<td>CAGCTGAATATCGGCAATGTT</td>
</tr>
<tr>
<td>Scramble siRNA</td>
<td></td>
<td>CAGGGTATCGACGATTACAAA</td>
</tr>
<tr>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer</td>
<td>Targeted gene (Accession No.)</td>
<td>Sequence</td>
</tr>
<tr>
<td>----------------------</td>
<td>-------------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Forward primer</td>
<td>Beta-Actin (NM_007393)</td>
<td>5’-CAAGATCATTGCTCCTCCTGAGC-3’</td>
</tr>
<tr>
<td>Reverse primer</td>
<td></td>
<td>5’-GCAGCTCAGTAACAGTCCGC-3’</td>
</tr>
<tr>
<td>Forward primer</td>
<td>RPL8 (NM_012053)</td>
<td>5’-CATCTCCCACAACCCAGAGAC-3’</td>
</tr>
<tr>
<td>Reverse primer</td>
<td></td>
<td>5’-AGCCACAACACCAACACGG-3’</td>
</tr>
<tr>
<td>Primer Name</td>
<td>Sequence</td>
<td>Amplicon (bp)</td>
</tr>
<tr>
<td>----------------</td>
<td>-----------------------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>RPL2A - Primer A</td>
<td>5’-CTATCCTCATTCTCCCCACTGTGTAA-3’</td>
<td>640</td>
</tr>
<tr>
<td>RPL6B - Primer A</td>
<td>5’-GTGAAACACATAGTTGTGGTTATGC-3’</td>
<td>680</td>
</tr>
<tr>
<td>RPL16A - Primer A</td>
<td>5’-ATGCTAGACTTCTCCCTCTGTATCC-3’</td>
<td>573</td>
</tr>
<tr>
<td>RPL16B - Primer A</td>
<td>5’-GTACACTATAACACACACCCAAACCTCC-3’</td>
<td>701</td>
</tr>
<tr>
<td>RPL7A - Primer A</td>
<td>5’-CCAGTTACATCAGTTGAGTAGAGA-3’</td>
<td>598</td>
</tr>
<tr>
<td>RPL20A - Primer A</td>
<td>5’-GACGTAAATTGTACCATGCTGAATA-3’</td>
<td>545</td>
</tr>
<tr>
<td>MMS2 - Primer A</td>
<td>5’-CACCACATATTGTCTATTGTACTG-3’</td>
<td>630</td>
</tr>
<tr>
<td>UBC13 - Primer A</td>
<td>5’-ACAGGAATTTCCTTTCTTTAAACC-3’</td>
<td>536</td>
</tr>
<tr>
<td>SIR4 - Primer A</td>
<td>5’-ATATTTTTATCGTTGAGAACGAACG-3’</td>
<td>619</td>
</tr>
<tr>
<td>PFA3 - Primer A</td>
<td>5’-TTAAATATTCCGGTCAATTCTCTGGA-3’</td>
<td>667</td>
</tr>
<tr>
<td>RPS10B - Primer A</td>
<td>5’-GAAGCCTGAGTTTTTTCAAACAATA-3’</td>
<td>428</td>
</tr>
<tr>
<td>RPS28A - Primer A</td>
<td>5’-CTTGACAATCTCTACCTTTTTTA-3’</td>
<td>674</td>
</tr>
<tr>
<td>RPS29B - Primer A</td>
<td>5’-GAAAATATCTGTTGGCTAGCAGTGG-3’</td>
<td>597</td>
</tr>
<tr>
<td>RAD14 - Primer A</td>
<td>5’-AGGAAAGTGAAAAAGAGGGTAAAGGAA-3’</td>
<td>385</td>
</tr>
<tr>
<td>MAD1 - Primer A</td>
<td>5’-CAGTTTTGACATTTGTTGTAACCG-3’</td>
<td>527</td>
</tr>
<tr>
<td>KanB*</td>
<td>5’-CTGCAGCGGAGGACCCGTAAT-3’</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*KanB (the reverse primer for all amplifications), designed by the yeast deletion library project consortium, is based on the sequence of the mutant insert module that was used to replace the respective target gene in the mutants.*
<table>
<thead>
<tr>
<th>Yeast mutant (deleted gene name)</th>
<th>ORF</th>
<th>Mammalian Homolog</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPL6B</td>
<td>YLR448W</td>
<td>L6</td>
<td>A protein component of the large subunit; binds to 5S, 5.8S, and 28S rRNA, and initiator and elongator tRNA; Mutation caused resistance to aminoglycoside antibiotics; Overexpression in cancer cells; Upregulation led to resistance to anticancer drug-induced apoptosis through differential regulation of Bcl-2 family members.</td>
<td>Uchiumi, 1983; Ulbrich, 1982; Davies, 1998; Gou, 2010 Du, 2005.</td>
</tr>
<tr>
<td>RPL7A</td>
<td>YGL076C</td>
<td>L7</td>
