

**SYNERGISTIC ACTIVATION OF INTERLEUKIN-6 (IL-6) RELEASE BY HUMAN
LUNG FIBROBLASTS EXPOSED TO *MYCOPLASMA FERMENTANS* AND RESIDUAL
OIL FLY ASH (ROFA)**

by

Fei Gao

M.D., China Medical University, 1995

M.S., Fudan University Medical Center, 2000

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This dissertation was presented

by

Fei Gao

It was defended on

September 2, 2005

and approved by

James P. Fabisiak, PhD
Dissertation Advisor, Assistant Professor
Environmental and Occupational Health
Graduate School of Public Health
University of Pittsburgh

Bruce R. Pitt, PhD
Committee Chair, Professor
Environmental and Occupational Health
Graduate School of Public Health
University of Pittsburgh

Simon Barrat-Boyes, BVSc, PhD
Committee Member, Assistant Professor
Infectious Diseases and Microbiology
Graduate School of Public Health
University of Pittsburgh

Barry R. Stripp, PhD
Committee Member, Associate Professor
Environmental and Occupational Health
Graduate School of Public Health
University of Pittsburgh

Koichi Takimoto PhD
Committee Member, Assistant Professor
Environmental and Occupational Health
Graduate School of Public Health
University of Pittsburgh

James P, Fabisiak Ph.D.

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The adverse health consequences of air pollution are well recognized and range from minor upper respiratory system irritation to severe chronic lung disease. The identity and mechanisms of these pollutants, as well as how toxicity is influenced by additional risk factors are unclear. This study elucidates the relationship between air pollution and microbial agents and explores the mechanisms by which the two stimuli interact to cause adverse health effects with important public health relevance. *Mycoplasma fermentans* is a species of atypical bacteria with immune-regulatory properties and potential to establish chronic latent infections. Particulate matter (PM) is a complex and diverse component of air pollution associated with adverse health effects. The hypothesis of this study is that *M. fermentans* infection modulates the cellular responses induced by exposure to residual oil fly ash (ROFA), a type of PM particularly rich in metals. Using human lung fibroblasts (HLF) as an in vitro model I measured the release of the immune-modulating cytokine interleukin-6 (IL-6) as a biomarker of stress-induced cell activation after exposure to various chemical and microbial challenges alone or together. The synergistic interaction between live *M. fermentans* and ROFA to stimulate IL-6 release and gene expression in HLF was demonstrated. This effect was specific for PM that contains high amounts of water-

soluble metal and was recapitulated when NiSO₄ was substituted for ROFA. The potentiating effect of live infection was mimicked by exposure to *M. fermentans*-derived macrophage-activating lipopeptide-2 (MALP-2), a Toll-like receptor-2 agonist. Experiments with consecutive singular exposures to MALP-2 and NiSO₄ revealed that pre-treatment of cells with NiSO₄ facilitated MALP-2-induced IL-6 production, while pre-exposure to MALP-2 failed to influence the response to Ni. Facilitation of MALP-2 response by NiSO₄ depended, in part, upon Ni-induced activation of the ERK1/2 MAP kinase. These interactive effects were studied at the level of gene transcription using a series of IL-6 promoter-luciferase reporter constructs and mutants. Transcription factors NF-κB and NF-IL6 play an important role in regulating IL-6 promoter activity caused by MALP-2 and NiSO₄ co-exposure.

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1. INTRODUCTION

1.1. MYCOPLASMA AND M. FERMENTANS

Mycoplasma are a group of the smallest free-living and self-duplicating organisms with minute genomes (a total of about 500 to 2200 genes) (1). They can infect plants and animals including humans. Mycoplasma are not typical bacteria or viruses but a member of the mollicute family, having no cell wall, acting as virus-like infectious agents, and possessing complexity somewhere between viruses and bacteria. Mycoplasma utilize the host's machineries for enhanced replication and protein synthesis. They can grow in tissue fluids or in viable tissue cell cultures with / without causing tissue cell damage or pathogenicity. Therefore, mycoplasma may comprise a part of the normal mucosal flora of healthy persons, although blood, cerebrospinal, pleural, abdominal and joint fluids and bone from healthy persons are usually negative for mycoplasma. Mycoplasma can also reside intracellularly, unlike common bacteria which typically exist extracellularly. Thus, mycoplasma often evade detection by conventional laboratory diagnostic assays that use fluid samples. As mycoplasma do not always trigger an elevated white blood cell count, systemic mycoplasma infection will not show on a routine complete blood count (CBC) used to determine elevated white blood cells typically associated with a bacterial infection. In addition, they are difficult to detect by direct microbiological culture due to their slow growth and fastidious metabolic requirements. One of the most useful distinguishing features of mycoplasma is their peculiar fried-egg colony shape, consisting of a central zone of growth embedded in the agar and a peripheral one on the agar surface (2). The intracellular life cycle also allow these microorganisms to escape from the host's immunological challenges. Moreover, mycoplasma frequently alter their antigenic surface molecules and utilize

molecular mimicry to avoid the immune surveillance. Thus, these microbes are hard to detect and may be commonly present in human populations.

It has been shown that seven different species of mycoplasma are associated with various infections in humans. They are *M. pneumoniae*, *M. genitalium*, *Ureaplasma urealyticum*, *M. fermentans*, *M. pirum*, *M. hominis*, and *M. penetrans*. Mycoplasma infections have been implicated as causal triggering or complicating factors in many chronic illnesses including neurodegenerative disorders such as Amyotrophic Lateral Sclerosis (3;4). *M. pneumoniae* is the most common pathogen in lung infections among 5 to 35 year old patients. Infection with this organism remains an important cause of pneumonia and other airway disorders such as tracheobronchitis, pharyngitis and chronic asthma (5-9). This organism is also associated with extrapulmonary manifestations such as hematopoietic, joint, central nervous system, liver, pancreas and cardiovascular disorders (10-12). *M. genitalium*, which was originally isolated from urethral specimens of two men with nongonococcal urethritis, could be involved in pelvic inflammatory disease (13-15). *Ureaplasma urealyticum* is responsible for nonchlamydial, nongonococcal urethritis of males, meningitis in very-low-birth-weight infants, and chorioamnionitis (16;17). *M. pirum*, *M. hominis*, and *M. penetrans* have been proposed as human pathogens and possible cofactors in HIV infection-associated diseases (18-20). These organisms may contribute to the variation in the time from infection with HIV to the development of AIDS symptoms. Therefore, several mycoplasma species are directly involved in the induction of pathological changes. Unlike the above mentioned mycoplasma species, *M. fermentans* has been found in saliva and oropharyngeal of 45% of healthy adults and is considered to be a commensal in the human mucosal tissues. Despite this high prevalence in

healthy patients, this organism is associated with several human diseases, such as arthritis, chronic fatigue syndrome (21-24), fibromyalgia (25) and leukemia (26;27). It was also proposed to act as a human pathogen and cofactor in HIV infection-associated disorders (28;29). In addition, *M. fermentans* has been isolated from synovial tissues in patients with rheumatoid arthritis (30-32), which points to its possible implication in this clinical manifestation. Therefore, infection with *M. fermentans* may aggravate or facilitate pathological changes generated by other agents. One of the well documented effects of *M. fermentans* is its capacity to stimulate immunocompetent cells to produce pro-inflammatory cytokines or in particular cases, to lead them to cell death (33). Infection with *M. fermentans* also appears to inhibit enzymes, such as proteases and phospholipases and to increase production of oxygen radicals (33;34). Taken together, infection with *M. fermentans* may participate with other factors in the pathogenesis of various diseases. Yet, the molecular mechanisms underlying these effects remain uncertain.

1.2. MALP-2

The innate immune system relies on a vast array of pattern recognition receptors for the detection of pathogens. Pattern recognition receptors such as toll-like receptors (TLRs), bind conserved molecular structures shared by large groups of pathogens, termed pathogen-associated molecular patterns (PAMPs). PAMPs are conserved motifs that are unique to microorganisms and are essential for their metabolism and thus survival (35). They have the following characteristics. First, PAMPs are produced only by microbes and not by host cells, enabling the innate immune system to distinguish between self and nonself. Second, as PAMPs are essential for microbial survival, mutations in or loss of patterns can be lethal, and therefore these patterns are not subject

to high mutation rates. And third, PAMPs are invariant between microorganisms of a given class, which implies that only a limited number of germ line-encoded pattern recognition receptors are needed to detect the presence of a microbial infection (35). Unlike most bacteria, mycoplasma have no cell wall, and thus their lipid bilayer membrane is the only structure that regulates the interaction with the external environment. The Mollicutes membrane classically consists of two bi-lipid sheets and membrane proteins which include the integral membrane proteins and peripheral proteins. Outer peripheral proteins, also named membrane lipoproteins, are thought to include the immuno-dominant antigens of mycoplasmas and to be directly involved in the interaction with host cells (36;37). They are currently the subject of substantial interest because they are presumed to be involved in several important functions, such as interaction with host cells, invasion into host cells and avoidance of immune surveillance. Crude lipoprotein extracts, purified lipopeptides or fatty components from mycoplasmas have all been demonstrated to stimulate the production of cytokines by monocytes and to induce B-cell proliferation (37-40). Three forms of a mycoplasma lipopeptide / protein with the ability to modulate the host immune system were independently identified and named macrophage-activating lipopeptide 2 (MALP-2), P48 and M161Ag (identical to MALP-404) (41). Most lipoproteins extracted from bacteria are triacylated at the NH₂-terminal cysteine residue, molecular motif recognized by toll-like receptors (TLRs) 1 and 2. In contrast, mycoplasma-derived macrophage-activating lipopeptide 2 (MALP-2) is only diacylated. It is a 2-kDa lipopeptide originally isolated as an inducer of nitric oxide (NO) at picomolar concentrations from macrophages (39). The amino acid sequence of MALP-2 is entirely consistent with the N-terminal amino acid sequences of M161Ag and P48. It is probably derived from the larger lipoprotein M161 Ag by posttranslational cleavage (40;42). Studies shows that the amino acid

sequence of MALP-2 is *S*-(2,3-bisacyloxypropyl) cysteine-GNNDESNISFKEK, with 1mol of C_{16:0} and a further mol of a mixture of C_{18:0} and C_{18:1} fatty acid per lipopeptide molecule (43). In addition to its effect on NO, it induces chemoattractant mediators, such as macrophage inflammatory protein 1 α and 2 (MIP-1 α and 2) and monocyte chemoattractant protein 1 (MCP-1) in mouse and human cells (44). MALP-2 exhibits potent endotoxin-like activity and its lethal toxicity is comparable to that of LPS (45). Synthetic MALP-2 produces no oncogenic activity (43) and activates TLR2 and TLR6 due to its unique diacylated N-terminal (41;46-48). Coexpression of TLR2 and TLR6 is absolutely required for MALP-2 responsiveness because TLR2 and TLR6 cooperate to recognize MALP-2 and TLR6 appears to confer the ability to discriminate between the NH₂-terminal lipoylated structures of MALP-2 from other lipopeptides derived from other bacteria (47).

MALP-2 induces AP-1 and NF- κ B activity and cytokine secretion in macrophages via the activation of mitogen-activated protein kinase (MAPK) pathways (49). Its stimulatory effects on macrophages were fully comparable with those previously found with *M. fermentans* lipoproteins, suggesting that the lipopeptides derived from *M. fermentans* might constitute excellent tools for better understanding the immunomodulation and other pathogenic properties induced by these microorganisms.

1.3. RESIDUAL OIL FLY ASH AND NICKEL

Particulate matter (PM) is one of the six common air pollutants besides ozone, carbon monoxide, nitrogen dioxide, sulfur dioxide and lead that have air quality standards set by the United State EPA. It is the term for particles found in the air, including dust, dirt, soot, smoke, and liquid droplets. Particle size and composition varies widely according to their origin and generation

process. Some are directly emitted into the air from cars, trucks, buses, factories, construction sites and burning of wood, others may be formed in the air from the chemical conversion of gases. Depending on the wind and particle size, they stay in the air for different periods of time and are spread to varying distances. Particulate matter can be divided into several levels according to their size. Total suspended particles (TSP) are those with diameters less than 100 μm , inhalable particles are also called PM_{10} since they are less than 10 μm in diameter, respirable particles ($\text{PM}_{2.5}$) have a diameter less than 2.5 μm and ultrafine particles are smaller than 0.1 μm in diameter (50). Particles with a diameter of 2.5 μm or less are potentially more harmful than larger particles because they can travel over hundreds of miles from their origin and have relatively longer atmospheric lifetime than do larger particles. They penetrate more readily into cells and through tissue barriers, have greater surface area per unit of mass (toxic reactions presumably occur at the surface) and dissolve more rapidly than do larger particles, thus enhancing the bioavailability of solubilized agents.

In addition to particle size, particulate matter also can be categorized by its composition. Two widely studied particulate matters are diesel exhaust particles (DEPs) and residual oil fly ash (ROFA). DEPs are products of the combustion system of diesel engines and a known environmental pollutant. It is a complex mixture of particulate materials primarily composed of black carbon, often referred to as elemental carbon, a soiling agent. It also contains many organic compounds including polycyclic aromatic hydrocarbons (PAHs) that are potential carcinogens (51). DEPs have larger surface areas, ranging from 30 to 100 m^2 / g , on which a wide range of organic compounds are absorbed. Nearly all DEPs fall within the $\text{PM}_{2.5}$ -size range, with mass median diameters ranging from 0.05 to 0.3 μm . (52). ROFA is a combustion

product of fuel oil. Fly ash from fossil fuel combustion contributes more than 2.5×10^5 tons annually to the ambient air PM burden in the United States. Although size may vary with time, origin and other factors, ROFA is frequently less than $2.5 \mu\text{m}$ in diameter. It consists of mostly inorganic materials with variable compositions depending on the fuel grade and temperature of combustion. Compared to other ambient pollution particles, ROFA is chemically complex and includes sulfates, silicates, carbon-and nitrogen-containing compounds, contaminants of the fuel, and additives (53). One of the outstanding characteristic of ROFA is that it is rich in transition metals, such as iron, zinc, magnesium, vanadium, copper and nickel (54;55). Rich in metals, with little in the way of organic components, make it a particularly useful surrogate for ambient air PM to test how metals mediate the biologic effects of air pollution particles. ROFA has been used in a number of *in vitro* and *in vivo* toxicologic studies (56-59). The soluble transition metals (especially metal sulfates) play an important role in mediating biological effects of ROFA (60-64). Copper ions may cause some of the biologic effects of inhaled particulate air pollution in the Provo region of the United States, and may provide an explanation for the sensitivity of asthmatic individuals to Provo particulates that has been observed in epidemiologic studies (65;66). Some studies also found that vanadium, another transition metal rich in ROFA, mimicked the effects of intact ROFA for most of the production and release of inflammatory mediators by airway epithelial cells (60;67). Potential mechanisms whereby ROFA induces its injurious effects may involve development of an oxidative burden generated by the high content of transition metals in ROFA and by depletion of antioxidant defenses in target cells and tissues. Thus, the oxidant-sensitive transcriptional factors such as nuclear factor κB (NF- κB) and activator protein-1 (AP-1) can be activated (68;69). In human airway epithelial cells, NF- κB was proposed to mediate the expression of interleukin-6 (IL-6) in response to ROFA exposure (70).

Nickel is another transition metals found in ROFA released from power plants that is associated with adverse health effects. In the lower airways, nickel exposure is associated with immunological sensitization, epithelial dysplasia, asthma, lung cancer, and fibrosis (71-74). At the cellular level, nickel stimulates signaling cascades in airway epithelium that increase expression of inflammatory cytokines like IL-8, the profibrotic gene, plasminogen activator inhibitor-1, and genes involved in hypoxic responses (75). Also, the existence of multiple metals may provoke some synergistic effects on exposed cells (75).

1.4. INTERLEUKIN-6

IL-6 was first cloned during an effort to isolate and characterize the virus-induced protein interferon- β (76). It belongs to the family of IL (interleukin)-6-type cytokines which comprises IL-6, IL-11, LIF (leukaemia inhibitory factor), OSM (oncostatin M), CNTF (ciliary neurotrophic factor), CT-1 (cardiotrophin-1) and CLC (cardiotrophin-like cytokine) (77). IL-6-type cytokines exert their action via the signal transducers gp130 (glycoprotein 130 KDa), LIF and OSM receptors leading to the activation of the JAK / STAT (Janus kinase / signal transducer and activator of transcription) and MAPK cascades (78;79). IL-6 is well recognized for its role in the acute phase inflammatory response, which is characterized by production of a variety of hepatic proteins termed acute phase proteins (80). In addition to its role in the acute phase response, IL-6 is important for the development of specific immunologic responses. It may be an important mediator of several infectious and autoimmune diseases (81-83). These include human immunodeficiency virus, rheumatoid arthritis, Castleman's disease, and the paraneoplastic symptoms associated with cardiac myxoma (84-87). IL-6 production has been documented in a seemingly endless variety of lymphoid and nonlymphoid cells, such as T-cell lines (88;89), B

cells, fibroblasts (90), endothelial cells (91), keratinocytes (92), monocytes / macrophages (88;93), mast cells (94;95), and a variety of tumor cell lines (96). Among the proinflammatory cytokines, IL-6 is considered to contribute to the initiation and extension of inflammatory process. It may play a role in regulating the cellular recruitment in the lungs during an inflammatory response, with dramatic consequences for the cellular profile in the bronchoalveolar lavage and the subsequent fibrosis (97). In vitro infection of human lung fibroblasts with *M. fermentans* represents a potent stimulus for the production of inflammatory cytokines IL-6 (98). Recent studies also found that endogenous IL-6 plays a critical role in the progress of lung inflammation / injury associated with exposure to air pollutants in various animal and cell culture experiments (99-101). Since IL-6 is considered a critical and interesting cytokine in inflammatory process, it has been used as a prototype cytokine to study the possible inflammatory response caused by *M. fermentans* and particulate matters by human lung fibroblasts.

1.5 HYPOTHESIS

Since simultaneous microbial and chemical stimuli would be commonplace in the real world situation, it is important to study the interaction between the two divergent stimuli. Here, I hypothesize that microorganisms such as *M. fermentans* modulate cellular response to environmental stimuli such as atmospheric particulate matters to provoke an inflammatory response greater than the sum of the two stimuli. Unfortunately, there is no well-established animal model for the study of *M. fermentans* infection, so in vitro approach using co-exposure to *M. fermentans* or its derived lipopeptide MALP-2 and particles is the main method used to investigate the interaction between those two stimuli. To study the immune-modulating effect of *M. fermentans*, lung fibroblasts were used for the study instead of phagocytic bactericidal cells

like macrophages in order to achieve a chronic infection. Also, previous lab studies found that human lung fibroblasts isolated from lung transplant patients during the follow up phase have high *M. fermentans* infection rate. Those infected cells released high amount of immunomodulating cytokine such as IL-6 and tumor necrosis factor (TNF) (98) . To further study the interaction between *M. fermentans* and PM, human lung fibroblasts were used and immunomodulating cytokine IL-6 was measured as the endpoint.

2. SPECIFIC AIMS

The overall goal of this project is to characterize the synergistic interactions between *M. fermentans* and residual oil fly ash (ROFA) to amplify IL-6 production in human lung fibroblasts and identify the mechanism of this synergistic reaction.

The specific aims of the project are:

1) To establish and characterize the synergistic interaction of *M. fermentans* and ROFA to stimulate the production of the immune-modulating cytokine IL-6 by HLF.

Human lung fibroblasts were deliberately infected or uninfected with *M. fermentans* and then exposed to ROFA fine particles at various concentrations. IL-6 release was determined at various times following exposure to ROFA. Particulate matters from various origins were compared for their ability to stimulate IL-6 release. The ability of the *M. fermentans*-derived lipopeptide MALP-2 to substitute for live infection with the microorganism was tested. Water soluble and insoluble fractions of ROFA, as well as a number of its known metal constituents, were compared for their ability to amplify IL-6 release.

2) To explore the involvement of MAPK pathway in the IL-6 production by MALP-2 and NiSO₄ from HLF.

Time course of NiSO₄ and MALP-2 co-exposure suggested that one stimulus may facilitate the subsequent response to the other in producing IL-6. A pre-treatment paradigm was used to test for conditioning effects of one compound to influence IL-6 release by the other chemical. MAPK pathway specific inhibitors were used to explore which pathways play an important role in regulating IL-6 production caused by NiSO₄ and MALP-2. NiSO₄ was found to activate

ERK1/2 and JNK in HLF and its facilitation effects on MALP-2 in producing IL-6 can be partially abolished by ERK1/2 but not JNK specific inhibitor. ERK1/2 specific inhibitor PD98059 also partially abolishes IL-6 production by NiSO₄ and MALP-2 co-exposure.

3) To determine the effects of *M. fermentans-derived* macrophage activating-lipopeptide (MALP-2) and NiSO₄ on IL-6 gene transcription.

Luciferase reporter gene expression driven by the promoter region of human IL-6 promoter gene was used to determine transcriptional activation of the gene. Site-directed mutagenesis was used to generate constructs that lacked binding sites for NF-κB, NF-IL6 or AP-1. Treatment with NiSO₄ and MALP-2 alone or in combination were used to test their ability to affect IL-6 promoter activity.

