INVESTIGATING THE IMMUNOMODULATORY POTENTIAL OF METALS PRESENT IN SUDBURY PARTICULATE MATTER (SPAM)

By

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Abstract

The incidence of allergic disease has risen dramatically over the last century. This increase cannot simply be attributed to genetics, and so environmental contributions to allergic disease must be considered. Airborne particulate matter is recognized as a contributing factor in the initiation and exacerbation of allergic respiratory diseases due to its ability to act as an adjuvant and irritant. Particulate matter is heterogeneous in composition, and different components contribute to its adjuvant effects, including metals. This thesis investigates whether metals contained in Sudbury Particulate Matter (SPaM) are potential immunomodulators. We investigated the ability of metals in SPaM to influence inflammation both *in vivo* and *in vitro*. We demonstrate that the metals are able to alter the inflammatory profile after physiologically relevant exposure levels, suggesting that some of the metals present in SPaM are immunomodulatory in nature and may influence allergic disease.

Key Words

Particulate matter, inhalation tolerance, Sudbury particulate matter, nickel, copper, estrogen, adjuvant

Co-Authorship Statement

Both of the manuscripts contained in this sandwich-style thesis are multi-authored, and so this statement documents the contributions each of the authors made to this work:

For Chapter 2, my contributions to the work were as follows:

- Principal responsibility for the design, execution, analysis, and interpretation of all experiments, with consultation and input from my supervisor, Dr. Stacey Ritz;
- Principal responsibility for the writing and preparation of the manuscript, with consultation and input from Dr. Ritz.
- Dr. Sandhya Khurana was available for consultation and assisted with the blood and tissue collection from the *in vivo* experiments in addition to preparing the splenocyte cultures.
- Sebastien Lefebvre assisted with the daily *in vivo* exposures in addition to tissue collection and splenocyte culture preparation.

For Chapter 3, my contributions to the work were as follows:

- Principal responsibility for the design, execution, analysis, and interpretation of all experiments, with consultation and input from my supervisor, Dr. Stacey Ritz;
- Principal responsibility for the writing and preparation of the manuscript, with consultation and input from Dr. Ritz.

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CHAPTER 1: INTRODUCTION

1.1 Adverse health effects of air pollution: setting the stage

The London Smog of the 1950s shone a light on the dangers of air pollution and prompted an active interest in studying its detrimental health effects. Over 2400 people died from the complications due to the increased levels of black smog that covered the city [1]; thirty years later, epidemiological studies linked the mortality rate primarily with the presence of sulfur dioxide (SO_2) levels present in the smog [1]. For many years, researchers and air quality control measures' focus remained on SO_2 levels, smog, and acid rain, but over time, the adverse effects of particulate matter (PM) became more difficult to ignore. The first regulation for particulate air pollution in Canada was the National Ambient Air Quality Objective (NAAQO) for Total Suspended Particles (TSP) which was established in the 1970s [2]. Similarly, the United States Environmental Protection Agency (US EPA) was born to enforce the new regulations regarding pollution [3] under the Clean Air Act. In 2006, the World Health Organization (WHO) updated the guideline exposure for PM_{10}, reducing the annual guideline exposure to the lowest values to date [4].

In 2008 it was estimated that 25 000 Canadian deaths annually were associated with environmental pollutants [5]. In Ontario alone, the Ontario Medical Association (OMA) estimates that in general, 5900 deaths annually can be attributed to environmental pollutants, including ambient air pollution [5]. Further to this considerable loss of life, there is a large economic burden on the health care system as a result of diseases that are attributed to environmental hazards. In Canada approximately 9 billion dollars in health care costs are attributable to the health effects of environmental pollutants each year [5]. Worldwide, large cohort studies have consistently demonstrated that there is a significant correlation between

increases in PM and cardiopulmonary morbidity and mortality [6]. PM has also been linked to lung cancer, heart disease, chronic obstructive pulmonary disorder (COPD), asthma, allergies, decreased lung development, myocardial infarctions [6] and various autoimmune diseases such as rheumatoid arthritis and lupus [7]. While the correlation is not as strong, there are emerging studies that suggest that PM levels increase the risk of suicide [8, 9] and a positive trend regarding the exacerbation of schizophrenia [10] . Of particular interest for this study, many investigations have examined the relationship between exposure to ambient air pollution and atopic (IgE mediated) diseases such as allergy. European studies have consistently found an increased incidence of allergies and asthma in urban populations compared to those from rural areas [11, 12]. Epidemiological studies have shown a correlation between traffic air pollution, which makes up a large portion of air pollution, and the development of atopic disease and allergic sensitization in children [13, 14]. Thus it is clear that despite years of regulations and research, PM continues to be a major contributor to increased economic burden and increased morbidity and mortality for a variety of health outcomes.