<td>A protein component of the large subunit; A major autoantigen in autoimmune disease; Upregulated in cancer cells; Overexpression induced apoptosis;</td>
<td>Neu, 1997; Wang, 2000; Neumann and Krawinkel, 1997;</td>
</tr>
<tr>
<td>RPL2A</td>
<td>YFR031C-A</td>
<td>L8</td>
<td>Core element of the large ribosomal subunit; Binds to rRNA of the large subunit and participates in an inter-subunit bridge with small subunit rRNA; Mutant affected peptidyl-tRNA binding and peptidyltransferase activity; Depletion of L8 caused cell cycle arrest and apoptosis induction;</td>
<td>Meskauskas, 2008 Li, 2010</td>
</tr>
<tr>
<td>Yeast gene name</td>
<td>ORF</td>
<td>Mammalian Homolog</td>
<td>Function</td>
<td>References</td>
</tr>
<tr>
<td>----------------</td>
<td>-----------</td>
<td>-------------------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>RPL20A</td>
<td>YMR242C</td>
<td>L18a</td>
<td>Protein component of the large ribosomal subunit; Overexpressed in transformed and metastatic tumor cell line.</td>
<td>Dong, 1997;</td>
</tr>
<tr>
<td>RPL16A</td>
<td>YIL133C</td>
<td>L13a</td>
<td>Protein component of the large ribosomal subunit; Binds to 5.8S rRNA; Inhibits IFN-gamma-activated inflammatory genes; Depletion impaired rRNA methylation and cap-independent translation; Overexpression induced apoptosis.</td>
<td>Mukhopadhyay, 2008; Chaudhuri, 2007 Chen and Ioannou, 1999 Chen, 1998</td>
</tr>
<tr>
<td>RPL16B</td>
<td>YNL069C</td>
<td>L13a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UBC13</td>
<td>YDR092W</td>
<td>UBE2N</td>
<td>Both encode ubiquitin-conjugating enzymes; UBC13 interacts with MMS2 to assemble ubiquitin chain at the Ub Lys-63 residue; Hetermeric complex of UBC13, MMS2, and RAD5 is responsible for polyubiquitination of PCNA in error-free DNA synthesis; UBC13 involved in TRAF-dependent signaling pathway, haploinsufficiency of UBC13 have reduced cytokine secretion and lethality induced by lipopolysaccharide, as well as inhibition of NF-kB, JNK, and p38 MAPK; UBC13 recruits p53 to polysome and affects p53 organization within polysomes;</td>
<td>Hofmann and Pickart, 1999; Brusky, 2000; Fukushima, 2007; Laine, 2006</td>
</tr>
<tr>
<td>MMS2</td>
<td>YGL087C</td>
<td>UBE2V2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast gene name</td>
<td>ORF</td>
<td>Mammalian Homolog</td>
<td>Function</td>
<td>References</td>
</tr>
<tr>
<td>----------------</td>
<td>-------</td>
<td>-------------------</td>
<td>----------------------------------------------------------------------------------------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>SIR4</td>
<td>YDR227W</td>
<td>none</td>
<td>Involved in assembly of silent chromatin domains at telomeres; Involved in DNA double-strand break (DSB) repair; Loss of SIR4 led to checkpoint adaption and survival following induction of persistent DSB; Inactivation of SIR4 abrogated reactive oxygen species accumulation and apoptosis in yeast cells;</td>
<td>Moazed, 1997; Ubersax, 2003 Lewis, 1999 Orlandi, 2004</td>
</tr>
<tr>
<td>PFA3</td>
<td>YNL326C</td>
<td>none</td>
<td>Palmitoyltransferase for Vac8p (a protein involved in vacuolar fusion); Required for vacuolar membrane fusion and for vacuolar integrity under stress conditions;</td>
<td>Smotrys, 2005; Hou, 2005</td>
</tr>
</tbody>
</table>
Table 5. Information on the yeast mutants showing common sensitivity to VA, RA, and SCMT