3. RESULTS

3.1. Chapter 1

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Microbial Stimulation by *Mycoplasma fermentans* Synergistically Amplifies IL-6 Release by Human Lung Fibroblasts in Response to Residual Oil Fly Ash (ROFA) and Nickel

Fei Gao, Aaron Barchowsky, Antonia A. Nemecek and James P. Fabisiak

Department of Environmental and Occupational Health, Graduate School of Public Health,
University of Pittsburgh, Pittsburgh, Pennsylvania 15260

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3.1.3. Notes

To whom correspondence should be addressed at Department of Environmental and Occupational Health, Graduate School of Public Health, University of Pittsburgh, 3343 Forbes Avenue, Pittsburgh, PA 15237. Fax: (412) 383-2123. E-mail: fabs@pitt.edu .

3.1.4. Abstract

Mycoplasmas (MP), such as the species *M. fermentans*, possess remarkable immuno-regulatory properties and can potentially establish chronic latent infections with little signs of disease. Atmospheric particulate matter (PM) is a complex and diverse component of air pollution associated with adverse health effects. We hypothesized that MP modulate the cellular responses induced by chemical stresses such as residual oil fly ash (ROFA), a type of PM rich in transition metals. We assessed the release of interleukin-6 (IL-6), a prototypic immune-modulating cytokine, in response to PM from different sources in human lung fibroblasts (HLF) deliberately infected with *M. fermentans*. We found that *M. fermentans* and ROFA together synergistically stimulated production of IL-6 compared to either stimulus alone. Compared to several other PM, ROFA appeared most able to potentiate IL-6 release. The potentiating effect of live MP infection could be mimicked by *M. fermentans*-derived macrophage-activating lipopeptide-2 (MALP-2), a known Toll-like receptor-2 agonist. The aqueous fraction of ROFA also contained potent IL-6 inducing activity in concert with MALP-2, and exposure to several defined metal salts indicated that Ni and, to a lesser extent V, (but not Cu) could synergistically act with MALP-2 to induce IL-6. These data indicate that microorganisms like MP can interact with environmental stimuli such as PM-derived metals to synergistically activate signaling pathways that control lung cell cytokine production and, thus, can potentially modulate adverse health effects of PM exposure.

Key Words: residual oil fly ash; mycoplasma; interleukin-6; particulate matter; MALP-2; human lung fibroblasts; nickel; transition metals; innate immunity; cytokines.

3.1.5. Introduction

Atmospheric particulate matter smaller than 10 μm (PM_{10}) is listed as one of the six "criteria pollutants" for which the USA EPA has set air quality standards. Epidemiological studies show that PM is associated with a variety of adverse health effects (102-105). Particulate matters arise through variety human and natural activities and thus give rise to a myriad of PM types of diverse chemical composition and physical properties. While the biological effects of PM differ widely according to their size, composition, and quantity, certain entities including metals (60;67), organic aromatic hydrocarbons (106), and microbial-derived biomolecules (107) have all been specifically implicated in the toxic effects. Numerous *in vivo* and *in vitro* studies reveal that PM exposure results in activation of inflammatory responses accompanied by elaboration of immune-modulating cytokines (108-110). Residual oil fly ash (ROFA), a combustion product of fuel oil rich in transition metals, possesses potent biological effects (53) and has been useful as a model particulate to study how metals mediate the biologic effects of air pollution particles.

Mycoplasmas (class *Mollicutes*) are a class of cell wall-free bacteria that represent the simplest self-replicating microorganisms known (111). Due to their extremely small genome (0.58–2.20 Mb) and their limited metabolic options for replication and survival, these fastidious microorganisms have adopted a strict parasitic lifestyle in intimate relationship with a variety of animal and human hosts. With the exception of *M. pneumoniae* (112), these microorganisms are not usually considered severely pathogenic; however, they may modulate host defense mechanisms (113;114). *Mycoplasma* spp. are commonly found on various mucosal surfaces of healthy individuals, however, systemic dissemination and opportunistic growth of these

microorganisms has been detected in patients with a variety of chronic inflammatory conditions including asthma (9;115). *M. fermentans* serves as a good example of these "stealth" pathogens (21;29;116-118).

We have previously observed that several early passage fibroblast cell lines derived from human lung were infected with *M. fermentans* (98). Infection induced production of immune-modulating cytokines such as IL-6 and strongly potentiated the ability of known inducers such as TNF- β . Since exposure to PM, in general, and ROFA, in particular, can serve as potent stimuli for cytokine-dependent inflammatory responses *in vitro* and *in vivo*, we sought to test the hypothesis that *M. fermentans* exacerbates release of immune-modulatory factors induced by these chemical agents. We used our model of human lung fibroblasts (HLF) deliberately infected with *M. fermentans* to examine the ability of various components of PM to induce the induction of a prototypic immuno-modulatory marker cytokine, IL-6. *M. fermentans* and ROFA synergistically interacted to increase the production of IL-6. These effects were mimicked with concurrent exposure to the Toll-like receptor-2 (TLR-2) agonist, *M. fermentans*-derived macrophage-activating lipopeptide-2 (MALP-2), and nickel. Thus, exposure to microbial-derived agents can strongly determine the cellular response to specific chemical stresses and warrants that the microbial ecology be taken into consideration in the risks and mechanisms of toxicity posed by atmospheric pollutants such as particulate-derived metals.

3.1.6. Material and Methods

Materials

Cell culture medium, fetal bovine serum (FBS), and TRIzol™ were from Invitrogen (Gaithersburg, MD). Tissue culture plasticware was from Falcon (Becton-Dickinson, Franklin Lakes, NJ). SP-4 mycoplasma growth media (119) was formulated with mycoplasma broth base, peptone, tryptone, yeastolate, yeast extract (all from Becton-Dickinson Microbiological Systems, Sparks, MD), phenol red (Invitrogen), CMRL-1066 media, and Penicillin G (Sigma-Aldrich Chemical, St. Louis, MO). Calf thymus DNA, Hoechst 33258, and various metal salts were from Sigma (St. Louis, MO). Low endotoxin bovine-serum albumin (BSA) was from Intergen (Purchase, NY) and *M. fermentans*-derived macrophage-activating lipopeptide-2 (MALP-2) was from Alexis Biochemicals (San Diego, CA). Bradford protein assay reagent was from Bio-Rad (Hercules, CA). Murine leukemia reverse transcriptase, RNase inhibitor, deoxynucleotides, oligo-dT and Taq DNA polymerase were from Promega (Madison, WI). Preparations of PM were provided by Dr. Andrew Ghio, U.S. EPA. ROFA was collected by Southern Research Institute (Birmingham, AL) downstream from a cyclone scrubber at a power plant in FL burning a low sulfur #6 oil (120). Other PM types included urban dust collected from Dusseldorf Germany (Dussel), volcanic ash from Mt. St. Helens (MSH), and an aqueous extract of PM collected from Provo Valley near Salt Lake City, Utah (SLC). The chemical and physical characteristics of these particles have been described in detail (107;120-122). The ROFA particles were devoid of endotoxin activity as measured by limulus amoebocyte assay.

Cell culture

Human lung fibroblasts (HLF) were isolated as outgrowths from explanted surplus transbronchial biopsy tissues obtained during routine follow-up bronchoscopy of lung transplant recipients as previously described (98) in accordance with a protocol approved by University of Pittsburgh Institutional Review Board. The individual cell lines used here were recovered from frozen stocks prepared at passage three and used for experiments over no more than eight additional subcultures. Greater than 95 % purity of fibroblasts was determined by positive immunohistochemical staining for vimentin and negative staining for cytokeratin A3 or Factor VIII. Cells were maintained in Minimal Essential Medium (MEM) supplemented with FBS (10 %, final concentration), glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg / ml), and Fungizone (1.25 µg / ml) in a humidified incubator at 37 °C with 5 % CO₂ / 95 % air. All cultures were negative for mycoplasma as determined by fluorescent microscopy using Hoechst 33258 dye (123) prior to the deliberate introduction of *M. fermentans*.

Isolation and culture of M. fermentans

M. fermentans was isolated from previously described de novo infected HLF cell lines (98) by inoculating 50 ml of SP-4 media (119) with 10 ml of spent tissue culture medium obtained from MP-infected HLF. Cultures were incubated in airtight flasks at 37 °C until a red to yellow color change was observed indicative of microbial growth. Aliquots of MP cultures were cryopreserved by addition of 0.8 ml of culture to 0.2 ml glycerol and freezing at -80 °C. The total amount of MP was quantified by fluorometric determination of DNA content using a modification of Hoechst 33258 assay (124). The amount of viable organisms recovered from the frozen stocks was determined for each infection by determining the number of color changing

units (ccu) measured with a limiting dilution assay (125). The isolated strain of MP showed classical "fried-egg" morphology typical for the genus when grown on solid media and PCR positivity using *M. fermentans* sequence-specific primers (data not shown). In addition, the *M. fermentans* species was verified using a species-specific monoclonal antibody. All experiments presented here utilized a single strain of *M. fermentans* derived from one infected cell line.

In vitro infection with M. fermentans

Uninfected HLF were seeded into T-75 flasks (6×10^5 cells / flask) and one P60 dish (2×10^5 cells / dish) and incubated for 24 h. At the time of infection, MP was rapidly thawed, centrifuged $12,000 \times g$ for 15 min and washed twice with 0.25 M NaCl. Final pellet was resuspended in complete tissue culture, and each T-75 flask and P60 dish received 450 ng or 150 ng of mycoplasma DNA, respectively. Uninfected control cells were set up under identical conditions but did not receive mycoplasma and were maintained in a separate incubator. At 4 days post-infection cells were trypsinized, counted, and seeded into appropriate plates for experiments. Mycoplasma infection was verified by staining the P60 dish with Hoechst 33258 dye. Based on organism load expressed as ccu / ml and DNA content these conditions represent the introduction of ~100 viable organisms and ~300 total organisms per cell.

Cell viability

Cell viability was measured by quantifying the reduction of the fluorogenic dye, Alamar Blue (Biosource, Camarillo, CA). Briefly, MP-infected or uninfected cells were seeded into 24-well plates (4×10^4 / ml) and allowed to attach for 24 h. Cells were then exposed to various concentrations of ROFA for 24 h. Media was then removed, cells washed once with PBS, and

media replaced with 0.9 ml serum-free MEM. Alamar Blue (0.1 ml of a 10 % solution made in serum-free MEM) was then added to each well, and cells returned to the incubator for 3 h. Fluorescence in each well was measured using a Cytofluor 2300 fluorescence plate reader using excitation 530 ± 25 nm and emission 590 ± 25 nm.

In vitro exposures and IL-6 measurement

M. fermentans-infected cells and uninfected cells were seeded into either 6-well plates ($3\text{--}4.5 \times 10^5$ cells / 3 ml / well) or 24-well plates ($0.6\text{--}1 \times 10^5$ cells / 1 ml / well) and cultured for 48 h. Media was removed and replaced with the same volume of serum-free media containing 0.1 % BSA with or without various stimuli. Conditioned media was collected at indicated time points and stored at -80 °C until further analysis. IL-6 content of conditioned media was measured using a specific enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. For experiments in 6-well plates, IL-6 content was normalized to the producing cell number measured by nuclei counts obtained after lysis in 0.1% citric acid / 0.1 % crystal violet (98). For experiments in 24-well plates, IL-6 was normalized to the DNA content of the monolayer measured using Hoechst 33258 fluorescence (124).

Measurement of cytokine mRNA

Total cellular RNA was isolated using TRIzol™ reagent (Invitrogen, Gaithersburg, MD) according to the manufacturer's instructions. cDNAs were generated from 0.5 µg total RNA by reverse transcription in a 30 µl reaction mixture containing Ambion 103 first strand buffer, 10 U RNase inhibitor, 0.33 mM each dNTP, 1.7 mM oligo dT, and 100 U M-MLV reverse

transcriptase. Reactions were incubated at 44 °C for 60 min in a MJ Research PTC-100 thermocycler. cDNA was stored at -20°C until further analysis. Specific primer pairs for IL-6 (forward 5'-GCCCAGCTATGAACTCCTTCTC; reverse 5'-GACTTGTCATGTCCTGCAGCC), IL-8 (forward 5'-ATGACTTCCAAGCTGGCCGTGGCT; reverse 5'-TCTCAGCCCTCTTCAAAAATTCTC) and β -actin (forward 5'-GGGACCTGACCGACTACCTC; reverse 5'-GGGCGATGATCTTGATCTTC) were used to amplify the specific cDNAs. Specific cDNAs were amplified using 5 μ l aliquots cDNA mixed with 1.25 μ l of IL-6, IL-8, or β -actin forward and reverse primers, 2.5 U of Taq DNA polymerase, and 0.125 mM dNTP in Ambion complete PCR buffer in a final volume of 50 μ l. PCR reactions were carried out for 20 s at 95 °C, 30 s at 55 °C, and 40 s at 72 °C for 19 cycles for β -actin or 24 cycles IL-6 and IL-8. The number of cycles was demonstrated to be within the linear amplification range for each product. PCR products were either detected on 2 % agarose gels stained with ethidium bromide or quantified in real-time fashion during the PCR amplification using the double-strand DNA fluorescent dye PicoGreen at 430 nm emission and 525 nm excitation. IL-8 and IL-6 mRNA expression were normalized to the housekeeping gene β -actin by determining the ratio of the IL-8 or IL-6 fluorescent signals to that for β -actin.

Statistical analysis

Data presented are expressed as mean \pm SEM collected from at least three or four individual experiments for studies employing pooled HLF or cells derived from individual donors, respectively. Comparisons were made using a one-way ANOVA followed by appropriate group comparisons such as Dunnett's multiple comparison to control or Bonferonni's correction for multiple *t*-tests. Significance of response to a range of concentrations to single agents was

determined by a test for linear trend across the applied concentrations. To control for variability between multiple individually derived cell lines repeated measures ANOVA and post hoc tests were applied with pairing of data collected within a specific cell line. When variability between cell lines precluded the assumption of a normal distribution, nonparametric Wilcoxon signed rank tests were used to compare groups. To compare observed dose-response relationships with combined stimuli to a theoretical additive model, concentrations were transformed to log scale, and a predicted additive response was derived as the algebraic sum to similar concentrations of each agent alone. Log-dose response curves were subjected to linear regression and the slopes compared by analysis of covariance. Statistical analyses were performed using GraphPad PRISM™, version 3.0 (GraphPad™ Software, San Diego, CA), and differences were considered significant at the $p < 0.05$.

3.1.7. Results

***M. fermentans* and ROFA Synergistically Stimulate IL-6 Release from HLFs**

To explore possible interactions between ROFA and *Mycoplasma fermentans* (MP), I first exposed MP-infected and uninfected HLF to various concentrations of ROFA for 24 h. Production of the immune-modulating cytokine IL-6 during this period was then measured (Fig. 1). In the absence of ROFA, IL-6 release in uninfected cells were 54 ± 11 pg / 10^5 cells and about 5-fold greater in cells infected with MP (445 ± 127 pg / 10^5 cells). Exposure of uninfected cells to ROFA alone produced a dose-dependent increase in IL-6 release that, at the highest dose tested (603 ± 141 pg / 10^5 cells at 50 μ g ROFA / ml), approximated that seen in the presence of

MP alone. When MP-infected cells were exposed to ROFA, however, the response was dramatically enhanced even at the lowest concentration of ROFA used (2 $\mu\text{g} / \text{ml}$), with IL-6 production ranging from 21 to 50 times greater than the basal production seen in uninfected cells. In order to more clearly characterize the synergistic nature of this interaction, the observed dose-response curve to ROFA in MP-infected cells was compared to that predicted by an additive model for these two stimuli (IL-6 release from MP alone + IL-6 released by each concentration of ROFA applied to uninfected cells). Figure 1B shows the observed and predicted log-dose response curves. Linear regression of each line was performed and comparison revealed a highly significant difference between the slopes of the observed and predicted lines ($p < 0.02$). Thus, the observed response to ROFA plus MP was always greater than that predicted in an additive model, and this difference grew in magnitude as the concentration of ROFA increased. This represents a synergistic interaction between these two stimuli.

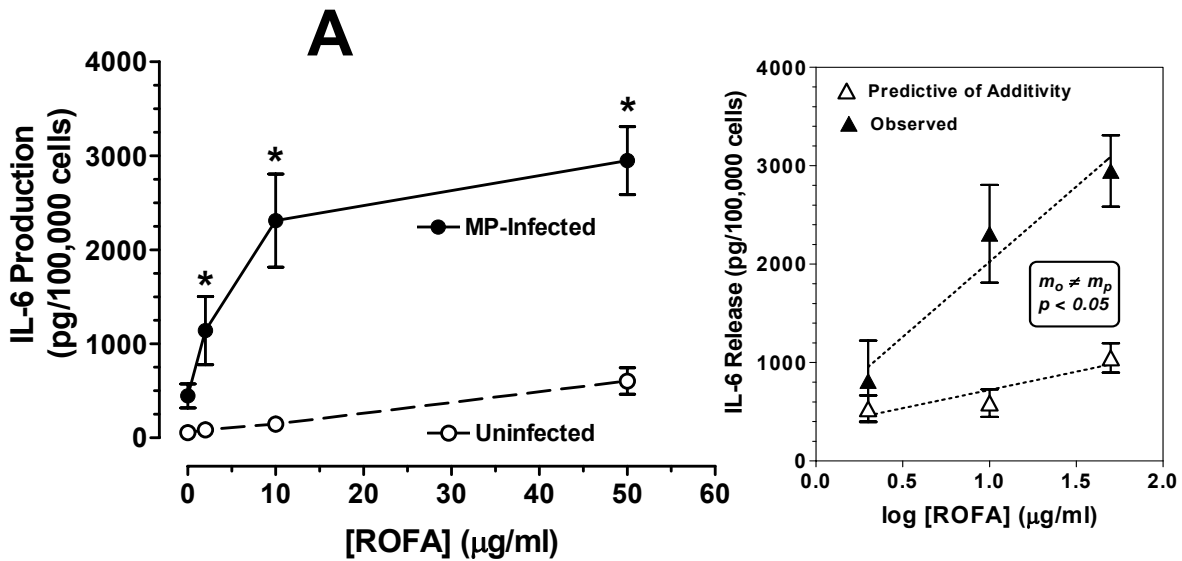


Figure 1: *M. fermentans* synergistically amplifies the ability of ROFA to induce IL-6 production in human lung fibroblasts (HLF).

I also measured the viability of HLF after exposure using the vital reduction of Alamar Blue. The LD₅₀ was approximately 100 µg / ml and did not appear to be altered by MP-infection, nor was it influenced by the presence or absence of 10 % FBS during the ROFA exposure period (data not shown). The observed LD₅₀ was considerably higher than concentrations sufficient to produce a synergistic effect of IL-6 production (Fig. 1). Thus, the synergistic interactions between ROFA and MP on induction of IL-6 production do not occur in the context of differential sensitivities to the overall toxic effects of ROFA.

Comparison of ROFA to PM from Other Sources

I next examined whether other types of PM might similarly augment IL-6 release from MP-infected cells. I measured IL-6 release during a 24-hour exposure of MP-infected and control uninfected HLF to equivalent concentrations (20 µg / ml) of urban dust collected from Dusseldorf, Germany (Dussel); aqueous extract of PM obtained from Provo Valley near Salt Lake City, Utah (SLC); Mt. St. Helens volcanic ash (MSH); as well as ROFA. Figure 2A shows that 20 µg / ml ROFA significantly increased IL-6 release about four-fold above that seen in untreated uninfected cells. In contrast, PM derived from other sources had little effect on IL-6 release at this same concentration. The slight increase observed with SLC did not reach statistical significance. When these same exposures were carried out on MP-infected cells, it was again observed that the combination of ROFA nearly tripled the release of IL-6 relative to untreated MP-infected cells alone (Fig. 2B). In keeping with the synergistic response, it is important to note that the amount of IL-6 release seen with the combination of ROFA plus MP is nearly 20-fold and 100-fold greater than that seen in uninfected cells in the presence and absence

of ROFA, respectively. Figure 2B also shows that the other PM types at this concentration lacked sufficient ability to induce IL-6 in MP-infected cells similar to uninfected cells. Thus, the observed potency of ROFA to induce IL-6 release and interact with MP appears to arise from some unique property of this PM or relative enrichment of some chemical component compared to other PM.

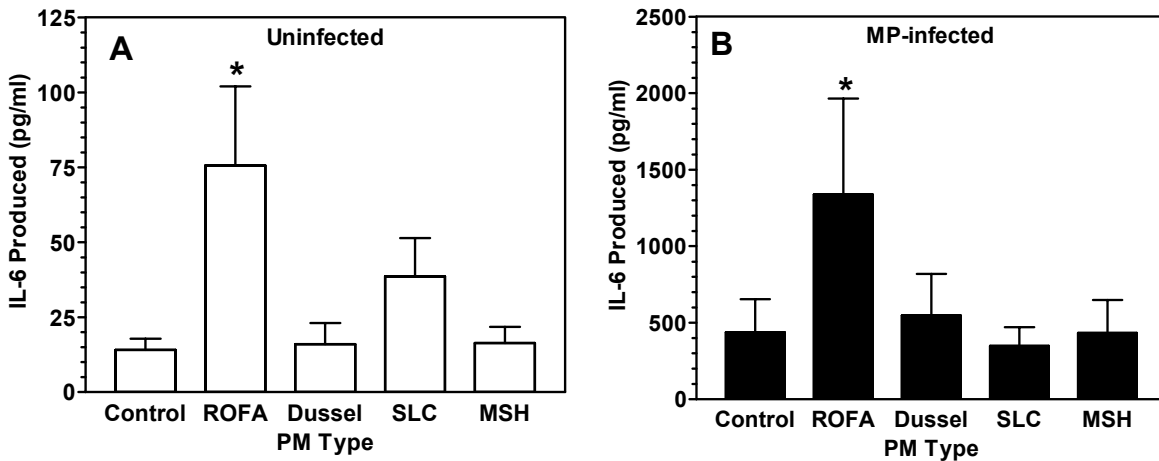


Figure 2: Comparison of various PM types on IL-6 production in control and MP-infected HLF. Uninfected control (panel A) and *M. fermentans* infected cells (panel B) were exposed to various PM types.