1.2 Particulate matter: small but mighty - definitions and sources

Ambient particulate matter (PM) is made up of both solid and liquid particles from natural and anthropogenic sources and is a major component of air pollution [15]. PM is created by one of two methods: either emitted directly from the source (primary) which includes automobiles, combustion and industrial processes, or produced by chemical reactions in the atmosphere (secondary) [15].

PM is often categorized by size. Coarse PM (PM_{10}) is the respirable fraction of total suspended particulates (TSP), and is defined as having a diameter equal to or smaller than 10 μm in size and is able to deposit in the upper branches of the bronchioles, where it is usually expelled as a cough, spit or swallowed. PM_{10} is composed of fly ash (combustion-generated particles), dust from a variety of sources, and oxides elements from the Earth's crust [16]. Epidemiological studies focusing on PM_{10} have consistently demonstrated an association between increases in morbidity and mortality and levels of PM [17], with an average 1% increase in total mortality for every 10 $\mu g/m^3$ increase in ambient PM_{10} concentrations [18].

Fine particulates ($PM_{2.5}$) are those having an aerodynamic diameter of 2.5 μm or less. $PM_{2.5}$ is made up mostly of metals, sulfate, nitrate, ammonium and carbonaceous materials that are adsorbed to the particle surface [19]. Worldwide $PM_{2.5}$ is estimated to be the cause of 800 000 premature deaths annually and 3% of all cardiopulmonary deaths [20]. $PM_{2.5}$ is thought to have a greater potential for toxicity than PM_{10} because its smaller diameter allows it to penetrate deeper into the lung and deposit in the alveolar areas. In addition, the smaller diameter also results in a greater surface area-to-mass ratio, so that a higher concentration of toxic chemicals can be transported per unit mass.

Ultra fine particles (UFP) are defined as those having a mean aerodiameter of less than 0.15 μm. Similar to $PM_{2.5}$, because of the greater surface area per unit mass, it is believed that more chemicals can adsorb to UFPs resulting in their toxicity and adjuvanticity [21]. Their small size also allows them to penetrate deep within the lung in greater amounts which would also add to the toxicity of these particles. In addition, there is evidence to suggest that UFPs are able to cross the alveolar membrane and enter the bloodstream directly [1] and travel to organs [22]. Particles that are able to enter the bloodstream may increase the activity of clotting factors which can lead

to an increased risk of ischemic heart disease [1], and thereby contribute directly to cardiovascular morbidity and mortality associated with ambient PM [22].

1.3 Models of PM used in research

There are several different models that are commonly used in experimental laboratory investigations of PM including but not limited to diesel exhaust particles (DEP), Ottawa dust (EHC-93), and residual oil fly ash (ROFA). One of the major differences between these models of particulates is their chemical composition, which is also a driving influence regarding their adjuvant capabilities for environmental antigens. DEP are generated from the exhaust of diesel engines, and are comprised mostly of carbon which is different than other particulates such as EHC-93, SPaM and ROFA which have extensive levels of metals present. DEP has been shown to have different composition and inflammatory profile depending on the engine source of the particulate [23]. EHC-93 is ambient particulate matter collected from the filters of the Environmental Health Centre in Ottawa ON [24], and has been shown to cause inflammation, which is due to the soluble metals [25], such as zinc [26], which makes up a large percentage of EHC-93. ROFA has a different composition profile which can be attributed to the oil fuel burning industry where ROFA is collected [27]. ROFA tends to be metal rich with high levels of iron and nickel making it another nickel rich model of PM. Despite the differences in metal concentrations, and chemical profiles for each of the models used, they are all able to induce inflammation and pulmonary toxicity and act as adjuvant for allergic sensitization among other health effects in animal models.

1.4 Sudbury Particulate Matter (SPaM)

Sudbury, Ontario has a global reputation for its nickel mining, refining, and smelting industry. Nickel is produced from sulfidic Ni/Cu ores using the Orford process [28]. Airborne emissions generated from this process contribute to the unique chemical profile of airborne PM in Sudbury. A sample of this 'Sudbury Particulate Matter' (SPaM) was collected in 2006 [29]. Total PM was collected, sieved to isolate the fraction less than 38 μm, and physically and chemically characterized [29]. Not surprisingly, compared to other types of PM, SPaM had a distinct chemical profile, with increased levels of metals such as nickel, copper, iron and cobalt (Table 1.1). In these studies, we decided to focus on Ni and Cu because of the relatively high levels that are present in SPaM as compared to other types of PM commonly used in research (Table 1), and because of community concern about local pollutants.