<table>
<thead>
<tr>
<th>Gene name</th>
<th>ORF</th>
<th>Mammalian Homolog</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPS28A</td>
<td>YOR167C</td>
<td>S28</td>
<td>Protein component of small ribosomal subunit; Involved in decoding activity of ribosome and control of translation accuracy;</td>
<td>Synetos, 1996</td>
</tr>
<tr>
<td>RPS29B</td>
<td>YDL061C</td>
<td>S29</td>
<td>Protein component of small ribosomal subunit; Overexpression of S29 expression was shown to induce quiescence and apoptosis;</td>
<td>Khanna, 2000; Coppock, 2000</td>
</tr>
<tr>
<td>RPS10B</td>
<td>YMR230W</td>
<td>S10</td>
<td>Protein component of small ribosomal subunit; Involved in assembling of ribosomes, protein synthesis, and optimal cell proliferation;</td>
<td>Ren, 2010</td>
</tr>
<tr>
<td>RAD14</td>
<td>YMR201C</td>
<td>XPA</td>
<td>Recognizes and binds to damaged DNA during nucleotide excision repair;</td>
<td>de Laat, 1999</td>
</tr>
<tr>
<td>MAD1</td>
<td>YGL086W</td>
<td>MAD1L1</td>
<td>Encodes a component of the spindle checkpoint; Member of the Myc/Max/Mad network of transcriptional regulators, which antagonizes Myc functions; Interferes with Fas-, TRAIL-, c-Myc, and UV-induced apoptosis; Reduced apoptosis induced by serum withdrawal</td>
<td>Hardwick and Murray, 1995; Gehring, 2000; Bejarano, 2000;</td>
</tr>
</tbody>
</table>
Fig. 1.