***M. fermentans*-Derived 2 kDa Lipopeptide (MALP-2) Mimics the Effect of *M. fermentans* to Synergistically Interact with ROFA and Stimulate IL-6 Release**

I next sought to further define the signaling pathways that might be involved in IL-6 production during live infection with MP and hypothesized that activation of innate immune responses via

activation of specific Toll-like receptor pathways could play a role. Activation of TLR-4 initiates the well-described induction of inflammatory cytokines by macrophages and other cells in response to lipopolysaccharide (LPS) derived from gram-negative bacteria cell walls. Mycoplasma, however, lack a characteristic cell wall and hence are devoid of endotoxin. Several reports demonstrated that membrane lipoproteins derived from *M. fermentans* exhibit macrophage-stimulatory activities and induce the production of proinflammatory cytokines (43;126). Macrophage-activating lipopeptide-2 (MALP-2), a 14 amino acid N-terminal fragment containing a unique diacylated cysteine, has been characterized and shown to induce the production of proinflammatory cytokines, as well as nitric oxide, via specific interaction with TLR-2 and TLR-6 (42;48). I wondered if MALP-2 could recapitulate the effects of live infection and synergistically interact with ROFA to induce cytokine release. To test this possibility, I exposed HLFs to various concentrations of MALP-2 in the presence and absence of ROFA (40 $\mu\text{g} / \text{ml}$). Conditioned media was collected after both 24 h exposure (Fig. 3A) and 48 h exposure (Fig. 3B) and analyzed for IL-6 content. Exposure of HLF to MALP-2 alone induced a moderate concentration-dependent increase in IL-6. The effect of MALP-2 alone was first observed at 300 pg / ml and was further increased at 600 pg / ml . The amount of cytokine released relative to unstimulated cells after 48 h exposure to the highest concentration of MALP-2 alone (385 ± 277 with MALP-2 vs. 9 ± 4 $\text{pg} / \mu\text{g}$ DNA unstimulated) was approximately 2-fold higher than that observed after only 24 h (150 ± 105 vs. 4 ± 1 $\text{pg} / \mu\text{g}$ DNA), suggesting a fairly linear increase in IL-6 accumulation within the medium over this time. ROFA itself in the absence of MALP-2 had a very minimal effect on IL-6 release that reached 11 ± 4 and 45 ± 21 at the 24 (Fig. 3A) and 48 (Fig. 3B) hour time points, respectively. The inclusion of MALP-2 in combination with ROFA, however, markedly stimulated the release of IL-6. The between cell line variability was

sufficiently high to preclude a normal distribution of our data points. At each concentration of MALP-2, however, each of the six cell lines tested showed an enhanced response to combination of ROFA and MALP-2 ($p < 0.05$, Wilcoxon's signed rank test). At 24 h (Figs. 3A) and 48 h (Figs. 3B), the highest concentration of MALP-2 (600 pg / ml) in combination with ROFA released three to six fold more IL-6, respectively, compared to the same concentration of MALP-2 alone. I also compared the observed MALP-2 + ROFA response to values predicted by an additive model by summing the responses seen to ROFA alone and MALP-2 alone for each cell line (dotted lines in Fig. 3A and 3B). At both time points the response observed with both 300 and 600 pg / ml MALP-2 in combination with ROFA was significantly more than predicted from an additive model. To further demonstrate the synergistic nature of these interactions, I analyzed log-dose response relationships by linear regression using those concentrations of MALP-2 having a demonstrable effect (100–600 pg / ml) (Fig. 3A and 3B, insets). The slope of the observed response to MALP-2 plus ROFA was three-fold and seven-fold greater at the 24 and 48 h time points, respectively, compared to that predicted by an additive model; thus it is clear that the addition of ROFA dramatically synergized the effect of MALP-2 to induce IL-6.

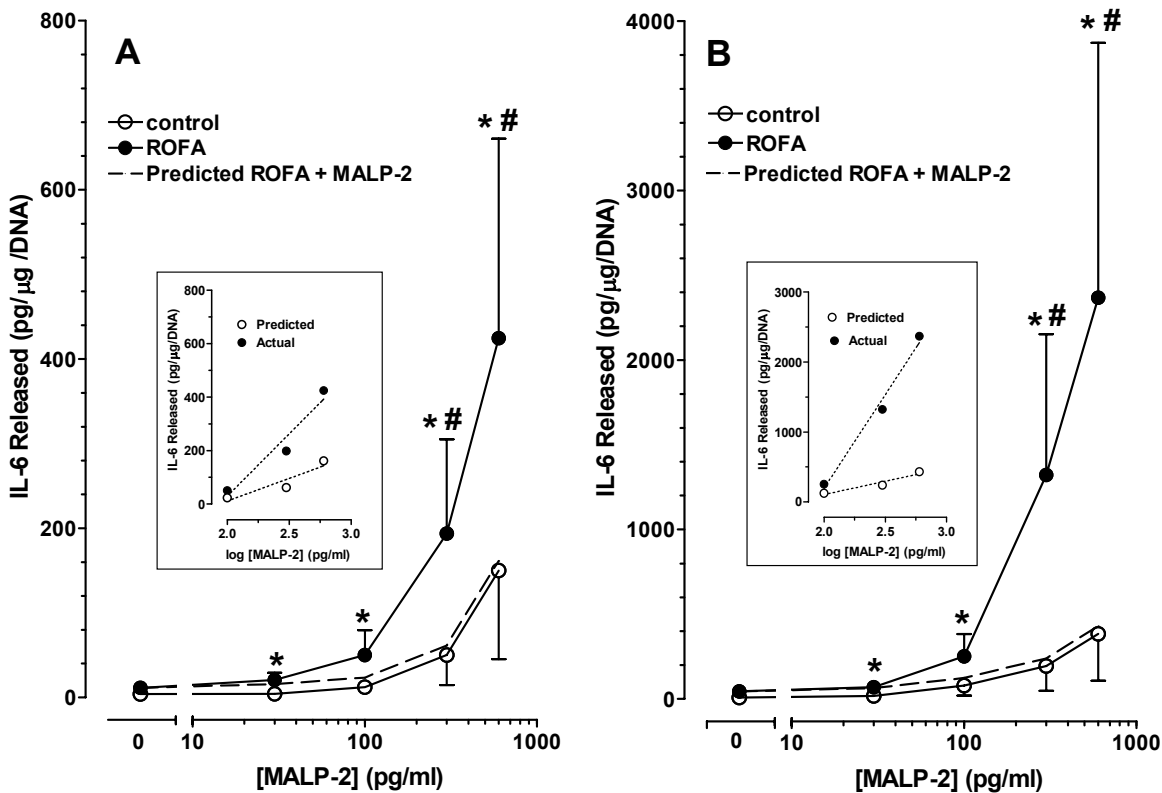


Figure 3: *M. fermentans*-derived macrophage activating peptide (MALP-2) and ROFA synergistically stimulate IL-6 release from HLF.

The Effect of ROFA Is Mimicked by Its Water-Soluble Fraction and Specific Metal Constituents

Airborne products derived from the combustion of fossil fuels are complex mixtures containing an elemental carbon core with adsorbed organic hydrocarbons and various metal salts and oxides depending on the origin of the fuel. ROFA is extremely rich in its metal content, most notably vanadium (V), iron (Fe), and nickel (Ni), among others. Many studies have implied that ROFA's soluble metallic components are responsible for its bioactivity. To first evaluate the effect of

soluble components in ROFA, we prepared a standard solution of ROFA particles in serum-free tissue culture media as used in the experiments described above. Half of this mixture was filtered through a 0.22 μm filter to essentially obtain an aqueous extract of ROFA. I exposed HLF to either untreated ROFA (20 and 40 $\mu\text{g} / \text{ml}$) or the soluble fraction corresponding to the same amounts of whole ROFA. Table 1 shows the IL-6 response to soluble ROFA (filtrate) and total ROFA in the presence and absence of MALP-2 (600 pg / ml) after treatment for 24 and 48 h. As expected, exposure of the cells to ROFA or MALP-2 alone has very minimal effects of IL-6 release at either time point. When cells were exposed to the lowest concentration of total or soluble ROFA in combination with MALP-2 there was no enhancement at the 24 h time point, but by 48 h cells exposed to either total ROFA or soluble ROFA in the presence of MALP-2 released approximately ten-fold more IL-6 than cells exposed to any stimulus alone. With this small number of observations the response observed with 20 $\mu\text{g} / \text{ml}$ ROFA filtrate plus MALP-2 failed to reach statistical significance, however; no significant difference was detected compared to the response seen with the same concentration of total ROFA plus MALP-2. When a higher concentration of ROFA (40 $\mu\text{g} / \text{ml}$) was used, significant synergy was observed between MALP-2 and total ROFA at both time points. The response seen with 40 $\mu\text{g} / \text{ml}$ ROFA filtrate plus MALP-2 was about 50 % and 75 % of that seen with total ROFA plus MALP-2 at the 24 and 48 h time points, respectively. Thus, these results show that soluble components of ROFA can synergistically interact with MALP-2 in producing IL-6 in a manner similar to that seen with exposure of HLF to the entire particle. Although the synergy seen with ROFA filtrate is slightly weaker than ROFA as a whole, there is no statistically significant difference between intact ROFA and its soluble components.

Table 1: Water-Soluble Components of ROFA Mimic the Intact Particle to Induce IL-6 in HLF in the Presence of MALP-2

Treatment	IL-6 Produced (pg/ μ g DNA)	
	24 h	48 h
Untreated	3 \pm 0.4	21 \pm 13
MALP-2 alone (600 pg/ml)	33 \pm 14	64 \pm 22
20 μ g/ml ROFA		
Total alone	11 \pm 4	57 \pm 25
Total + MALP-2	38 \pm 15	773 \pm 249 ^a
Filtrate alone	11 \pm 7 ^b	39 \pm 29
Filtrate + MALP-2	36 \pm 10	544 \pm 281
40 μ g/ml ROFA		
Total alone	25 \pm 15	91 \pm 39
Total + MALP-2	499 \pm 235 ^a	1097 \pm 338 ^a
Filtrate alone	39 \pm 30	42 \pm 13
Filtrate + MALP-2	196 \pm 71	836 \pm 185 ^a

Since ROFA's soluble metallic components may be responsible for this synergy, I next sought to define which metal elements found in ROFA might alone synergize with MALP-2 to induce IL-6 release from HLFs. The ROFA preparation used in the previous experiments contains high levels of vanadium, nickel, iron, and copper. Therefore, I first treated HLFs with three concentrations of various metallic salts in the presence or absence of 600 pg / ml MALP-2 (Fig. 4). The most dramatic interaction for stimulation of IL-6 production was seen with NiSO₄ and MALP-2. Exposure of HLF to 200 μM NiSO₄ by itself produced about a ten-fold increase in IL-6 production and was about the same magnitude as that seen with MALP-2 alone. The combination of MALP-2 along with 200 μM Ni, however, stimulated IL-6 release to a level over 5 times that seen with either stimulus alone and over 50 times greater than that observed in unstimulated cells. Exposure of cells to lower concentrations of Ni alone (2 and 20 μM) was ineffective and did not modulate the response to MALP-2. Exposures of HLF to similar concentrations of CuSO₄ had no stimulatory effect by themselves and, in fact, decreased the response to MALP-2 alone. The two vanadium compounds, vanadyl (V⁴⁺) sulfate (VOSO₄) and sodium vanadate (V⁵⁺) (Na₃VO₄) induced small increases in cytokine production when applied at 200 μM in the absence of MALP-2 that were similar to that seen with the same concentration of Ni alone. While the addition of MALP-2 to 200 μM Na₃VO₄ further increased IL-6 release, the observed response appeared was much less than that observed with Ni + MALP-2. The response to 200 μM VOSO₄ in combination with MALP-2 appeared only slightly greater than the additive effects of each stimulus alone and also was substantially less than that seen with nickel.

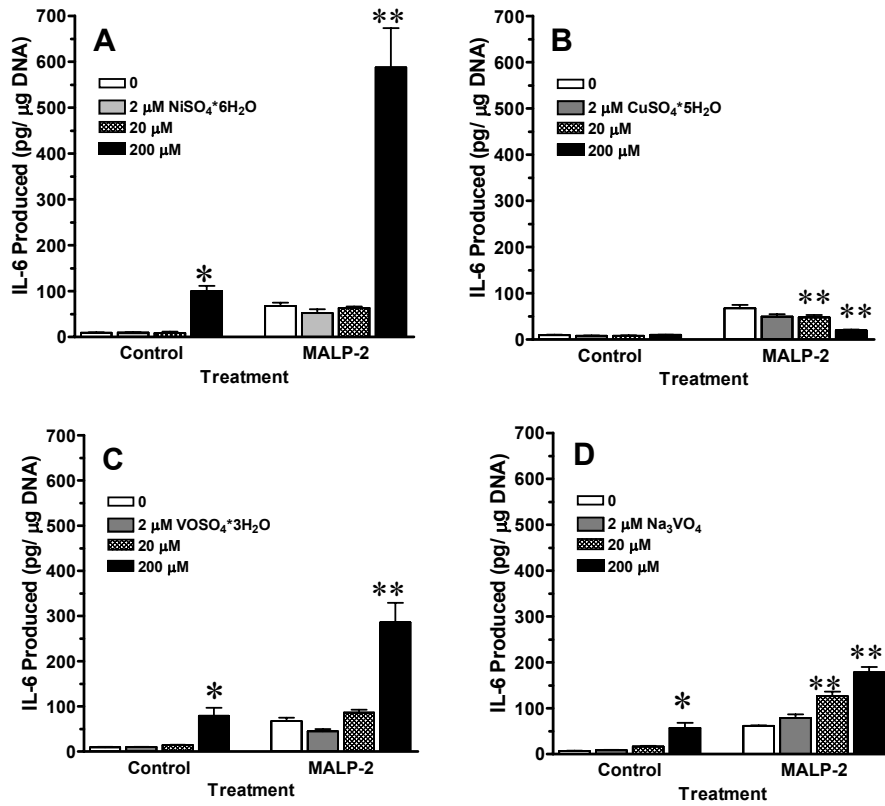


Figure 4: Effect of several soluble metal salts on IL-6 release in the presence and absence of MALP-2.

To explore the dose-response relationships of Ni in more detail and further demonstrate the synergistic relationship between NiSO₄ and MALP-2, I treated HLFs with various concentrations of NiSO₄ between 20 and 200 μM in the presence or absence of 600 pg / ml MALP-2 (Fig. 5). The effects of nickel alone were extremely small, with IL-6 release remaining below 100 pg / μg DNA at the highest concentration for both the 24 h (Fig. 5A) and 48 h (Fig. 5B) time points. NiSO₄, however, dramatically increased IL-6 release in a dose-dependent manner in the presence of MALP-2 to a level that was ten times that seen with MALP-2 or the highest concentration of Ni alone. At the 48 h time point in the presence of MALP-2, even the lowest concentration of Ni provided enhancement over 20 μM Ni alone (101 ± 19 vs. 6.1 ± 0.7 pg IL-6 / μg DNA) and was nearly twice that predicted from an additive model (62.3 ± 11.7 pg IL-6 / μg DNA). The synergistic interaction between Ni and MALP-2 was apparent when the slope of the observed dose-response to Ni plus MALP-2 was compared to that predicted by adding the observed responses to each agent alone, where the slope of the observed response was ten-fold greater than that predicted by an additive model. Thus, soluble nickel ions may be one of the primary components in ROFA capable of synergistically interacting with MALP-2 to stimulate IL-6 production from HLF.

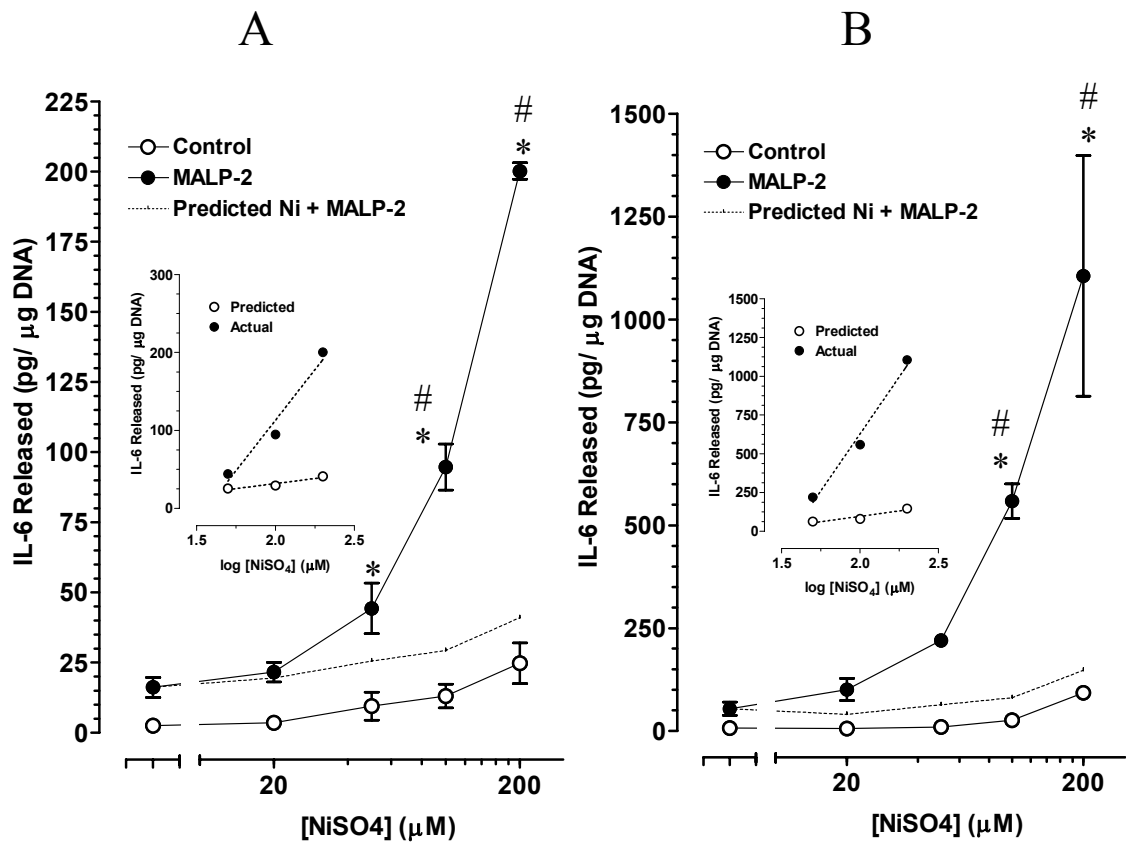


Figure 5: NiSO₄ and MALP-2 synergistically stimulate IL-6 release from HLF. IL-6 was measured at both 24 (panel A) and 48 h (panel B) after exposure.

The effects of ROFA and Ni were further investigated at the level of cytokine mRNA and compared for their ability to modulate gene expression of IL-6 and another cytokine, interleukin-8 (IL-8). IL-8 is a potent inflammatory cytokine with neutrophil chemotactic activity whose regulation shares many similarities to that of IL-6. The steady-state levels of both IL-6 and IL-8 mRNA in HLF were measured after 24 h stimulation with Ni (200 μ M) or ROFA (40 μ g / ml) in the presence or absence of MALP-2 using RT-PCR, and β -actin gene expression was assessed as a control. Figure 6A shows a representative experiment and the specific ethidium bromide-stained PCR products obtained after agarose gel electrophoresis. Note that both IL-6 and IL-8 transcripts were barely detectable in unstimulated cells. Increased expression of IL-6 and IL-8 was observed after 24 h treatment with either NiSO₄ or ROFA alone and was similar in magnitude to that seen after MALP-2 alone. The combinations of MALP-2 / ROFA or MALP-2 / NiSO₄, however, produced a dramatic accumulation of mRNA for both cytokines that was much greater than that seen with any single agent alone. The level of each cytokine mRNA species was measured using quantitative real-time RT-PCR, normalized to the amount of β -actin transcript, and then compared between each treatment (Figs. 6B and 6C). The steady-state level of IL-6 mRNA was induced about ten-fold above unstimulated control when cells were exposed to any of the stimuli as single agents; in contrast, the abundance of IL-6 transcripts were approximately 50-fold greater with the combinations of MALP-2 + ROFA and MALP-2 + NiSO₄. A similar pattern of induction was also seen for IL-8, although the difference between MALP-2 + NiSO₄ and NiSO₄ alone just failed to reach statistical significance, in part, because of the unequal variances between the combination and single-stimuli exposure groups.

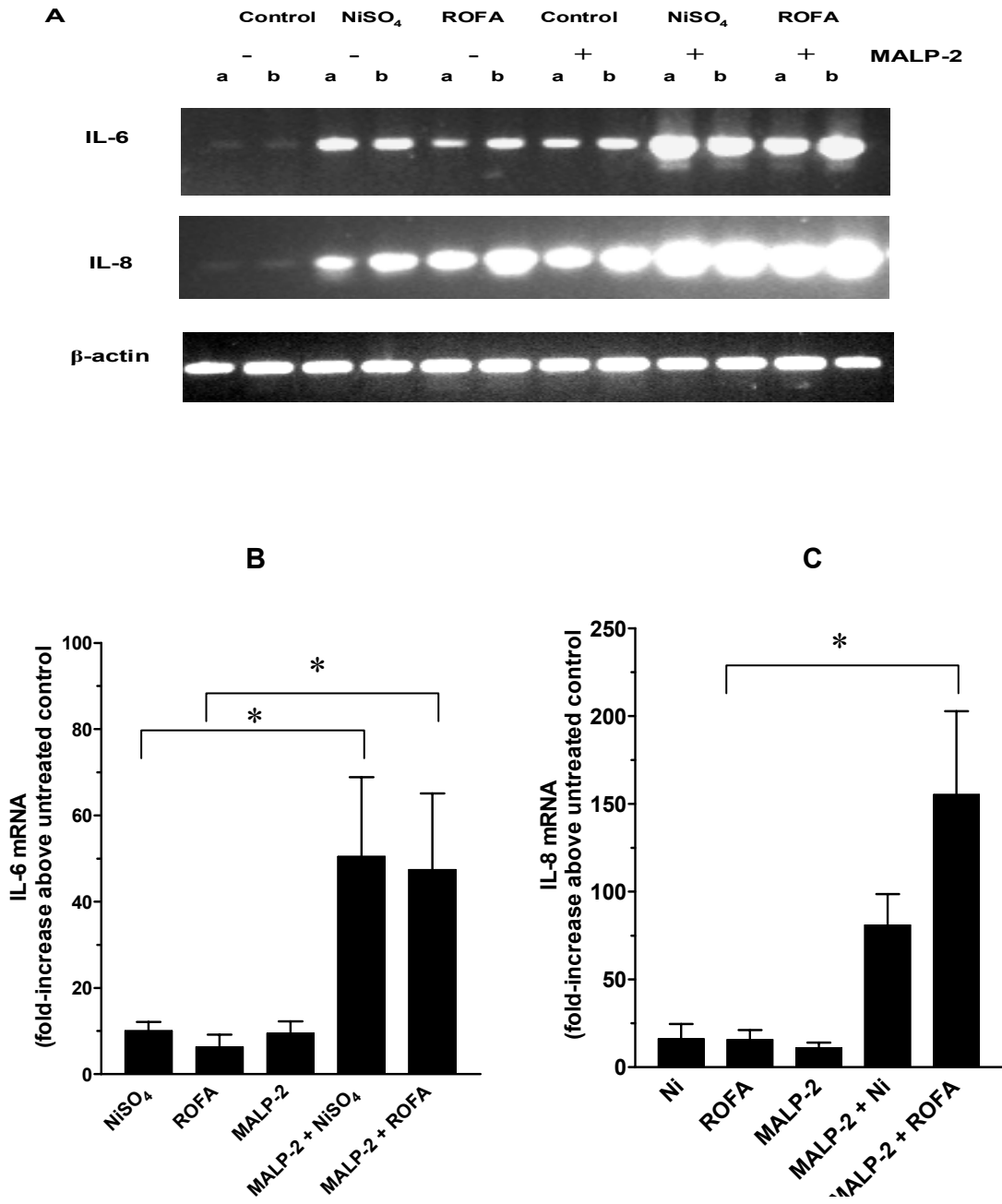


Figure 6: Effects of MALP-2 plus ROFA or NiSO₄ on steady-state level of IL-6 and IL-8 mRNA.

3.1.8. Discussion

This study is the first to observe a potent and significant interaction between a particular PM, ROFA, and exposure to the microorganism, *M. fermentans*. Using IL-6, a prototypic immunomodulating cytokine, as a biomarker for cellular activation of inflammatory responses, I demonstrate that levels of chemical stress and microbial stimulation that produce minimal response alone produce profound cell-activating effects when applied together. These interactions are clearly synergistic since, (1) the observed response to simultaneous chemical and microbial exposure is greater than that predicted by the algebraic sum of each stimulus alone, and (2) the shape and slope of the dose-response curves to each agent can be reciprocally modulated by minimally-effective concentrations of the other.

Dose-response relationships determined in specific pathogen-free animals and sterile cell culture models are often used to set risk assessment guidelines for various environmental agents. This rarely, however, mimics relevant "real-life" exposures, where individuals may be exposed in the presence of microbial infection/colonization. Such infection can range from clinically evident disease (pneumonia, tracheobronchitis), to subclinical latent chronic infection, to colonization with various "harmless" commensals. Moreover, ambient PM itself is a complex mixture that contains particulates from many sources, including those of biological origin. In fact, bacterial endotoxin and other microbial products have been implicated in some of the biological activity of ambient PMs (109). Thus, the synergistic interactions between different components of the PM mixture and concurrent presence of microorganisms need to be considered when evaluating their overall toxicity.

M. fermentans, serves as an example of a mycoplasma with potential to establish chronic subclinical infection. Originally isolated from the genitourinary tract (127), *M. fermentans* has also been documented in joint fluid of patients with rheumatoid arthritis but not those with other arthrides (32;118), leukemic bone marrow (128), and a disproportionately high percentage of blood samples from those with chronic fatigue syndrome (21). Recently, *Mycoplasma* spp. were detected in the airways of humans in absence of symptoms of acute infection, and the incidence was greater in asthmatics (9). Using sensitive PCR-based detection methods, high incidences of *M. fermentans* positivity have been noted in saliva (116), blood (29), and urine (129) from apparently normal healthy subjects. Data regarding the presence of *M. fermentans* within the human lung or its ability to establish chronic "symptomless" pulmonary infection, however, are severely limited.

It remains unresolved as to what particular components of PM contribute to its adverse health effects. ROFA is frequently used as a surrogate to study the adverse effects of PM, particularly in the context of particulate-derived metal (53); however, it represents a very small component of total air PM and does not accurately reflect the complex composition of ambient particulate mixtures. Several studies have pointed to the importance of the "fine" (<2.5 μm) and "ultrafine" (<0.1 μm) fractions in mediating ambient PM toxicity (130;131). Although these size fractions represent only <50 % and <10 %, respectively, of the total mass fraction of PM_{10} , these fractions are enriched in their metal content. Multiple sources of PM-derived metals exist in addition to ROFA. Whether ambient PMs are capable of interacting with microbial stimuli in a manner analogous to ROFA will undoubtedly depend on their sources, chemical composition, size distribution, and physical properties.

The ability of ROFA to produce cell and tissue injury and stimulate inflammatory cytokines has been linked to its high metal content (60;67;132;133). In much the same way, our data support a role for particulate-derived metal in mediating the synergistic effects with MP. Vanadium and iron have been implicated as the primary transition metals in ROFA capable of inducing oxidative stress and activating inflammatory responses such as cytokine release. In contrast, my data point to an important role for Ni in mediating the effects observed here. The preparation of ROFA used here contains 37.5 mg Ni/gm, which if completely soluble would produce from 6 to 25 μM Ni with the ROFA concentrations used in Figure 1 (10–40 $\mu\text{g} / \text{ml}$). It should be pointed out, however, that the threshold for the effect of NiSO_4 was between 20 and 50 μM ; therefore, it is likely that other metals or alternate forms of Ni also participate in the overall effects of ROFA. Ni is most often considered as an occupational hazard in exposed workers (74), although considerable exposure does occur in the general population, often in the form of ultrafine metal-rich combustion-derived PM. Nearly 1 million to 200 million people living in the vicinity of Ni-emitting sources are exposed to median concentrations of 200 and 50 $\text{ng Ni} / \text{m}^3$ respectively (134). Assuming an ambient level of 100 ng / m^3 , 50 % deposition, negligible elimination, and normal respiration, we estimate a daily dose of 864 ng Ni that if uniformly distributed within the noncellular volume of the lung (22 ml) would produce $\approx 1 \mu\text{M Ni}$. While this value is below that necessary to produce synergy, a small change in breathing parameters, potential for uneven distribution, and possible accumulation over time could produce Ni levels closer to those used in our experiments. Using data from the Six Cities studies, Laden *et al.* (131) demonstrated that Ni was positively associated with daily deaths. In animal studies, Ni synergistically interacts with V to initiate untoward cardio-vascular effects following inhalational exposure (63).

My data do not speak directly to the cellular and molecular mechanisms that account for the synergistic interactions between ROFA and MP. Since ROFA contains a variety of transition metals (Fe, V, Ni, among others) it is possible that enhanced formation of reactive oxygen species (ROS) during metal and microbial exposure can subsequently modulate signal transduction pathways culminating in IL-6 release. The SLC PM, however, was inhibitory towards MALP-2-induced IL-6 despite the fact that this PM is particularly rich in redox-active Cu (65). Although Ni leads to formation of ROS (135-137) it is not considered as redox-active as other transition metals. It is possible, however, that Ni coordinated to specific ligands enhances its redox activity (137;138) and / or imparts an intracellular regio-specificity that is critical for these effects.

Numerous transcription factors such as NF- κ B and AP-1 play well-established roles in the regulation of cytokine gene expression following diverse microbial and chemical stressors. Activation of these pathways by ROFA (70;139) and MALP-2 (140) can occur through activation of protein kinase-dependent and oxidant-sensitive mechanisms. Other mechanisms of Ni-dependent regulated gene expression include stabilization of hypoxia-inducible factor (HIF-1 α) (141), as well as other novel, as yet defined, transcription factors (142). Other possibilities for mechanisms of regulation-include metal-dependent inactivation of specific signal-transducing protein tyrosine phosphatases (133;143) and modulation of mRNA stability (144). Thus, it is possible that synergy arises via amplified activation of one or more of these specific transcription factors or signaling pathways or, alternatively, that each stimulus produces a unique profile of signaling events that converge to govern gene expression in an interactive way.

HLF are useful as *in vitro* model for these studies since they (1) support live MP infection, (2) represent a relatively normal untransformed human lung cell type without artifacts of prolonged tissue culture, and (3) play an active role in the cytokine and fibrotic response following tissue injury. Other cells such as macrophages and epithelial cells, however, may arguably be more relevant since they are amongst the first to encounter PM and infectious stimuli. Since regulation of cytokine production in diverse cell types likely follows common mechanisms, I anticipate that microbial stimuli and particulate-derived metals will similarly interact to activate other cell types within the lung.

In summary, my studies demonstrate the potential for profound synergistic interactions between microbial products and PM in the ability of HLF to produce immune-modulating / inflammatory cytokines. These effects occur in absence of cytotoxicity and with concentrations of stimuli that produce minimal effects by themselves. Since it appears that mycoplasma are signaling through TLR-dependent mechanisms, the phenomenon can likely be extended to a range of diverse microorganisms. My studies provide an experimental model to further examine the cellular and molecular mechanisms by which these microbial and chemical stimuli interact to modulate the expression of immune-modulating cytokines and other gene products important in the response to various environmental stresses.

3.2. Chapter 2

In preparation

MEK / ERK1/2 MAP kinase pathway is involved in the synergistic stimulation of IL-6 cytokine production by nickel sulfate and MALP-2.

Fei Gao and James P. Fabisiak

Department of Environmental and Occupational Health, Graduate School of Public Health,
University of Pittsburgh, Pittsburgh, Pennsylvania 15260

3.2.1. Abstract

Exposure to nickel sulfate (NiSO₄) causes dramatic IL-6 release by human lung fibroblasts (HLFs) in combination with *M. fermentans*-derived macrophage-activating lipopeptide-2 (MALP-2). The mechanisms however for these effects are not well understood. Therefore, cell culture experiments with HLFs were conducted to identify pathways involved in the synergistic interaction between MALP-2 and NiSO₄. This study found that MALP-2 and NiSO₄ co-exposure induces both IL-6 mRNA and protein production primarily at later time points suggesting that one stimulus potentiates the effect of another. Pre-treatment of cell with NiSO₄ alone facilitates subsequent MALP-2-induced IL-6 production and this facilitation effect is partially blocked by PD98059, a specific inhibitor of the MAP Kinase MEK / ERK1/2 pathway. Western blot analysis shows that NiSO₄ alone caused both ERK1/2 and JNK phosphorylation at about 10 minutes post-exposure and tapered away after 1 hour. Further study found that MEK/ERK inhibitor PD98059 but not JNK inhibitor SP600125 partially abolished the synergy between MALP-2 and NiSO₄ co-treatment in IL-6 production at both the protein and mRNA level. These findings support the idea that the MEK/ERK1/2 pathway but not JNK activation induced by NiSO₄ plays a role in the synergistic production of IL-6 in HLF.

3.2.2. Introduction

Inhalational exposure to airborne particulate matter (PM), even at ambient levels, has been linked to multiple adverse respiratory and cardiovascular health consequences including premature death. Although current regulations and environmental controls have substantially reduced the atmospheric release of larger mass fractions, amounts of PM of smaller size (less than 2.5 μm) remains high. These fine particulates are considered especially hazardous since a higher percentage of fine particulates are retained in the lung compared to larger particulates (145). Moreover, $\text{PM}_{2.5}$ and “ultrafines” contain the highest content of a variety of heavy metal toxins compared to larger particulate fractions. Fugitive fly ash derived from the combustion residual fuel oil (residual oil fly ash, ROFA) is now widely used as a surrogate to study the biological effects on metal-containing atmospheric particulates. The exposure to ROFA produces significant injury of airways and lungs. While devoid of significant amounts of organic components, ROFA does contain relatively high quantities of transition metals, such as iron (Fe) and vanadium (V) mostly in the form of soluble sulfates, and these are often implicated in the acute toxicity of ROFA (53;132). It is clear from numerous animal and in vitro studies that the toxicity from ROFA, as well as other PM, involves initiation of inflammatory responses within the lung; however the precise cellular and molecular mechanisms regulating the changes in gene expression that underlie these toxic pulmonary effects are still not completely understood. Activation of multiple stress-induced signal transduction pathways and transcription factors, in part through metal-catalyzed oxidative stress, have been demonstrated in many studies (146-148).

Numerous other metal species are also present in ROFA, even though the relative amount of each metal can vary widely depending on the source of the particles. Nickel (Ni) is frequently a major component of ROFA (149;150) and needs to be considered as a factor in the biological effects of PM. Ni exposure in the lower airways is associated with immunological sensitization, epithelial dysplasia, asthma, and fibrosis (151;152). Moreover, Ni is a well-established respiratory carcinogen in man (153-155) and appears to synergistically interact with other metals to amplify their toxic effects (75;156). Animal studies show that exposure of mice to nickel subsulfide by inhalation resulted in inflammation, hyperplasia, and fibrosis in the lung and inflammation and atrophy of the olfactory epithelium in the nose (157). More recent studies demonstrate that it is water-soluble nickel that is responsible for much of the pulmonary injury caused by ROFA (158). At the cellular level, Ni modulates gene expression by stimulating multiple signaling cascades in airway epithelium including, hypoxia-inducible factor (HIF-1 α), AP-1, and other novel pathways (142;159;160). We previously reported that both ROFA and nickel sulfate could interact with microbial stimuli during *in vitro* mycoplasma infection to markedly amplify the release of the immunomodulatory cytokine, IL-6, by human lung fibroblasts. The specific mechanisms responsible for this interaction remain to be identified and it is not clear if both stimuli are required simultaneously or if one stimulus can modulate the subsequent responsiveness to the other. Since other heavy metals did not show the same effects as nickel sulfate when co-exposed with MALP-2, I hypothesize that nickel sulfate plays the major role by facilitating the action of MALP-2 in producing IL-6 in HLF.

Mitogen-and stress-activated protein kinase (MAPK / SAPK) pathways are among the best studied signaling routes that play crucial roles in many aspects of inflammatory responses.

Several MAPK pathways (p38, ERK1/2 (p42/p44) and c-Jun N-terminal protein kinase (JNK)) in murine macrophages are activated by soluble factors derived from *M. fermentans* such as macrophage-activating lipopeptide 2 (MALP-2) and were required for induction of AP-1 and NF- κ B activity, as well as cytokine secretion in these cells (49). Similarly, several of the metals present in ROFA stimulate MAPK activation by acting as potent inhibitors of protein tyrosine phosphatase activity (161;162). In line with its role as a contact sensitizer, Ni-induced activation of dendritic cells is associated with activation of the p38 MAPK module (163-165). Hence, I sought to further define the molecular signaling mechanisms responsible to the synergistic interactions between Ni and microbial stimuli and hypothesized that specific MAPK pathways might play roles in the amplified production of IL-6 by HLF exposed to NiSO₄ and MALP-2.

3.2.3. Material and Methods

Materials

Cell culture medium and fetal bovine serum (FBS) were from Invitrogen (Gaithersburg, MD). Tissue culture plasticware was from Falcon (Becton-Dickinson, Franklin Lakes, NJ) with the exception of 60 mm² dishes from Costar (Corning, NY). Calf thymus DNA, Hoechst 33258 and nickel sulfate were from Sigma (St. Louis, MO). Low endotoxin bovine serum albumin (BSA) was from Intergen (Purchase, NY) and *M. fermentans*-derived macrophage-activating lipopeptide-2 (MALP-2) was from Alexis Biochemicals (San Diego, CA). Bradford protein assay reagent was from Bio-Rad (Hercules, CA). Murine leukemia reverse transcriptase, RNase inhibitor, deoxynucleotides, oligo-dT and Taq DNA polymerase were from Promega (Madison, WI). MAP kinase inhibitors SB203580, PD98059 and SP600125 were from Alexis Biochemicals. IL-6 specific enzyme-linked immunosorbent assay (ELISA) kits were from R&D Systems (Minneapolis, MN).

Cell culture

Human lung fibroblasts (HLF) were isolated and maintained as previously described (98) in accordance with a protocol approved by University of Pittsburgh Institutional Review Board. The individual cell lines were recovered from early passage frozen stocks and six different cell lines were pooled together and used for experiments over no more than eight additional subcultures. Cells were maintained in Minimal Essential Medium (MEM) supplemented with FBS (10 %, final concentration), glutamine (2 mM), penicillin (100 U / ml), streptomycin (100

µg / ml) in a humidified incubator at 37 °C with 5 % CO₂ / 95 % air. All cultures were negative for mycoplasma as determined by fluorescent microscopy using Hoechst 33258 dye (123).

mRNA Measurement

Conditioned media were collected for ELISA to verify the synergistic interaction before mRNA analysis. Total cellular RNA was harvested using RNeasy Mini Kit (Cat. No. 74104 from Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcription-polymerase chain reaction (RT-PCR) was performed with 1 µg of the resulting RNA, as described previously (166). Specific primer pairs for IL-6 (forward 5'-GCCAGCTATGAACTCCTTCTC; reverse 5'-GACTTGTCATGTCCTGCAGCC), β-actin (forward 5'-GGGACCTGACCGACTACCTC; reverse 5'-GGGCGATGATCTTGATCTTC), were used to amplify the specific cDNAs. PCR reactions were carried out for 20 s at 95 °C, 30 s at 55 °C, and 40 s at 72 °C for 17 cycles for β-actin and 24 cycles for IL-6. PCR products were either detected on 2 % agarose gels stained with ethidium bromide or quantified in real-time fashion during the PCR amplification using the double-strand DNA fluorescent dye PicoGreen at 430 nm emission and 525 nm excitation. IL-6 mRNA expression were normalized to the housekeeping gene β-actin by determining the ratio of the IL-6 fluorescent signals to those for β-actin.

Western Blot Analysis

Cells were washed twice with cold 1x phosphate-buffered saline (PBS) and collected with 40 µl/dish RIPA buffer (150 mM NaCl, 1 % NP-40, 0.25 % sodium deoxycholate, 1 mM EDTA, 50 mM Tris, pH7.4) supplemented with leupeptin, aprotinin, pepstatin (1 µg / ml each), 1 mM

phenylmethylsulfonyl fluoride, 1 mM sodium fluoride and 1 mM sodium orthovanadate . Lysates were rocked on ice for 15 min and clarified by centrifugation at 12,000 x g for 7 min. The obtained supernatants were aliquoted, flash frozen in EtOH / dry ice and stored at -80 °C. Samples were analyzed for protein according to Bradford assay (Bio-rad, Hercules, CA). Total cell proteins were prepared by using RIPA buffer, 0.1 M DTT and 6X LB (0.35 M Tris-HCl (pH 6.8 @ 25 °C), 0.35 M SDS, 30 % glycerol, 0.175 mM bromphenol blue). Samples were heated at 95 °C for 3 minutes and 60 µg of protein were loaded onto 12 % SDS polyacrylamide gels. After separation, proteins were transferred to PVDF membrane (NEN life Sciences, Boston, MA). Ponceau S Staining was applied to verify that equal amounts of protein per lane were transferred to the blot. Immunoblots were developed using the MAPK pathway immunodetection kits (Cell Signaling technology, Beverly, MA). Briefly, PVDF membrane with transferred proteins was washed with 25 ml of 1 x Tris-buffered saline (TBS) and blocked for 1 hour at room temperature with 25 ml of Blocking Buffer (5 % w / v nonfat dry milk in 1x TBS supplemented with 0.1 % Tween-20). Membrane was washed 3 times with 15 ml of 1x TBST for 5 min each after blocking and incubated overnight at 4 °C with 1:1000 dilution of primary antibody in 10 ml primary antibody in Blocking Buffer. After incubation, membrane was washed for 3 times with 15 ml 1 x TBST and incubated with 1:2000 dilution of anti-rabbit IgG and 1:1000 dilution of anti-biotin for one hour at room temperature in 10 ml Blocking Buffer. Membrane was washed 3 times with 15 ml 1 x TBST for 5 min each and incubated for about 1 min with 10 ml of Lumiglo and peroxide reagents solution and chemiluminescent image was then captured using Epi chemi II darkroom (UVP Inc., Upland, California). Membranes were probed sequentially with phospho-specific MAPK primary antibodies to measure the activated

form of each isoform, followed by an antibody detecting total (phosphorylated and unphosphorylated) MAPK protein. Data were analyzed by Labwork software.