A

\[ Y = 5 \times 10^8 X + 0.0471 \]

\[ R^2 = 0.9966 \]

B
Fig. 2.
Fig. 3.
Fig. 4.
References


Al-Ahmad M, Manno M, Ng V, Ribeiro M, Liss GM, Tarlo SM. Symptoms after mould exposure including *Stachybotrys chartarum*, and comparison with darkroom disease. Allergy. 2010 Feb; 65 (2) :245-55.


- 137 -


Kruber P, Trump S, Behrens J, Lehmann I. T-2 toxin is a cytochrome P450 1A1 inducer and leads to MAPK/p38- but not aryl hydrocarbon receptor-dependent interleukin-8 secretion in the human intestinal epithelial cell line Caco-2. Toxicology. 2011 Jun 18; 284 (1-3) :34-41.


- 140 -


Chapter V

Discussion and conclusions

In this study on interaction of *Stachybotrys chartarum* trichothecenes with alveolar macrophages, we first profiled the toxicity effect at various doses and times of exposure, with the aim of finding the most significant endpoints of this interaction. We used the SC macrocyclic trichothecenes mixture (SCMT) extracted from the macrocyclic trichothecene chemotype of *S. chartarum*, to mimic the real-world exposure scenario (Wang and Yadav 2006). Alveolar macrophages function as an important host innate defense system in the exposed lung. Recent rodent exposure studies have imaged AMs phagocytosing toxin-containing SC spores (Gregory et al 2004). Generally, macrophages respond to exogenous pathogens or endogenous aging or dying cells by endocytosis. This is followed by amplification of inflammatory and or immune response by secretion of cytokines and chemokines to recruit other immune cells. Alternatively, if overwhelmed by the challenge of the pathogen, macrophages may undergo apoptosis (Dörger and Krombach 2002). In this study, apoptosis was the major endpoint that resulted from SC toxin treatment and no inflammatory response (measured in terms of cytokines) was detectable.

To figure out the mechanisms underlying the SCMT-induced apoptosis, selected apoptotis-associated targets and signaling pathways were examined. Since there is an epoxide moiety in the chemical structure of trichothecenes which is critical in governing their potent toxicity (Thompson and Wannemacher 1986), and the fact that epoxide addition to aflatoxin B$_1$ during its activation leads to DNA adduct formation (Choy 1993), we examined
the DNA damage potential of the SCMT toxin. It turned out that, similar to the immediate ribosome-binding ability of trichothecenes (that usually happening within 5 minutes of exposure), the SCMT toxin caused DNA damage (as evidenced by Comet assay) within the same time frame, before any signs of apoptosis were detectable. Subsequently, probing of the stress responsive MAPK pathway and DNA damage-responsive p53 pathway confirmed the activation and involvement of these signaling events in the interaction of SCMT toxin and alveolar macrophages (Wang and Yadav 2006). In our studies thus far on SCMT toxin-macrophage interactions, three sets of molecular events have been captured, one occurring at the initial level (ribosome-binding, and DNA damage), the other coinciding with the intermediate level (MAPK and p53 signaling), and of course, the last one, representing the endpoint (apoptosis).

The next step focused on investigating the signal transduction targets which connect the dots of these three sets of events. To assess the individual pathways/targets underlying the big picture on the toxin-macrophage interaction, we took advantage of the powerful tool of genome-wide microarray, to profile the transcriptional changes associated with this interaction. Two time points were selected to perform the analysis, a pre-apoptosis time-point (2 hours) and an advanced apoptosis time-point (24 hours), with an intention to capture the transcriptional changes before and after the onset of apoptosis. As expected for a macromolecule synthesis inhibitor, both up- and down- regulation of hundreds of genes were observed in both time points (Wang and Yadav 2007). Genes involved in a variety of cellular processes and multiple signaling pathways were impacted during the SC toxin treatment of macrophages. Notably, in agreement with our previous toxicity endpoint data (Wang and
Yadav 2006), the genes involved in mitochondrial - and death receptor-mediated apoptotic pathways were identified. Overall, the microarray results confirmed that multiple pathways, including stress-responsive pathway, DNA-damage response pathway and oxidative stress pathway, among others are involved in the interaction (Wang and Yadav 2007).

Further exploration of the upstream targets that work immediately downstream of the ribosomal binding and DNA damage events was undertaken. This was accomplished using another valuable tool, the yeast deletion library, wherein each one of the over 4800 genes is deleted in a haploid mat-a yeast background. This genome-wide library was screened for potential targets, with emphasis on ribosomal and DNA damage/repair-related genes. Given the facts that both ribosome and DNA damage/repair related events are highly conserved in eukaryotes, that each of the yeast ribosomal genes has its mammalian homolog, and that trichothecene inhibits growth in yeast, the outcome was expected to provide us the relevant macrophage targets for upstream signaling events during programmed cell death (apoptosis).

The relationship between trichothecene and ribosomes has drawn extensive research efforts since 1980s, especially on T-2 toxin (Middlebrook and Leatherman 1989). However, all those studies have been focusing on either the binding kinetics or the steps disrupted during mRNA translation and de novo protein synthesis (Murthy et al 1985, Yagen and Bialer 1993); no direct data showing the binding sites or interacting molecules in the ribosome are available.