Statistical analysis

Data presented are expressed as mean \pm SEM collected from at least three individual experiments. Comparisons were made using a one-way ANOVA followed by appropriate group comparisons such as Dunnett's multiple comparison to control or Bonferonni's correction for multiple *t*-tests. Statistical analyses were performed using GraphPad PRISMTM, version 3.0 (GraphPadTM Software, San Diego, CA), and differences were considered significant at $p < 0.05$.

3.2.4. Results

NiSO₄ and MALP-2 Co-exposure Synergistically Induces IL-6 mRNA and Protein Production.

My previous study (166) observing the synergistic interactions between chemical (ROFA, NiSO₄) and microbial stimulation was restricted to times following 24 and 48 hrs of concurrent exposure. In order to more carefully delineate the time course and correlate the temporal relationship between changes in IL-6 mRNA gene and protein expression, IL-6 mRNA in cells and IL-6 protein in media were both measured at varying times after simultaneous exposure to NiSO₄ (200 μM) and MALP-2 (600 pg / ml). Cells were seeded into 6 well plates at 4-6 x 10⁵ cells / 4 mls / well and incubated for 2 days. Confluent cells were washed once with 1 x PBS and changed to fresh serum-free MEM with 0.1% BSA. Cells were then exposed to NiSO₄ (200 μM) or MALP-2 (600 pg / ml) or both for a varying periods of time. Conditioned media were collected for ELISA and total cellular RNA was harvested using RNeasy Mini Kit. Figure 7A shows a typical gel of IL-6 and β-actin RT-PCR products obtained from HLF. IL-6 mRNA was barely detectable in any samples in the absence of any stimulus and both Ni and MALP-2 alone appeared to produce a measurable response of approximately equivalent magnitude. The combination of Ni and MALP-2 together, however, produced a robust signal that was much greater than either single stimulus alone at all of the selected time points. IL-6 mRNA levels were quantified using real-time RT-PCR and normalized to the expression of β-actin. Figure 7B shows that the Ni / MALP-2 combination produced an early, approximately 12-fold increase in IL-6 mRNA that was substantially greater than the 3 – 4 fold increases seen with MALP-2 or Ni

alone at this same time. This early increase was transient with a consistent fall in mRNA at the 4 hr time point, however, as time progressed between 4 and 30 hrs, a prolonged and persistent accumulation of IL-6 transcripts was observed only in the cells exposed to Ni / MALP-2 combination while IL-6 mRNA in response to either agent alone remained unchanged. When IL-6 protein in conditioned media was measured by ELISA (Figure 7C) similar levels of IL-6 accumulated over time in response to Ni or MALP-2 alone. While significantly more IL-6 was measured at all time points for the combined stimulation, the most robust enhancement of IL-6 release offered by combined exposure occurred only after 8 hrs of stimulation and correlated with late prolonged phase of mRNA accumulation seen in Figure 7B.

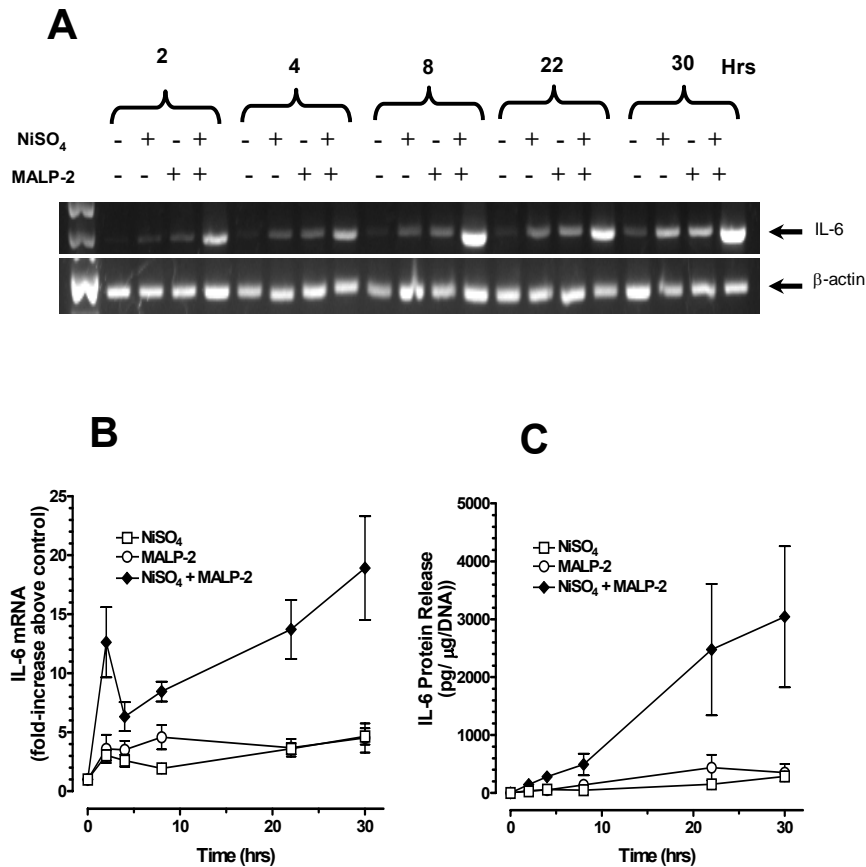


Figure 7: NiSO₄ and MALP-2 co-exposure induces synergistic increases in IL-6 mRNA and protein.

Nickel sulfate Pre-treatment Facilitates MALP-2 Effects on IL-6 Production.

The synergistic stimulation of IL-6 expression by nickel and MALP-2 may require simultaneous presence of both stimulants. Alternatively, the delay between exposure and manifestations of maximal synergy suggests that one stimulus may condition cells to respond to subsequent challenge with the other stimulus. To test if single exposure to either agent could modulate the subsequent response to the other we pretreated cells with either NiSO₄ or MALP-2 alone for 24 hours. A control group of cells was incubated in serum-free MEM (w / 0.1 % BSA) with no added stimuli. Media was then replaced with fresh serum-free media with cells from each pretreatment group receiving NiSO₄, MALP-2, NiSO₄ / MALP-2 combined or no addition. Cells were allowed to condition the media for 24 hrs at which time it was collected and assayed for IL-6 content by ELISA. Figure 8 shows that nickel sulfate-pretreated cells produced a significantly larger amount of IL-6 in response to subsequent stimulation with MALP-2 alone or NiSO₄ / MALP-2 combined than did non-pretreated control cells. In these Ni-pretreated cells, secondary stimulation with simultaneous NiSO₄ and MALP-2 produced a greater response compared to MALP-2 alone. In contrast, pretreatment with MALP-2 produced no significant difference in IL-6 production in response to any subsequent challenge stimulus compared to control non-pretreated cells. These data suggest that exposure to NiSO₄ can enhance the ultimate response of HLF to the microbial product, MALP-2.

Nickel sulfate Induces ERK and JNK Phosphorylation

The next step was to define mechanisms by which NiSO₄ potentiates MALP-2 effects on IL-6 production. Mitogen activated protein kinases (MAPK) are known to play a crucial role in many aspects of immune-mediated and inflammatory responses. Ni has been demonstrated to activate MAPK pathway activation in human dendritic cells (163;164), whereas synthetic MALP-2 also induces cytokine secretion in macrophages via activation of MAP kinases, p38, ERK1/2 (p42 / p44) and c-Jun N-terminal protein kinase (JNK) (167). My biphasic pre-treatment/treatment protocol allowed temporal separation of the signaling events used by each stimulus. Therefore, we could specifically address the role of MAPK in response of each stimulus. To test if Ni-mediated facilitation of MALP-2 depended on Ni-induced activation of specific MAPK, the time course of MAPK activation was followed by monitoring the phosphorylation status of the specific MAPK isoforms (ERK1/2, p38, JNK) after exposure to 200 μM NiSO₄ alone. Western blot analysis using antibodies specific for the phosphorylated forms of each MAPK subtype show that exposure to nickel sulfate alone leads to phosphorylation of ERK1/2 and JNK in HLF. ERK1/2 activation was very rapid with peak signal observed at 10 minutes and then diminishing to control levels by 2 hrs. The pattern for the 46 kD form of JNK appeared similar to that seen with ERK1/2 with the exception that levels of phospho-JNK-46 appeared elevated relative to control at even longer times. For the 54 kD form of JNK the time course of activation was somewhat slower with peak phosphorylation observed at 1 hr post-stimulation. The levels of phosphorylated p38 remained very low or undetectable at all times following Ni exposure.

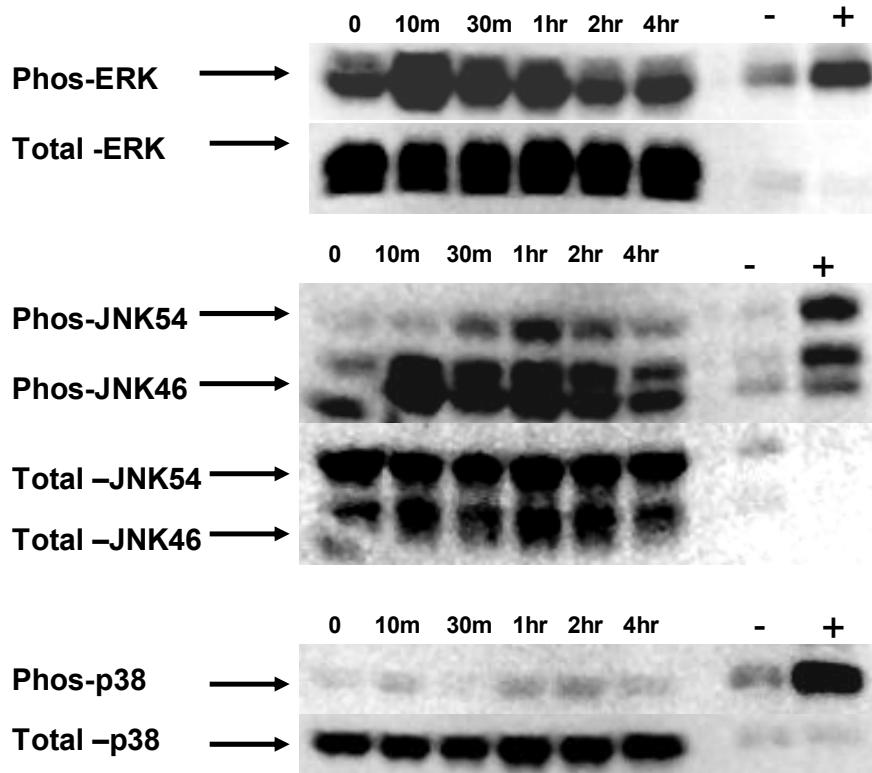


Figure 9: Nickel sulfate induces ERK1/2 and JNK phosphorylation while phosphorylation level of p38 is undetectable.

Inhibition of MAP Kinase MEK/ERK1/2 or p38 Reduce the Ability of NiSO₄ to Potentiate MALP-2-induced IL-6 Production

I next tested whether the enhanced MALP-2 response observed after Ni-pretreatment was dependent upon the ability of Ni to activate specific MAPK pathways. To this end I used three specific MAPK inhibitors: PD-98059, a synthetic compound that specifically inhibits the ERK-activating MAPK kinase MEK-1 (168;169), SB203580, a p38 pathway inhibitor (170;171), and SP600125, a c-Jun N-terminal protein kinase (JNK) inhibitor (172;173). Since the synergistic interactions between Ni and MALP-2 can be reproduced with sequential treatment of Ni followed by MALP-2, experiments were set up to examine involvement of MAP kinases at both the Ni-pretreatment phase, as well as the MALP-2 challenge phase. In one set of experiments, HLF were first pretreated with NiSO₄ for 24 hours in the presence or absence of each MAPK inhibitor followed by challenge with MALP-2 in the absence of the inhibitor for an additional 24 hours. In another set of experiments, I omitted the MAPK inhibitors during the pretreatment phase with NiSO₄, but included them during the second phase with MALP-2. It should be noted that cells were washed and replenished with fresh serum-free medium prior to the initiation of the MALP-2 challenge so that the IL-6 response represents the amount released only during the 24 hr MALP-2 treatment. Figure 10 shows the ability of various MAPK inhibitors to modulate MALP-2-induced IL-6 release when applied during the Ni pretreatment phase or the MALP-2 challenge itself. I found application of PD 98059 or SB 203580 during the NiSO₄ pre-treatment phase alone decreases the subsequent MALP-2 induction of IL-6. This effect was specific for Ni pretreatment since application of the same concentrations of MAPK inhibitors (20μM) during the MALP-2 treatment phase was without effect. The JNK inhibitor SP600125 failed to

attenuate IL-6 release when applied at either time. These results suggest that Ni-induced activation of specific MAPK isoforms, like ERK1/2 and perhaps p38, are important determinants in the Ni-dependent modulation of cytokine release following MALP-2 exposure.

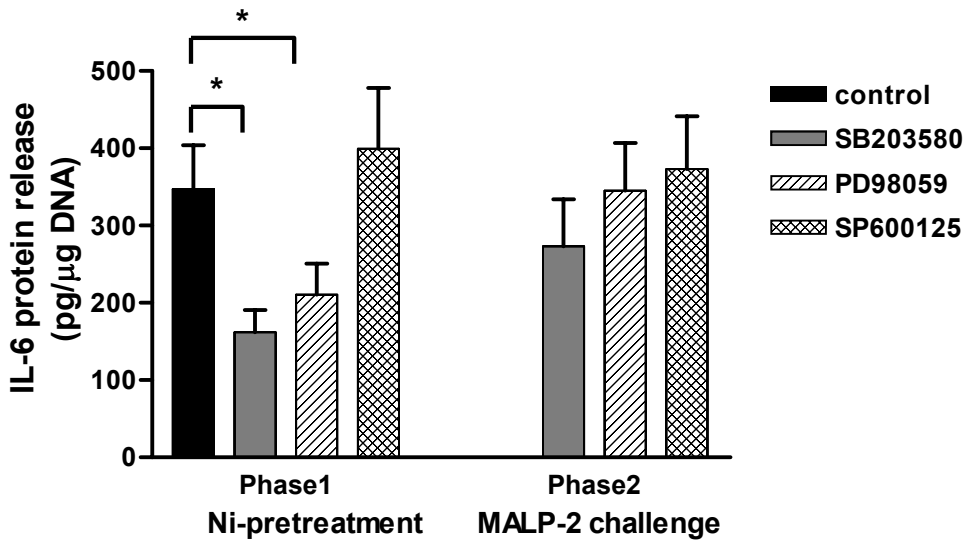
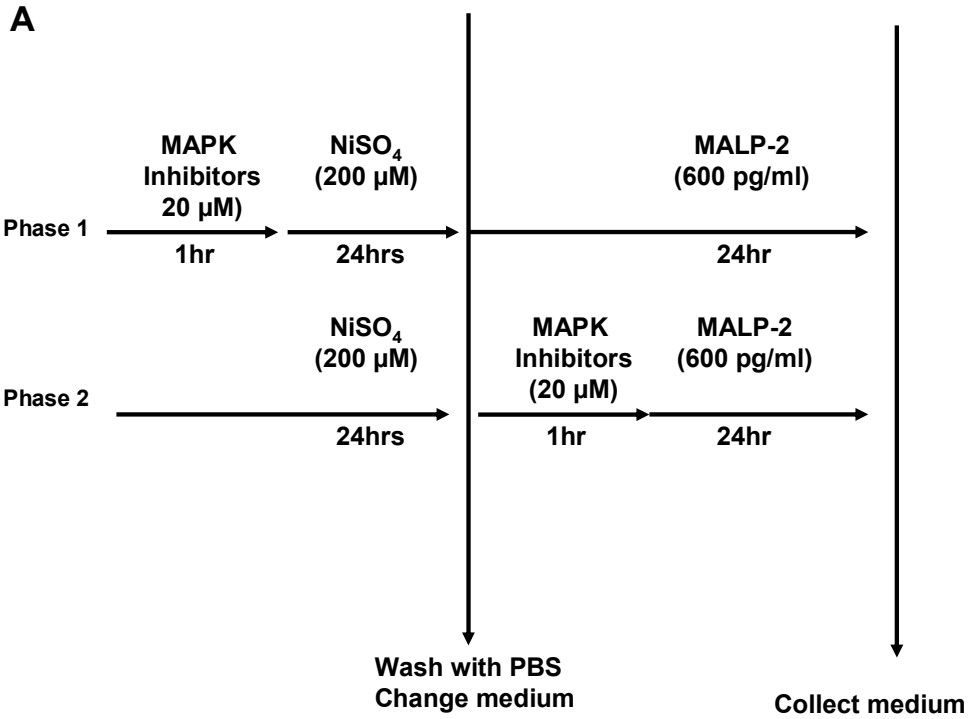


Figure 10: Blocked of MAP Kinase MEK/ERK1/2 and p38 pathways reduce the ability of NiSO₄ to enhance MALP-2-induced IL-6 production.

MEK/ERK1/2 is Involved in the Synergistic Upregulation of IL-6 Protein and mRNA Expression during Concurrent MALP-2 and Nickel Sulfate Coexposure.

I next wanted to determine how various MAPK inhibitors would impact the synergistic interactions between Ni and MALP-2 when the two stimuli were applied simultaneously and whether the pattern of inhibition was distinct from the ability to modulate the response to either single stimulus alone. In addition, since the results in Figure 11 show less than complete inhibition for any one single MAPK antagonist, the effects of various combinations of MAPK inhibitors were also determined. For this, HLF were treated with MAPK inhibitors (20 μ M), alone or in combinations for one hr prior to the addition of NiSO₄ (200 μ M), MALP-2 (600 pg / ml), or Ni + MALP-2. Incubations were continued in the presence of MAPK inhibitors for 24 hrs at which time medium was collected and analyzed for IL-6 by ELISA. Figure 11A shows that when the MAPK inhibitors were applied as single agents during combined Ni / MALP-2 exposure, only PD98059 produced significant inhibition (33 %) whereas the p38 and JNK inhibitors did not. In addition, no further enhancement of PD98059 was observed when it was applied in combination with SB203580. With the exception of SP + PD combination, which may reflect a slight stimulatory effect of JNK inhibitor (see Figure 11B), PD98059 treatment always produced a significant attenuation of the MALP-2 + Ni response either alone or in combination with other antagonists. In addition, I also observed that the simultaneous presence of the p38 and JNK inhibitors significantly inhibited IL-6 release in response to MALP-2 + Ni, although either agent alone was without effect. Figure 11B and 11C show the responses to Ni

and MALP-2 alone, respectively. Note the differences in scale of the y-axes compared to Figure 11A, which reflects the synergistic interaction when Ni and MALP-2 are combined. In contrast to that seen with Ni + MALP-2, inhibition of the MEK/ERK1/2 pathway did not attenuate IL-6 release to either stimulus alone. None of the combinations of MAPK inhibitors affected the response to Ni alone with the exception of SP600125, which produced a slight but significant enhancement of IL-6 release. The response to MALP-2 alone was reduced in the simultaneous presence of all three MAPK inhibitors, however most likely reflects the effects of combined SB203580 and SP600125 which was similar in magnitude to effect of SB + SP + PD, but narrowly failed to meet statistical significance. Therefore, it appears that inhibition of ERK1/2 pathway of MAPK can attenuate the response to the combination of Ni and MALP-2 and that this effect is unique to the combined stimuli since similar patterns of inhibition were not observed when PD 98059 was applied with either Ni or MALP-2 alone. I also observed that joint inhibition of p38 and JNK could attenuate the response to MALP-2 + Ni response, whereas inhibition of either one alone was insufficient. The effect was not specific for the combined stimuli since a similar pattern was observed in response to MALP-2 alone.

The role of ERK1/2 was further substantiated in an experiment that separately assessed IL-6 gene expression at the mRNA level. In this experiment HLFs were stimulated Ni + MALP-2 in the presence or absence of PD 98059, SB 203580, and SP 600125 (20 μ M each). Since the time course of mRNA accumulation (see Fig. 7B) revealed a biphasic response in IL-6 transcript accumulation, cells were harvested and RNA prepared at both 2 and 24 hrs post-treatment to correspond to the early and late mRNA response. Figure 12 shows the amount of IL-6 mRNA normalized to β -actin present in HLF at 2 and 24 hrs after stimulation. In the absence of any

MAPK inhibitor the amount of mRNA was about three-fold higher at the 24 hr time point compared to 2 hrs after stimulation. The accumulation of mRNA at the early 2 hr time point was essentially unchanged by any of the MAPK inhibitors. In contrast, the ERK1/2 pathway inhibitor produced a significant reduction in the mRNA response observed at 24 hrs, whereas the remaining 2 inhibitors, SP 600125 and SB 203580, were without effect. Taken together, my data indicate that MEK-ERK1/2 MAP kinase pathway plays an important role in mediating the synergy between NiSO₄ and MALP-2 in augmenting IL-6 gene expression.

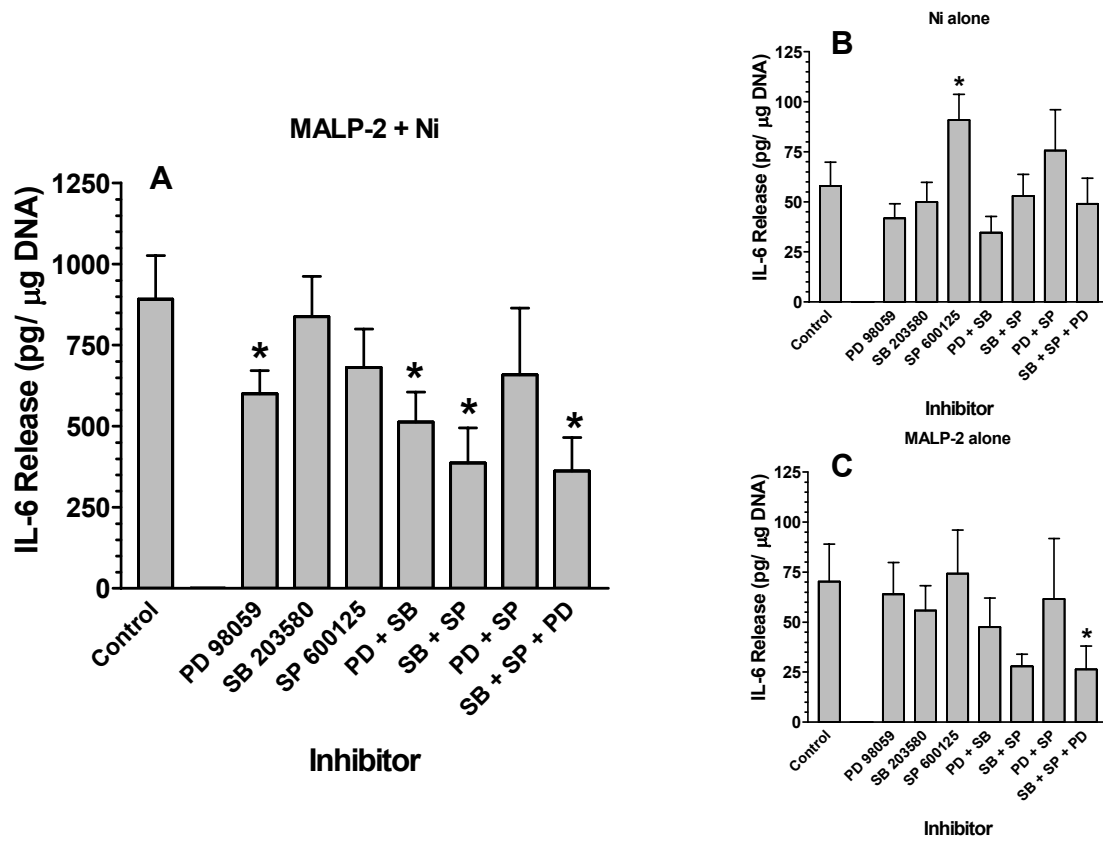


Figure 11: MAP kinase MEK/ERK1/2 pathway inhibitor PD98059 partially inhibits the synergy between MALP-2 and nickel sulfate in IL-6 production by HLF.

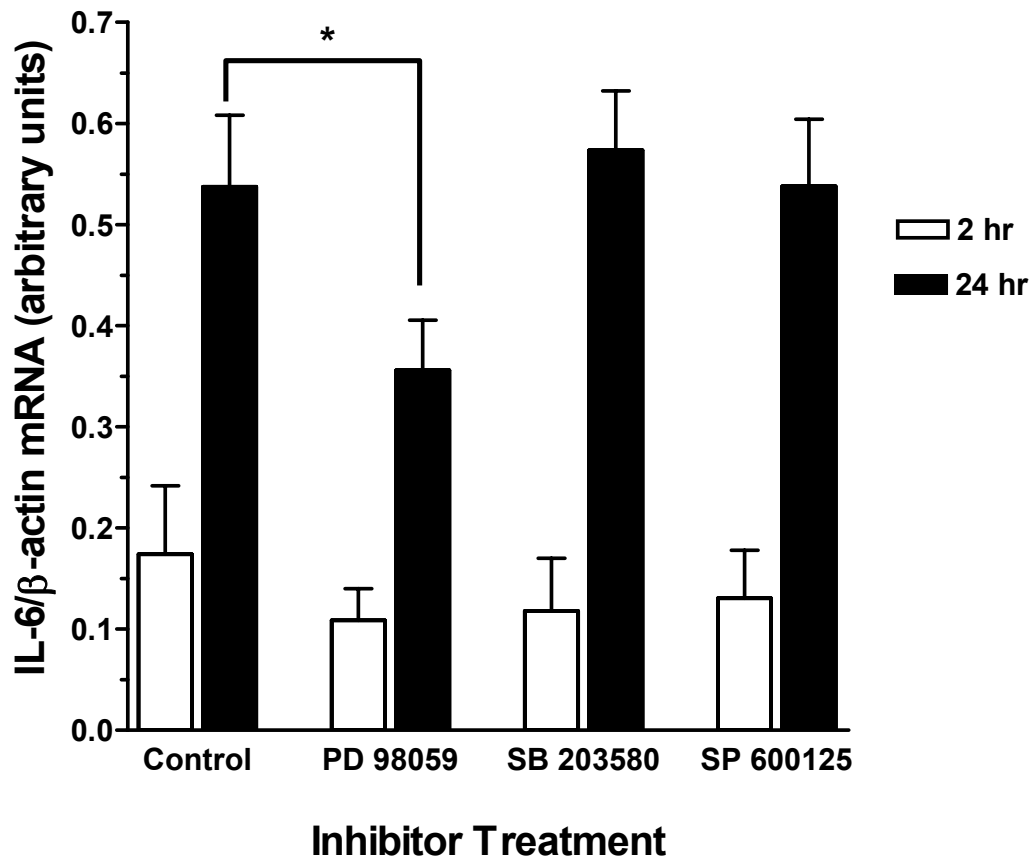


Figure 12: MAP kinase MEK/ERK pathway inhibitor PD98059 partially inhibits the synergy between MALP-2 and nickel sulfate in IL-6 mRNA production by HLF.

3.2.5. Discussion

This study is the first to explore the mechanisms of the unique synergistic interaction between NiSO₄ and MALP-2 in human lung fibroblasts. MAP kinase pathways are important targets for ROFA-induced cellular and biochemical responses, while the mechanism by which ROFA activates these signaling pathways is not known. One mechanism of ROFA-induced MAP kinase activation could be the induction of an oxidative stress due to the high content of bioavailable metals, predominantly vanadium and nickel. In vivo study shows that ROFA induced activation of pulmonary ERK1/2, and the ERK1/2 activation was inhibited at the cellular, biochemical, and functional levels by dimethylthiourea (DMTU), an oxygen radical scavenger, which supports the role for oxidative stress in this response (148). These results are consistent with a number of in vitro studies that have demonstrated the ability of oxidative stress generated by ROFA-associated metals to induce MAP kinases (174;175). Another possible mechanism for ROFA-induced activation of pulmonary MAP kinases could be by the inhibition of tyrosine phosphatase activity as shown by Samet et al. using human bronchial epithelial cells (143;176). In vivo studies show that there was a pronounced inflammatory response to ROFA instillation and a marked increase in levels of P-Tyr and P-MAPKs present within the alveolar epithelium and in the inflammatory cells, while the airway epithelium showed a prolonged increase in the expression of P-ERK1/2 (161). Nickel, one of the main metal component in ROFA, activates MAP kinase signaling cascades in different type of cells (159;164;165), but little is known about nickel's effect on MAP kinase in human lung fibroblasts. This study is the first to examine the possibility of nickel-induced MAPK activation in HLF. Acute exposure to nickel sulfate induced a rapid activation of two distinct MAPKs, ERK1/2 and JNK (Fig. 9), while blocking MEK /

ERK1/2, not JNK pathway can partially abolish nickel sulfate's facilitation effects on MALP-2 (Fig.10) in human lung fibroblasts.

The hypothesis that nickel sulfate potentiates MALP-2 effects on IL-6 production by activating MER / ERK1/2 pathway was verified in another experiment when MAP kinase inhibitors were given at different phases to examine the involvement of MAP kinase during both the Ni-pretreatment phase, as well as the MALP-2 challenge phase. The result of this experiment gives rise to two other issues. First, although nickel sulfate cause JNK phosphorylation, it is not responsible for its potentiating effects on MALP-2 since the corresponding JNK specific inhibitor failed to decrease the IL-6 production at either phase of the study. Secondly, although the levels of phosphorylated p38 remained very low or undetectable at all times following Ni exposure, the data from Figure 10 shows that blocking p38 by SB203580 can partially diminish nickel sulfate's effects on assisting MALP-2 on IL-6 protein release. Recently study by Numazawa et al. suggested that a steady-state level of the p38 MAP kinase activity is required for the nuclear translocation of ERK1/2 (177). Their study also strongly suggests that the p38 MAP kinase activity participates in ERK nuclear translocation and thereby positively regulates ERK-mediated signal transduction at multiple steps (177). SB 203580 has been widely used to specifically shut down the p38 MAP kinase-dependent pathway, its inhibition effects on IL-6 release in my study is probably due to the impaired ERK –mediated signal transduction caused by inactivation of basal p38 MAP kinase activity. However, there could be other possibilities such as the cross-talk between MAPK signaling pathways, involvement of other upstream kinases in addition to MAP kinase or even other signaling pathways activated in the interactive effects between nickel and MALP-2 since the inhibitor experiments cannot fully block the

effects of nickel sulfate or the combination effects on IL-6 production. My further studies suggest that even though there may be some cross-talk or uncertainty about the whole MAPK pathway effects on nickel sulfate and MALP-2 exposure, blocking MEK / ERK1/2 signaling pathway still can partially diminish the synergistic IL-6 mRNA and protein production by the combination of NiSO₄ and MALP-2 (Fig 11, 12). Another important issue is the relationship between MALP-2 and MAPK activation. Studies show that the inhibition of MEK / ERK1/2 by PD98059 treatment partially inhibited, in a dose-dependent manner, cytokine production by RAW 265.7 in response to MALP-2 (49). In addition, ERK1/2 and p38 pathway inhibitors, respectively, partially or completely inhibited cytokine production in the human cell line THP-1 stimulated with sMALP-2 or LPS (47). Little is known about MALP-2 and MAPK activation in human lung fibroblasts. My study shows that Nickel-induced MAPK activation leads to further MALP-2 related IL-6 production in HLF. MEK / ERK1/2 pathway was confirmed to play a role in the synergistic interaction between nickel sulfate and MALP-2 in IL-6 production, but p38 may also be involved. p38 inhibitor SB 203580 partially inhibited IL-6 production in the pre-treatment model, while it failed to inhibit the interaction of nickel sulfate and MALP-2 when both were given at the same time. This suggested that phosphorylation of MAPK pathways in different orders would affect the synergistic IL-6 production in HLF by the combination of Ni and MALP-2.

Another important issue about the mechanisms of nickel and MALP-2 inducing IL-6 production in human lung fibroblasts that has not been touched in this study is the IL-6 gene stability. Nickel sulfate and MALP-2 effects on IL-6 gene stability is another direction that needs to be revealed to better study the mechanisms of this unique effects.

3.3. Chapter 3

Transcriptional regulation of IL-6 gene by MALP-2 and NiSO₄ in Human Lung Fibroblasts

3.3.1. Introduction

IL-6 is a multi-functional cytokine that plays a central role in host defense mechanisms. It is produced by many cell types and influences function of immune and other types of cells. IL-6 elicits major changes in the biochemical, physiological and immunological status of the host (e.g. the "acute phase" plasma protein response). IL-6 enhances plasma protein gene expression not only in hepatocytes but also in monocytes, fibroblasts and lymphocytes (178). In addition, IL-6 is believed to play a role in the pathogenesis of pulmonary fibrosis (179;180), rheumatoid arthritis (181), systemic lupus erythematosus (182;183) and other immune-mediated diseases (184-186).

The expression of IL-6 is controlled at both transcriptional and post-transcriptional levels. While less is known about the mechanism for post-transcriptional regulation, several studies have identified sequence elements in IL-6 promoter that mediate its regulated expression by physiological stimuli and environmental insults. The human IL-6 promoter contains multiple regulatory elements including NF- κ B (-75 / -63), NF-IL6 (-158 / -145) and AP-1 (-283 / -277). AP-1 activity is induced by a variety of physiological stimuli and environmental insults such as growth factors, cytokines, neurotransmitters, polypeptide hormones, cell-matrix interactions, bacterial and viral infections and air pollution particles. These stimuli activate mitogen activated protein kinase (MAPK) cascades that enhance AP-1 activity through the phosphorylation of distinct substrates (187). In turn, AP-1 regulates a wide range of cellular processes, including

cell proliferation, death, survival and differentiation. NF- κ B is another crucial transcriptional factor that regulates IL-6 gene expression. NF- κ B belongs to the Rel family of transcription factors that form homo- or heterodimers through the Rel homology domain (188;189). In most cells, NF- κ B is present in the cytoplasm bound to a member of the I κ B family of inhibitors (190;191). Upon activation of the cells by cytokines, such as TNF- α and IL-1, viruses, bacteria, or mitogens, I κ B is phosphorylated and then rapidly degraded allowing the translocation of NF- κ B to the nucleus where it binds to specific DNA binding sites. Like NF- κ B, NF-IL6 is ubiquitously expressed but functionally regulated at translational and post-translational levels. NF-IL6 is a member of a C/EBP family. It not only regulates the expression of IL-6, but also induces various acute phase proteins (192). Several lines of evidence suggest that members of the NF- κ B, C/EBP, and AP-1 transcription factor families may work in concert to regulate the IL-6 promoter in a cell-type- or inducer-specific fashion (193-196). Indeed, a physical and functional interplay among all three classes of proteins has been widely demonstrated (197;198). It has been shown that air pollution particles induce activation of NF- κ B and other transcription factors in lung epithelial cells (199). Studies also show that MALP-2 induce cytokine production via NF- κ B and AP-1 activation (200;201). In the previous studies, I have demonstrated that NiSO₄ and MALP-2 synergistically induce pro-inflammatory cytokine IL-6 release by human lung fibroblasts. I also found that extracellular signal-regulated kinase (ERK1/2) plays a role in this synergistic interaction between MALP-2 and NiSO₄. These findings raise the possibility that MALP-2 and nickel sulfate synergistically increase IL-6 gene transcription by activating different transcriptional factors. I tested this possibility using luciferase-based reporter gene constructs containing various forms of the human IL-6 promoter. Three forms of wildtype

human IL-6 promoter of progressively increasing length along with a series of mutant promoters containing alterations in specific transcription factor binding sites were utilized for these studies.

3.3.2. Material and Methods

Materials

QIAEX II Gel extraction kit (Cat. No. 20021), High purity midiprep (Cat. No. 12143) and QIAprep spin miniprep kit (Cat. No. 27104) were from Qiagen (Valencia, CA). TOPO TA cloning kit (Cat. No. K4530-20) and LIPOFECTAMINE PLUS Reagent were from Invitrogen (Carlsbad, CA). pGL3-basic luciferase vector (Cat. No. E1751), the Dual-Luciferase™ Reporter Assay System (Cat. No. E1960) and Passive Lysis 5 X Buffer (Cat. No. E1941) were from Promega (San Luis Obispo, CA). Forward (5'-CATAGACGGATCACAGTGCA-3') and reverse primer (5'-TGTGGA GAAGGAGTTCATAGC-3') was purchased from Integrated DNA Technologies, Inc. (Coralville, IA). T4 DNA ligase (Cat. No.10481220001) was from Roche Applied Science (Indianapolis, IN). XL10-Gold® Ultracompetent cells (Cat. No. 200314) and QuikChange® XL Site-Directed Mutagenesis Kit were from Stratagene (La Jolla, CA).

Cell culture

Human lung fibroblasts (HLF) were maintained in Minimal Essential Medium (MEM) supplemented with FBS (10 %, final concentration), glutamine (2 mM), penicillin (100 U / ml), streptomycin (100 µg / ml) in a humidified incubator at 37 °C with 5 % CO₂ / 95 % air. All cultures were negative for mycoplasma as determined by fluorescent microscopy using Hoechst 33258 dye (123).

Luciferase reporter gene assays

Human lung fibroblasts were plated onto 12-well plates (2 x 10⁵ cells / well) in MEM containing 10 % FBS. Twenty-four hours later, the cells were washed with 1x PBS and fed 400 µl fresh

Dulbecco's modified eagle's medium (DMEM) without antibiotics and serum. Cells were transfected using Lipofectamine-Plus with 0.3 μg IL-6 or mutant promoter reporter construct and 0.1 μg pRL-tk containing Herpes simplex virus thymidine kinase promoter / well for 3 h according to the manufacturer's protocol. After the incubation, the medium was replaced with MEM medium containing 10 % FBS and incubated overnight. Cells were washed again with 1x PBS and changed to serum-free MEM medium containing 0.1 % BSA before being exposed to NiSO_4 (200 μM) and / or MALP-2 (600 pg / ml). IL-1 β (10 ng / ml) was used as a positive control. After the 24 hrs incubation, supernatants were collected and stored for ELISA and cells were harvested with 150 μl passive lysis buffer / well and collect into microcentrifuge tubes. After centrifuging at 14000 rpm for 10 min, supernatants were collected and dual luciferase activities were determined according to the manufacturer's protocol.

Statistical analysis

Data presented are expressed as mean \pm SEM collected from at least three individual experiments. Comparisons were made using a one-way ANOVA followed by appropriate group comparisons such as Bonferonni's correction for multiple *t*-tests. Statistical analyses were performed using GraphPad PRISMTM, version 3.0 (GraphPadTM Software, San Diego, CA), and differences were considered significant at $p < 0.05$.

3.3.3. Rationale

Making human IL-6 promoter-luciferase reporter construct (see Fig. 13)

An approximate 1.7 kb DNA fragment downstream from the IL-6 protein coding sequence was amplified from HEK293 genomic DNA by polymerase chain reaction (PCR) with primers (5'-

CAT AGA CGG ATC ACA GTG CA-3'(+13-+32) and 5'-TGT GGA GAA GGA GTT CAT AGC-3' (-1744- -1764). The PCR product was extracted by using QIAEX II Gel extraction kit and cloned into a pCR2.1-TOPO TA vector. Multiple clones were selected and grown by 6 hours incubation in LB broth, DNA from individual clones were extracted by QIAprep spin miniprep kit and digested by BamHI restriction enzyme for 30 minutes. After digestion, clones with the appropriate orientation were detected by running 1% agarose gel and grown overnight. Clones and PGL3-Basic vector were digested with endonuclease enzymes and ligated using T4 DNA ligase. Transformation was performed by using XL10-Gold Ultracompetent cells and DNA was purified by high purity midiprep. Three IL-6 promoter-luciferase reporter constructs (-1700IL-6luc, -860IL-6luc and -500IL-6luc) were then generated with pGL3-basic luciferase vector by using endogenous restriction enzyme sites on PCR-based methods. The region -500-0 contains the known AP-1, CREB (NF-IL6), and NF- κ B sites (see Fig. 14 and 15).

Making IL-6 promoter

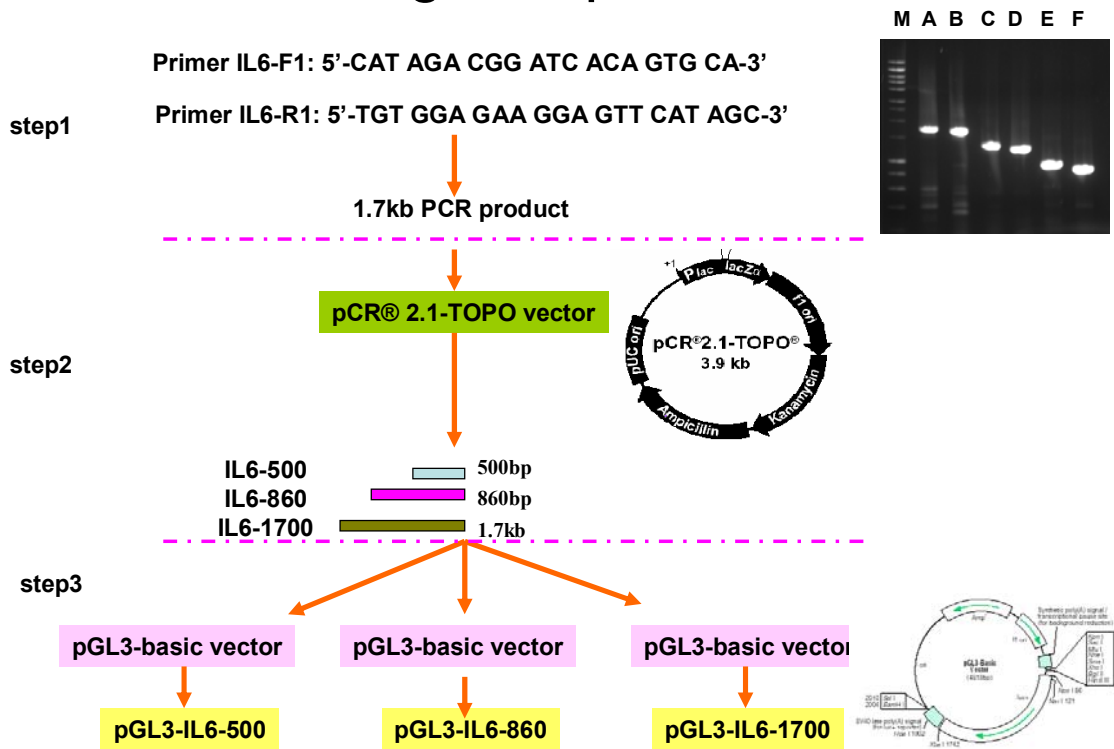


Figure 13: Rationale of making IL-6 promoter with various lengths.