Both SCMT toxin and pure commercially available macrocyclic trichothecenes (Verrucarin A and Roridin A) were employed to screen the yeast deletion library, in order to find the targets that are macrocyclic trichothecone (MT)-specific, by placing emphasis on the deletion mutants sharing sensitivity or resistance to all three trichothecene types.
In all, 9 ribosomal proteins were identified to have association with resistance or sensitivity to MT-induced growth inhibition in yeast. The 6 mutants (RPL2A, RPL6B, RPL7A, RPL16A, RPL16B, and RPL20A) showing increased resistance to MT were disrupted in the ribosomal large subunit proteins, whereas the 3 mutants (RPS10B, RPS28A, and RPS29B) showing increased sensitivity to MTs were all disrupted in ribosomal small subunit proteins. Among the 6 ribosome-related resistant mutants, 4 (RPL2A, RPL6B, RPL7A, and RPL20A) have been reported to bind to rRNA. Since MTs have the potential to bind to rRNA, we hypothesize that the binding of MTs to rRNA might therefore interfere with the normal interaction of ribosomal protein(s) with rRNA, which may lead to the protein conformational change or modification, and subsequent signal transduction event. Appropriate experiments will have to be designed to verify this assumption.

Deletion of 5 genes relevant in DNA damage response pathways were found associated with either resistance or sensitivity to MTs. Their encoded proteins are known to be associated with multiple cellular processes underlying genotoxic response, including post-replicative DNA repair (MMS2 and UBC13), nucleotide excision repair (RAD14), cell cycle checkpoint (MAD1), and telomere maintenance (SIR4). These results implicate the involvement of these potentially critical protein targets in MT-DNA interaction; however further studies would be needed to elucidate their specific role in the underlying mechanisms of this interaction.

Overall, the following conclusions were drawn in this study.

- *Stachybotrys chartarum* macrocyclic trichothecene mixture (SCMT) caused cytotoxicity as measured by lactate dehydrogenase release in murine alveolar macrophages in a dose- and time-dependent manner. However, no inflammatory
response in terms of cytokines or induction of nitric oxide was detectable in SCMT-treated macrophages. Interestingly, endotoxin (LPS)-pretreatment before SCMT exposure led to exacerbation of cytotoxicity in the alveolar macrophages.

- The SCMT-caused toxicity in alveolar macrophages was manifested as programmed cell death (apoptosis) as early as at 3h post-exposure.

- The study led to the first demonstration of DNA damage in SC macrocyclic trichothecene-treated macrophages (which was detectable as early as 15 minutes post-exposure based on single cell electrophoresis).

- SCMT treatment of alveolar macrophages caused perturbation in reduced and oxidized glutathione levels implying induction of oxidative stress.

- The SCMT-induced apoptosis was linked to MAPK signaling particularly via p38 and DNA damage response pathway as evidenced by p53 accumulation.

- Comparison of gene expression changes in pre-apoptotic (< 3h) and advanced apoptotic macrophages revealed the involvement of multiple signaling pathways in SCMT-alveolar macrophage interaction. Besides confirmation of the involvement of MAPK and p53 signaling, these studies revealed the involvement of both mitochondrial and death receptor-mediated apoptosis pathways in the SCMT-macrophage interactions.

- The upstream events underlying SCMT-macrophage interaction were investigated in a lower eukaryotic model (yeast) based on a chemical genomic approach involving screening of yeast deletion library for resistance or sensitivity to macrocyclic
trichothecenes (MTs). The results showed that 9 ribosomal proteins and 5 DNA damage response-related proteins govern the MT-caused toxicity in this model.

Knocking down of one of the identified ribosomal targets (RPL8) in alveolar macrophages rescued in part the cells from SCMT-induced cell death. This is the first report on the involvement of a ribosomal protein in mediating the MT-induced apoptosis in alveolar macrophages.
References for Chapter I and V


- 154 -