Human IL-6 promoter

-1683 TCGTCGAAAATACATAGACGGATCACAGTGCACGGCTGCGGATAGGGAGCAGTGGCTTCGTTTCATGCAGGAAAG
Forward primer
AGCAGCTTTTATGTATCTGCCTAGTGTACAGTGCCGACGCCTATCCCTCGTCACCGAAGCAAAGTACGTCCTTTC

-1608 AGAACTTGGTTCAGGAGTGTCTACGTTGCTTAAGACAGGAGAGCACTAAAAATGAAACCATCCAGCCATCTCCC
TCTTGAACCAAGTCTCACAGATGCAACGAATTCTGCTCTCGTGATTTTACTTTGGTAGGTCGGTAGGAGGG

-333 ACTTCGTGCATGACTTCAGCTTTACTCTTTGTCAAGACATGCCAAAGTGCTGAGTCACTAATAAAAAGAAAAAAG
AP-1
TGAAGCAGTACTGAAGTCGAAATGAGAAACAGTTCTGTACGGTTTCACGACTCAGTGATTATTTCTTTTTTTC

-258 AAAGTAAAGGAAGAGTGGTCTGCTTCTTAGCGCTAGCCTCAATGACGACCTAAGCTGCACTTTTCCCCTAGTT
GRE
TTTCATTTCTTCTCACCAAGACGAAGAATCGCGATCGGAGTTACTGCTGGATTCGACGTGAAAAGGGGGATCAA

-183 GTGTCTTGCATGCTAAAGGACGTCAATTGCACAATCTTAATAAGGTTTCCAATCAGCCCCACCCGCTCTGGCC
NF-IL6
CACAGAACGCTACGATTTCTGCAGTGTAACGTGTTAGAATTATTCCAAAGGTTAGTCGGGGTGGGCGAGACCGG

-108 CCACCCTCACCTCCAACAAAGATTATCAAATGTGGGATTTTCCCATGAGTCTCAATATTAGAGTCTCAACCCC
NF-κB
GGTGGGAGTGGGAGGTTGTTTCTAAATAGTTTACACCCTAAAAGGGTACTCAGAGTTATAATCTCAGAGTTGGGG

-33 CAATAAATATAGGACTGGAGATGTCTGAGGCTCATTTCTGCCCTCGAGCCCACCGGGAACGAAAGAGAAGCTCTAT
TATA element **Major RNA start site**
GTTATTTATATCCTGACCTCTACAGACTCCGAGTAAGACGGGAGCTCGGGTGGCCCTTGCTTTCTCTTCGAGATA

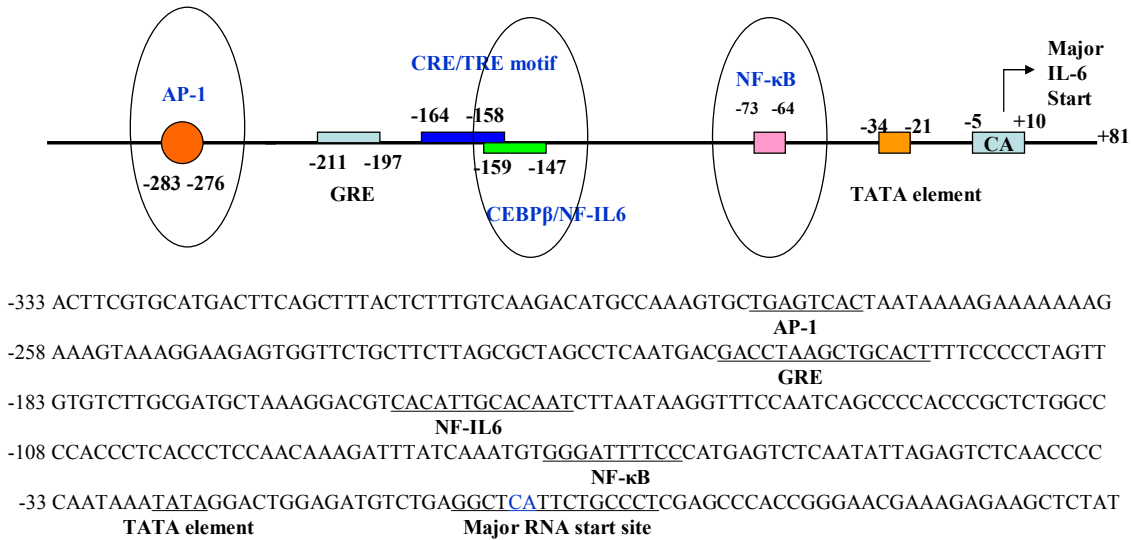
+43 CTCCCCTCCAGGAGCCAGCTATGAACTCCTTCTCCACAAGTAAGTGCAGGAAATCCTTAGCCCTGGAAGTCCCA
GAGGGGAGGTCCTCGGGT**CGATACTTGAGGAAGAGGTGT**TCAATCACGTCCTTAGGAATCGGGACCTTGACGGT
Reverse primer

Figure 14: IL-6 promoter gene sequence and important transcription factor sites

Making human IL-6 promoter-luciferase reporter mutants (Fig. 15)

IL-6 reporter gene constructs with a mutation in the NF-κB, the NF-IL6 or AP-1 site were generated by using QuikChange® XL Site-Directed Mutagenesis Kit according to the manual. The NF-κB site was disrupted by converting GGGATTTTCC to CTCATTTTCC, AP-1 site from TGAGTCAC to TGCAGCAC and NF-IL6 site from CACATTGCACAAT to

CACACCGTTCAATCT. Mutant IL-6 promoter-luciferase reporter constructs were generated within the context of the longest IL-6 promoter, -1700IL6luc.



Site-mutation:

AP-1: 5'-TGA-GTC-AC-3' → 5'-TGC-AGC-AC-3'
 NF-IL6: 5'-CAC-ATT-GCA-CAA-T-3' → 5'-CAC-ACC-GTT-CAA-TCT-3'
 NF-κB: 5'-GGG-ATT-TTC-C-3' → 5'-CTC-ATT-TTC-C-3'

Figure 15: Rationale of making IL-6 promoter mutants.

3.3.4. Results

NiSO₄ and MALP-2 Increase IL-6 Promoter Activity

To study human IL-6 gene promoter activity, three luciferase reporter constructs each containing ~1.7-kb, 860-bp or 500-bp fragment of the human IL-6 5' flanking region were made (Fig. 16). These constructs were transfected into human lung fibroblast cells and luciferase activities were compared following treatment of transfected cells with NiSO₄ or MALP-2 (Fig. 16). Results showed that NiSO₄ induced a very slight increase (Fig. 16) in IL-6 promoter activity compared to the basal level in untreated cells ranging from 26% with the shortest construct to 65% with the 1.7 kb promoter. Only the response of the 1.7 kb fragment achieved statistical significant compare to control. Similarly, MALP-2 induced minimal activation of IL-6 promoter activity (Fig.16). Similar to NiSO₄-induced effect, MALP-2 caused a larger increase in IL-6 promoter activity with the two longer fragments. Co-treatment with NiSO₄ and MALP-2 resulted in larger increase in IL-6 promoter activity compare to individual stimuli. MALP-2 and NiSO₄ co-exposure induced 1.9 ± 0.37 , 2.3 ± 0.54 and 2.8 ± 0.65 fold increases in IL-6 promoter activity with 500-bp, 860-bp and 1700-bp constructs, respectively and were all significantly different from untreated control. These relatively small effects were not due to the inability of transfected cells to respond to these stimuli, since simultaneous measurement of IL-6 release from the same preparations showed a dramatic synergistic enhancement of the cytokine production.

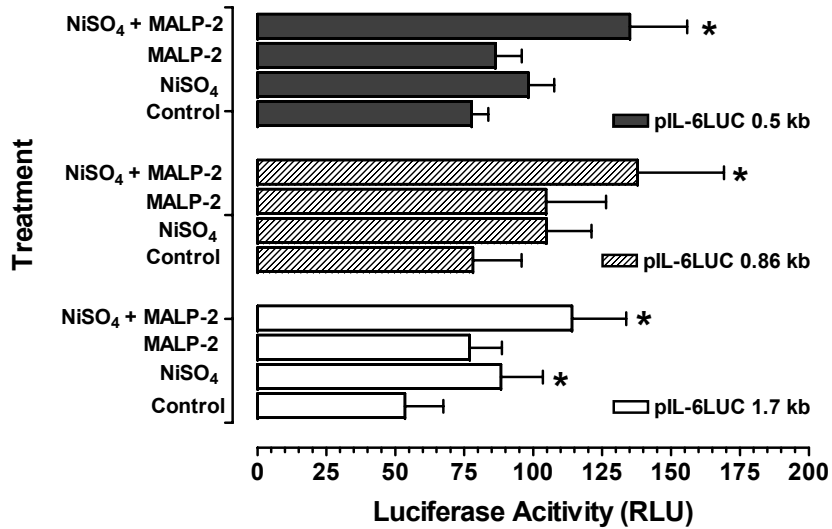
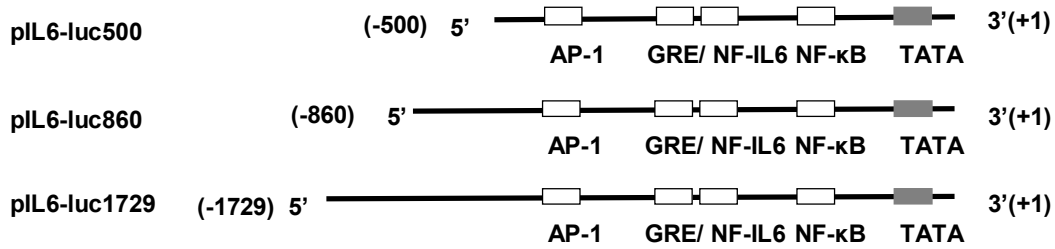


Figure 16: NiSO₄ and MALP-2 effects on IL-6 promoter activity.

NF-κB Element is Essential for Optimal Basal IL-6 Promoter Activity

To further investigate the importance of individual transcriptional factors in regulation of IL-6 gene transcription in lung fibroblasts, I introduced mutations in the known NF-κB, AP-1 and NF-IL6-binding sites in IL-6 promoter (Fig. 17). I used the luciferase reporter with the longest fragment of IL-6 promoter (1.7 kb) for this purpose, because this construct responded the best to NiSO₄ or MALP-2. Compared to the wild-type IL-6 construct, basal IL-6 gene expression in the

absence of any stimuli was dramatically decreased in NF-κB-mutant IL-6 promoter construct (87 % decrease compared to wild type). In contrast, much smaller reductions in the basal promoter activity were seen with AP-1 (44 %) or NF-IL6 (26 %) mutants. Further study also showed that enhancement of IL-6 promoter activity by any of the exogenous stimuli was also dramatically decreased in NF-κB mutant IL-6 promoter construct. IL-1-induced IL-6 gene expression was totally abolished in this mutant IL-6 promoter (Fig. 18). These findings suggest that NF-κB is essential for optimal IL-6 gene expression in human lung fibroblasts, however, it should be noted that a stimulatory effect of Ni could still be observed even in absence of a functional NF-κB cis-element (Fig.17).

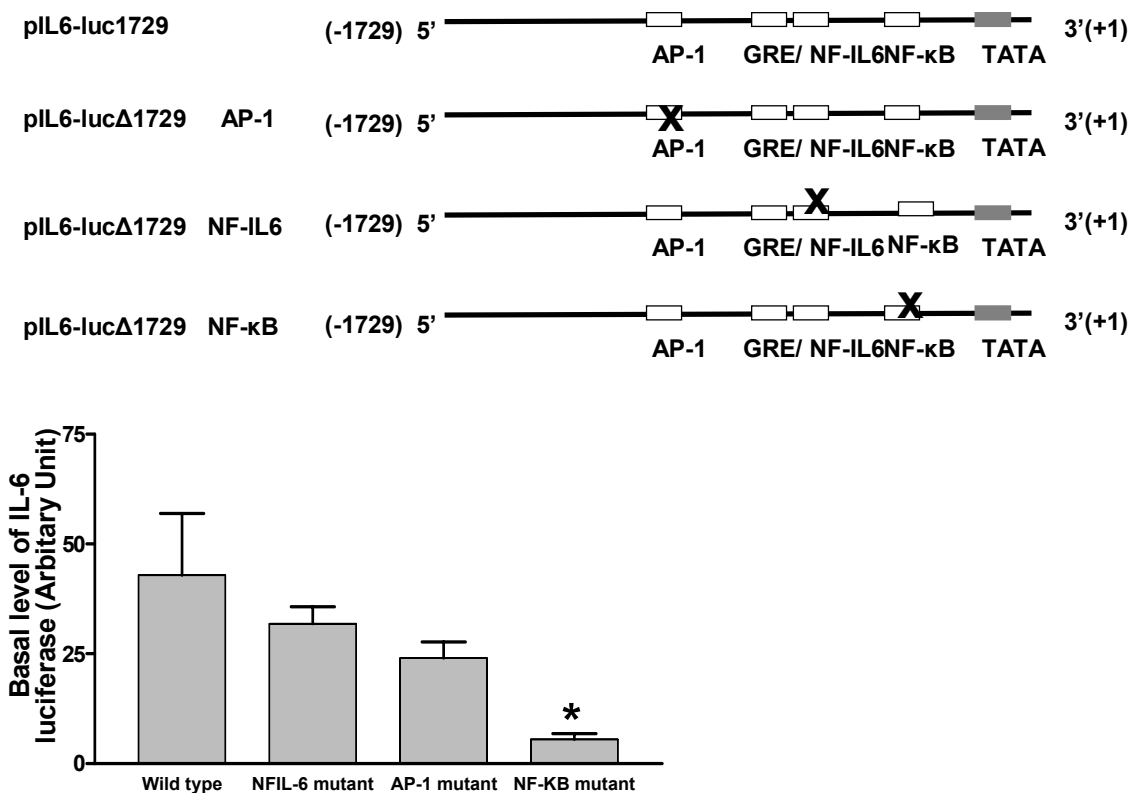


Figure 17: IL-6 promoter mutants and basal level of IL-6 promoter activity.

NF- κ B and NF-IL-6 are Involved in the Cooperative Interactions between MALP-2 and NiSO₄.

Next I tested whether the introduced mutations in transcription factor binding sites might influence IL-6 promoter activation in response to MALP-2 and NiSO₄. Using the 1.7 kb wildtype IL-6 promoter it was observed that the combination of Ni + MALP-2 approximately doubled the promoter activity ($p < 0.05$ relative to untreated control) and was of a similar magnitude to that seen with IL-1 β (Fig 18A). In contrast, the responses to Ni or MALP-2 alone were smaller and were not statistically significant when compared to control. As discussed above, both basal and induced activity of the NF- κ B mutant construct was substantially reduced (Fig. 18B). Ni alone, as well as Ni + MALP-2, however, still appeared to be able to drive this minimally active promoter to a level about 2-fold above control levels. The addition of MALP-2 to Ni failed to augment the stimulatory effect observed with Ni alone. While the overall magnitude of responses seen with the AP-1 mutant was reduced compared to the wildtype promoter, there remained a significant difference between the Ni alone and Ni + MALP-2 with greater induction by the latter (Fig.18C). Therefore, the cooperative interactions between MALP-2 and Ni did not appear to fully require a functional AP-1 binding site. In contrast, negation of NF-IL6 appeared to completely block any cooperation between Ni and MALP-2 since the responses to Ni alone and Ni + MALP-2 were indistinguishable from each other and not statistically different from untreated control. Therefore, it appears that the combination of Ni + MALP-2 represents a more efficacious stimulus for driving the wildtype IL-6 promoter compared to either of the two stimuli applied separately. Negation of either NF- κ B or NF-IL-6,

but not AP-1, binding sites eliminated any apparent functional cooperation between Ni and MALP-2. Thus, the ability of MALP-2 and Ni to interact to amplify IL-6 release from HLF may involve cooperative interactions between specific transcription factors such as NF- κ B and NF-IL6.

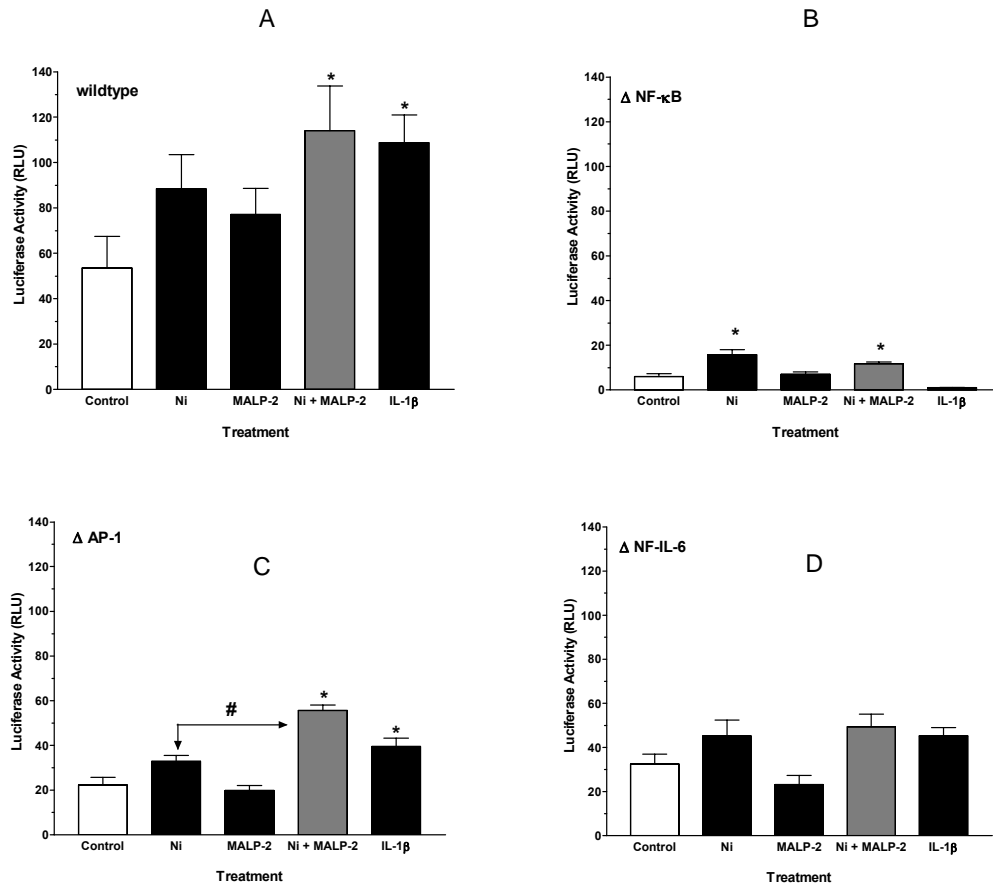


Figure 18: NiSO₄ and MALP-2 effects on wildtype and mutant IL-6 promoters by human lung fibroblasts.

3.3.5. Discussion

IL-6 production is positively or negatively regulated in various cell types by a variety of signals (202). In fibroblasts and certain tumor cell lines, multiple cytokines, including IL-1, tumor necrosis factor (TNF), platelet-derived growth factor, and interferon- β enhance IL-6 production. In the promoter region of the IL-6 gene five known functional *cis*-regulatory elements have been described, namely cAMP-responsive element, activator protein-1 (AP-1), nuclear factor IL-6 (NF-IL6), Sp1, and NF- κ B binding sites (188). Binding of specific nuclear transcription factors to such regulatory elements in the IL-6 gene promoter are known to play pivotal roles in the increased IL-6 production by cytokines and growth factors. Among the transcription factors, NF- κ B is a heterodimer consisting of two proteins encoded by *rel*-family genes (203), NF- κ B activation mediates critical cellular responses that control gene expression and programmed cell death (204). Although both NF- κ B and additional transcription factors such as AP-1 and NF-IL6/CEBP, are required to maximally induce IL-6 gene transcription, the contribution of the κ B regulatory element to the transcriptional activation of the IL-6 gene appears most prominent (205-209). For example, NF- κ B was found to be the most important for the transcriptional regulation of the IL-6 gene by IL-1 and TNF- α (210;211). Likewise, *in vivo* targeting of the p50 (NFKB1) subunit resulted in reduced expression of the IL-6 gene (212). Moreover, in human airway epithelial cells, activation of NF- κ B is associated with the expression of interleukin-6 (IL-6) in response to ROFA exposure (213). Activation of NF- κ B by nickel compounds was also found in mouse fibroblasts (3T3), human bronchoepithelial cells (BEAS-2B) and skin dendritic cell line (214;215). In the present study, I examined the roles of NF- κ B and other regulatory

elements in the increased IL-6 production exerted by MALP-2 and NiSO₄. Using site-directed mutagenesis and reporter gene assays, I have shown that the NF-κB element located near the transcription start site of the human IL-6 gene is essential for its basal expression in human lung fibroblasts as well as its activation by IL-1 or MALP-2. It is noteworthy, however, that significant activation could be measured in response to Ni suggesting the importance of other factors in mediating the effects of this metal on gene expression.

NF-IL6 belongs to a member of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors. These proteins comprise of a class of DNA-binding proteins called basic leucine zipper proteins that includes C/EBP α , C/EBP β (NF-IL6), C/EBP γ , and C/EBP δ (NF-IL6 β) (216). They are characterized by a leucine zipper domain and a DNA-binding basic region located in the C-terminus of the proteins. Members of the C/EBP family can associate through the leucine zipper domain to form homo- and heterodimers with each other and bind with similar affinity to various C/EBP-binding DNA sequences (217;218). NF-IL6 was shown to bind to the regulatory regions of various acute-phase protein genes and several other cytokine genes such as TNF, IL-8 and G-CSF. These observations indicate that NF-IL6 coordinates expression of not only the IL-6 gene but also other genes involved in acute-phase reaction, inflammation and hemopoiesis (219). Thus, NF-IL6 may be a pleiotropic mediator of many inducible genes involved in acute, immune, and inflammatory responses, similarly to NF-κB. Indeed, both NF-IL6- and NF-κB-binding sites are present in many inducible genes such as IL-6, IL-8, and several acute-phase genes (220). Transcription factors NF-IL6 and NF-κB synergistically activate transcription of the inflammatory cytokines such as IL-6 and IL-8 by a process that involve their direct physical interaction (221). My study shows that the disruption of an NF-IL6

site only mildly affects the basal promoter activity. But, this mutation blocked the NiSO₄ plus MALP-2-induced activation of IL-6 promoter activity. Hence, unlike NF-κB, NF-IL6 is not required for the basal IL-6 transcription in human lung fibroblast, whereas it mediates, in part, the MALP-2 and NiSO₄ induced IL-6 gene expression and importantly, is required for any cooperative interaction between these two stimuli.

AP-1 was one of the first mammalian transcription factors to be identified (222), but its physiological functions are still being resolved. AP-1 is not a single protein, but a mix of dimeric basic region-leucine zipper (bZIP) proteins that belong to the Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1 and Fra2) (223), Maf (c-Maf, MafB, MafA, MafG/F/K and Nrl) and ATF (ATF2, LRF1/ATF3, B-ATF, JDP1, JDP2) sub-families (224). AP-1 activity is induced by a plethora of physiological stimuli and environmental insults. In turn, AP-1 regulates a wide range of cellular processes, including cell proliferation, death, survival and differentiation. The induction of AP-1 by pro-inflammatory cytokines and toxic stress is mostly mediated by the JNK and p38 MAPK cascades (225). Once activated, the JNKs translocate to the nucleus (226), where they phosphorylate c-Jun and thereby enhance its transcriptional activity (227). AP-1 activity induced by nickel compound is varied in cells. Although NF-κB activation by nickel compounds was observed in mouse fibroblasts (3T3) and human bronchoepithelial cells (BEAS-2B), nickel treatment did not induce any activation of AP-1 in the same cells (228). In human airway epithelial cells, however, nickel-induced IL-8 expression was shown to be dependent on AP-1 activation through an oxidant-independent pathway. As a contact sensitizer, nickel sulfate activates the transcription factors NF-κB and AP-1 and increases the expression of nitric oxide synthase in a skin dendritic cell line (229).

In this study, my data indicate that NF-IL6 is also an important element upstream of the NF- κ B site that mediates NiSO₄ and MALP-2-induced upregulation of IL-6 gene transcription. These findings suggest that NiSO₄ and MALP-2 induced IL-6 gene expression is controlled by multiple transcription factors including NF- κ B, AP-1 and NF-IL6. Since transcriptional factor interaction may also be involved in the MALP-2 and nickel sulfate co-exposure, it seems important to study the IL-6 promoter with mutations in multiple sites to provide better information for studying this unique synergistic interaction in the future.

4. DISCUSSION

Experimental studies on the toxicologic effects of air pollutants primarily utilize exposure to single agents, while the real exposures in our normal life are rarely restricted to single stressful stimuli. The demonstration of biologic effects of single agents frequently requires the use of pollutant exposures at concentrations in great excess of those found in ambient atmospheres, thereby making the relevance of the experimental studies difficult to extrapolate to the human conditions. Recognizing this, an increasing number of inhalation studies are using more complex exposures to lower concentrations in order to mimic real world conditions (230;231). In this study, I first successfully established an in vitro model to demonstrate the synergistic interaction between mycoplasma live infection and ROFA particle in producing cytokine IL-6 in HLF. Later this model was extended into the synergistic interaction between nickel sulfate and toll-like receptor 2 agonist MALP-2 in IL-6 production. Further studies demonstrated the importance of MAPK pathway involvement in the synergistic interaction between NiSO₄ and MALP-2 and confirmed that several transcription factors like NF-κB and NF-IL6 played important roles in IL-6 production caused by Ni and MALP-2 co-exposure. However, lots of other possibilities still remain unknown and need further investigation. Here, I list some of the important issues that worth to be considered and studied in the future.

4.1. Oxidative stress

Mycoplasma and oxidative stress

It has been postulated that mycoplasma induce oxidative stress within infected cells. The production of reactive oxygen molecules is considered one of the major mechanisms by which

mycoplasmas produce cytotoxic effects. Mycoplasmas have a respiratory chain lacking cytochromes, and therefore superoxide and H₂O₂ are produced in significant amounts. Although the evidence for superoxide dismutase (SOD) in mycoplasmas is controversial, most of the species studied appear to express SOD (232). In normal human cultured cells, infection with *M. pneumoniae* causes a marked inhibition of intracellular catalase (233;234). Detoxification of elevated levels of H₂O₂ depends mostly upon the GSH redox cycle, and oxidative damage to the cells can be partially inhibited by adding vitamin E, an antioxidant (235;236). This characteristic of Mycoplasmas, in particular those found in the bloodstream of HIV-infected individuals (*M. fermentans* and *M. pirum*), may contribute to the observed oxidative stress, which would indirectly help activate HIV replication and increase the percentage of apoptotic cells (237;238). This suggests that *M. fermentans* induced oxidative stress may play a role in pathogenicity. Also, the activation of respiratory burst via NAD(P)H oxidase on inflammatory and possibly other cells in response to infection may contribute to the oxidative burden in infected cells (239).

ROFA and oxidative stress

On the other hand, evidence has accumulated that part of the injury induced by ROFA might be a result of oxidative stress (240;241). It has been reported that free radicals and aldehydes are generated after intratracheal instillation of ROFA in rats (242;243). In fact, oxidative stress is a central hypothetical mechanism for the adverse effects of PM₁₀ (244;245) via transition metal catalyzed redox cycling (121;246), ultrafine particle surfaces (247) and organic components (248;249). Particle-derived oxidative stress may play a role in initiating and prolonging inflammation, as well as in causing genotoxic effects. Since several of the components of PM₁₀ can generate oxidative stress, there is potential for additive or synergistic interaction between the

components. Evidence shows that there can be synergistic interactions between transition metals and ultrafine particles in causing oxidative stress and lung inflammation (66). One mechanism by which oxidative stress from PM₁₀ may induce the expression of proinflammatory mediators is via stimulation of intracellular signaling pathways that employ calcium (250;251). Intracellular calcium acts as a key signaling mechanism which, through interaction with a number of proteins such as calmodulin and enzymes such as protein kinases, is able to regulate the activation of a number of key transcription factors. Such transcription factors include nuclear factor of activated T cells (NFAT) and NF-κB (252;253). For this reason, the role of particle-induced oxidative stress in the calcium response was investigated using the antioxidants n-acetylcysteine and mannitol, both of which diminished the ultrafine carbon black-enhanced thapsigargin response (247). However, this inhibition was not absolute, suggesting that the effect may not be solely due to oxidative stress.

Since both *M. fermentans* and particulate matter, especially ROFA, which is rich in transition metals, can induce oxidative stress responsible for cell damage, it will be of interest to study the role of oxidative stress in the synergistic interaction between *M. fermentans* and ROFA in amplifying IL-6 production in HLF.

4.2. MAP kinase pathway

MAPKs are important targets for PM₁₀, however, the mechanism by which ROFA activates these signaling pathways is not known. One mechanism of ROFA-induced MAP kinase activation could be by the induction of an oxidative stress due to the high content of bioavailable metals, predominantly vanadium, iron and nickel. In vivo study shows that at the cellular, biochemical,

and functional levels, DMTU inhibition of ROFA-induced activation of pulmonary ERK1/2, supporting a role for oxidative stress in this response (148). These results are consistent with a number of in vitro studies that have demonstrated the ability of oxidative stress generated by ROFA-associated metals to induce MAP kinases (174;175). Another possible mechanism for ROFA-induced activation of pulmonary MAP kinases could be by the direct inhibition of tyrosine phosphatase activity as shown by Samet et al. using human bronchial epithelial cells (143;176). ROFA has a high content of vanadium, a potent tyrosine phosphatase inhibitor that leads to dysregulation of phosphotyrosine metabolism and persistent activation of mitogen-activated protein kinase (MAPK) signaling cascades in human airway epithelial cells (176). In vivo studies show that there was a pronounced inflammatory response to ROFA instillation and a marked increase in levels of P-Tyr and P-MAPKs present within the alveolar epithelium and in the inflammatory cells, while the airway epithelium showed a prolonged increase in the expression of P-ERK1/2 (161). Compared to other major metals components in ROFA like V and Zn, Ni, however, does not show very robust phosphatase antagonism. By itself Ni acts as a poor redox catalyst compare to Fe, Cu, or V, but its redox activity is increased by complex to various endogenous proteins (254). Ni activates mitogen-activated protein kinase signaling cascades in different type of cells (159;164;165). Nickel-induced oxidative stress plays an important role in the pathogenicity of nickel related cell injury (255-257). The induction of inflammatory mediators can be regulated by the activation of redox-sensitive transcription factors AP-1 and NF- κ B stimulated in response to reactive oxygen species (ROS). Both environmental and inflammatory cell-derived ROS can lead to increases in intracellular calcium, the activation and phosphorylation of the mitogen activated protein kinase (MAPK) family, including extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38

kinase, and PI-3K/Akt via sensitive cysteine-rich domains and the sphingomyelinase-ceramide pathway, leading to increased gene transcription (258-260). Activation of members of the MAPK family leads to the transactivation of transcription factors such as c-Jun, activating factor-2 (ATF2), and cyclic AMP response element binding proteins (CREB)-binding protein (CBP) (174;258). This eventually results in chromatin remodeling and expression of a battery of genes involved in inflammation, apoptosis, proliferation, transformation, and differentiation. H₂O₂, an oxidant stress causing cell injury and morphological transformation of epithelial cells, induced phosphorylation and activation of ERK at concentrations associated with the development of apoptosis in RPM cells, NIH/3T3 cells, bovine tracheal myocytes, and PC-12 cells (261-263). Crocidolite asbestos induces the phosphorylation and activation of ERK proteins, but not of JNK1, in RPM cells (264). All those suggest that ROFA-induced MAP kinase activation may be due to its oxidative stress effects.

4.3. Toll-like receptors

Another subject that can be investigated in the future is how the toll-like receptor family may be related to the interaction between MALP-2 and ROFA or nickel sulfate. *Toll* is a *Drosophila* gene essential for ontogenesis and antimicrobial resistance. Several homologues of Toll have been identified and cloned in vertebrates, namely Toll-like receptors (TLRs). TLRs are structurally characterized by a cytoplasmic Toll/interleukin-1R (TIR) domain and by extracellular leucine rich repeats. Toll-like receptors (TLRs) function to recognize pathogen-associated molecular pattern (PAMP) in mammals and play an essential role in the recognition of microbial components and activation of host defense. In response to microbial or environmental "danger" signals, represented by structural motifs not normally expressed by cells, Toll-like

receptors mediate intracellular signaling that leads to inflammatory gene expression by recruiting and/or activating TLR-specific adapter molecules. To date, four adapter proteins have been identified: MyD88, TIRAP/Mal, TRIF/TICAM-1, and TIRP/TRAM/TICAM-2. The interaction of the different TLRs with distinct combinations of adapter molecules creates a platform to which additional kinases, transacting factors, and possibly other molecules are recruited. These events ultimately lead to gene expression. More than ten members of the TLR family can be found in a search of human and mouse public genome databases (265;266) and ten members (TLRs 1–10) have been cloned and sequenced (267-271).

By far the most extensively characterized members of the TLR family are the proteins TLR-2 and TLR-4. TLR-2 acts as a PAMP receptor for various microbial products, including peptidoglycan, bacterial lipoproteins, lipoarabinomannan (LAM), glycosylphosphatidylinositol lipid from *Trypanosoma cruzi*, and zymosan, a component of yeast cell walls (272-275). TLR-2 does not recognize these PAMPs independently, but rather functions as a heterodimer with either TLR-1 or TLR-6 (276). A dimer of TLR-1 and TLR-2 (TLR-1/TLR-2) appears to recognize bacterial lipopeptides, whereas a TLR-2 / TLR-6 dimer responds to mycobacterial lipopeptides. These lipopeptides differ in the number of acyl groups they contain, attesting to the specificity in recognition by different TLRs. Lipoproteins are proteins containing lipid that is covalently linked to the NH₂-terminal cysteines; they are present in a variety of bacteria, including Gram-negative and Gram-positive bacteria and mycoplasmas. TLR-2 mediates the responses to lipoproteins derived from *M. tuberculosis*, *Borrelia burgdorferi*, *Treponema pallidum* and *Mycoplasma fermentans* (277-279). Most lipoproteins are triacylated at the NH₂-terminal cysteine residue, but mycoplasmal macrophage-activating lipopeptide 2 (MALP-2) is only diacylated. TLR-2 null cells are unresponsive to all lipoproteins, whereas TLR-6 null cells are

unresponsive to MALP-2 but responsive to other lipopeptides of bacterial origin (48). Coexpression of TLR-2 and TLR-6 is absolutely required for MALP-2 responsiveness since TLR-2 and TLR-6 cooperate to recognize MALP-2 and TLR-6 appears to confer the ability to discriminate between the NH₂-terminal lipoylated structure of MALP-2 and lipopeptides derived from other bacteria (266). Further investigation of TLR-2 and TLR-6 signaling is an important point for our future study since it is possible that alterations in TLR-2 expression or its signaling components by various environmental chemicals may facilitate the recognition and response to various microbial-derived components. This would render the exposed cells more sensitive to the cell-activating inflammatory properties of normal flora of pathogens compared to the cells in the absence of chemical exposure. The potential pitfall for this theory is that the effects observed with infection with live *M. fermentans* may be only partially TLR-2 dependent and additional TLRs may be involved. If the role of TLR-2 in mycoplasma-induced effects turns out to be small, the downstream signaling mechanisms common to all TLRs should be investigated. Among the four adaptor proteins, MyD88 is a good approach since it represents a common signaling protein recruited to all TLRs and is required for subsequent protein interaction that lead to NF- κ B activation.

4.4. Metal interaction

Another important issue to be discussed is the possible synergistic interaction between metals. ROFA does not contain significant amounts of organic components, but does contain relatively high quantities of transition metals. Metals cannot be destroyed by combustion, but are redistributed throughout the solid exhaust material. V, Fe, Ni, Zn, Cu and Co are typically the metals found at highest concentrations and appear to play a substantial role in acute toxic effects

on alveolar epithelial cells (176;280). Three metals, namely, Fe, V, and Ni, are present in the largest quantities, and the amount of each metal can vary depending on the source of the particles. Although particulate matters contain a mixture of chemicals, most toxicology studies investigate adverse responses to individual chemicals, not mixtures of chemicals. The traditional assumption, in the absence of further information, has been that if the individual chemicals have the same health end-point, then the chemical components of a mixture have mutually independent effects and the toxic response to multiple chemicals is additive. But in fact, some metal-metal interaction induces a synergistic effect related to health problems. Since our present study found that nickel component in ROFA is important to the end point of inducing proinflammatory cytokine release, we will discuss some metal-metal interactions related to nickel.

4.4.1. Nickel and Iron

In some ROFA preparation, nickel content can comprise up to 35% of the amount of all metals (149;150), and it was recently shown that it is water-soluble nickel that is responsible for the majority of pulmonary injury caused by ROFA (158). Moreover, pulmonary inflammation induced by ROFA was reproduced by intratracheal instillation of a mixture of soluble forms of nickel (132). Iron is another important PM component and some of the pathological effects after PM inhalation may be due to reactive oxygen species produced by iron-catalyzed reactions. For example, it has been suggested that iron could induce IL-8 production in A549 cells due to reactive oxygen species induction (132;281). Thus, it is clear that either iron or nickel alone can produce biological effects. However, there are very few investigations related to the effects of

co-exposure of these two metals. Recently it was found that nickel is an activator of heme oxygenase, the rate-limiting enzyme in heme catabolism. Ni^{2+} may also regulate and increase iron absorption through a mechanism that simulates hypoxic conditions in the tissues (282). But nickel can induce different signaling pathways with or without interference with iron metabolism and in some cases the excess of iron in PM could inhibit the effects of nickel (75).

4.4.2. Nickel and Cobalt

Nickel and cobalt are two metals that are frequently found together in workplaces such as mines, smelters, and cobalt alloys (283). For example, cobalt is a byproduct of nickel ore processing, cobalt alloys often include nickel (284), and cobalt shortages have forced hard metal manufacturers to substitute pure cobalt with mixtures of nickel and cobalt in the process of sintering (285). No epidemiology studies have established a link between inhalation of cobalt and lung cancer in humans, although cobalt can cause allergic asthma or ‘work-related wheeze’, which may lead to interstitial fibrosis (284). Of note is the fact that cross-reactivity between Ni and Co is often observed in workers with “hard metal asthma”. In animal models, cobalt sulfate (CoSO_4) aerosol can induce alveolar and bronchiolar tumor formation and can cause reductions in lung compliance (286). Although studies such as those mentioned above have investigated the toxicity of nickel and cobalt alone, little is known about the toxic effects of mixtures of nickel and cobalt on most tissues and organs. But still, some studies suggest that inhalation exposure to mixtures of nickel and cobalt chlorides affect certain aspects of pulmonary morphology in a synergistic manner (287;288).

4.4.3. Nickel and Vanadium

An *in vivo* study of the effects of ROFA-associated transition metals demonstrated that intratracheal instillation of V caused immediate effects, while instilled Ni was responsible for a delayed response (64). Moreover, certain interactive effects occurred when these metals were administered in combination, including an apparent synergism between Ni and V and an attenuating effect from Fe. Specifically, Ni exacerbated the immediate response to V in a more than additive manner, while Fe coexposure reduced the effects of both V and Ni + V instillation. Although the mechanisms of this synergy is not very clear, it is conceivable that at low lung burdens, Ni and / or V could produce oxidative stress that would not pose a significant problem for the defense and repair pathways of a healthy cell (63). However, generation of radicals by V could exacerbate the outcome of Ni-induced cell cycle arrest. Either or both of these mechanisms could play a role in causing the synergistic increases in pulmonary inflammation, bradycardia, hypothermia, arrhythmogenesis, and fibrinogen levels. These results indicate a possible synergistic relationship between inhaled Ni and V, and provide insight into potential interactions regarding the toxicity of PM-associated metals (63).

5. SUMMARY AND FUTURE DIRECTION

In the present study, a new discovery is the synergistic interaction between *M. fermentans* and ROFA in producing proinflammatory cytokine IL-6 release by human lung fibroblast cells. This discovery was later extended to include the interaction between *M. fermentans*-derived macrophage activating lipopeptide (MALP-2) and transition metal component nickel sulfate to stimulate IL-6 cytokine production to an extent greater than the additive sum of the responses to each stimulus alone. Part II of the thesis focuses on the potential MAP kinase pathway involvement in the synergistic interaction between the two stimuli. The very first study in this part is the time course of NiSO₄ and MALP-2 interaction which suggested that one stimulus potentiates another one to inducing IL-6 release in later time points. Later on, it was confirmed that NiSO₄ pretreatment facilitated MALP-2-induced IL-6 production and this potentiating effects is partially abolished by MER/ERK1/2 inhibitor. Even though NiSO₄ induced ERK1/2 and JNK phosphorylation, it seems only ERK phosphorylation is related to NiSO₄'s potentiation effects. In the third part of this study, IL-6 gene regulation was investigated in order to explore the possible mechanism of this unique synergistic interaction. Studies show that NF-κB is very important to the basal IL-6 gene expression in human lung fibroblasts, while NF-IL6 is important for the interaction between NiSO₄ and MALP-2. However, the same degree of synergistic interaction between NiSO₄ and MALP-2 seen with IL-6 protein is not observed at gene expression level, which suggests other mechanisms are likely involved. One of the possibilities is that either stimulus alone or together cause alterations in IL-6 mRNA stability. Other possibilities include the involvement of other signaling pathway kinases like MyD88 or TRAF6 that are separate from MAPK pathway since blocking MAPK pathway can not fully absolutely

abolish the synergistic interaction between NiSO₄ and MALP-2. Regulation of the expression of TLR family members is another issue to be addressed. It is possible that NiSO₄ or other metals facilitates MALP-2-induced IL-6 or other cytokine production by increasing toll-like receptor expression or function and need to be studied. Oxidative stresses induced by ROFA and *M. fermentans* may also play an important role in the interaction. Also, it will be important to develop an animal model that allows the in vivo study of the possible pulmonary injury / inflammation repair processes in response to the co-exposure of microbial and chemical stress.

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University of Pittsburgh

Pittsburgh, PA 15260

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