Table 1.1. Composition of DEP, EHC-93, SPaM (modified from [29]), **and ROFA** (modified from [30])**.**

	Diesel Exhaust Particles DEP (μg/g)	"Ottawa Dust" EHC-93 (μg/g)	"Sudbury PM" SPaM (μg/g)	Residual Oil Fly Ash ROFA (μg/g)
Cobalt	<20	5	77	Trace
Chromium	50	42	66	Trace
Copper	100	845	1066	Trace
Iron	300	14900	30202	122 000
Manganese	<10	483	230	Trace
Nickel	<50	70	1653	38 400
Titanium	50	929	952	n/a
Zinc	400	10400	1019	5340

Nickel

Nickel Ni(II) or Ni^{2+} is a ubiquitous metal found in the environment, but is not currently considered an essential element as it has not been found to be a part of any enzyme or protein in

complex organisms [28]. Ni is a component of many commercial products, and is also found in ambient PM from many locations, largely due to the Ni recycling industry [31] and due to industrial practices such as smelting and mining [28]. Ni can also be found in water, soil, and certain foods such as nuts, spinach and cocoa [32], resulting in Ni exposure being relatively unavoidable.

The toxicity of Ni is associated with its solubility. Soluble Ni(II) compounds are able to easily diffuse through the cell membrane, while insoluble forms are phagocytosed by macrophages [33]. Once in the bloodstream Ni is able to bind to albumin [34] and translocate from the initial source of absorption. In general, Ni(II) is able to facilitate the production of reactive oxygen species (ROS) which alters the GSH:GSSG levels within the cell, leading to oxidative stress. ROS are able to activate transcription factors that are related to inflammation. When exposed to soluble Ni(II), the IKK2/IκBα/NF-κB signalling pathway is activated [35], leading to the transcription of genes that result in the production of cytokines, chemokines, and adhesion molecules that are involved in immune responses [36, 37]. Indirect DNA damage can be caused by Ni(II)-induced inflammation and the release of oxygen radicals from immune cells [38], such as macrophages. In general, macrophages that have been exposed to Ni experience a decrease in phagocytic ability [33]. Ni is also able to alter immune responses by altering the expression of co-stimulatory molecules on the surface of murine macrophages resulting in more efficient antigen presentation and subsequent T cell activation [39]. Ni is also a known carcinogen in all forms except in its metallic form.

There are a variety of sulfidic nickel compounds that exist; in these studies, we focussed on nickel subsulfide, (Ni_3S_2) since it is the predominant form of Ni in SPaM due to the industrial methods used for the extraction and purification of Ni [18]. At the subcellular level, Ni_3S_2 has

been shown to be able to form an oxygen or hydrogen peroxide complex that is able to directly cause DNA damage [38] leading to toxicity. With respect to the respiratory tract, low levels of inhaled Ni_3S_2 were shown to induce leukocyte infiltration to the lungs and decreased the viability of alveolar macrophages [40] and inhibit their phagocytic ability [33].

Copper

Copper (Cu) is an essential element that is required for enzymes and cofactors, although excess Cu can also cause toxicity. Cu is found in several different kinds of ambient air pollution [26], and is at higher levels in SPaM than in other particulates often used in research (Table 1.1) [29]. The presence of Cu in ambient PM samples is believed to be partially due to brake wear from automobiles [41]. In experimental settings, soluble Cu as $CuSO_4$ is used to represent Cu found in ambient PM, focusing primarily on the impact of Cu toxicity in the lung [26, 41, 42]. Interestingly, Cu when encountered by the immune cells of the lung would more than likely be in an ion or particulate form, which differs from systemic Cu that would be bound to a complex or a transporter [43]. Free Cu as found in the lung, would allow Cu to participate in the Fenton Reaction, resulting in ROS and oxidative damage.

Part of the toxicity associated with Cu is due to its ability to switch between oxidation states, allowing Cu to participate in the Fenton Reaction. Products from the Fenton Reaction are oxygen radicals, resulting in oxidative damage. Cu has also been shown to increase levels of metallothionein 1 (MT-1) in the lung which is able to sequester free radicals [41]. Cu can also induce inflammatory responses through the activation of NF-κB, triggering the production of proinflammatory cytokines such as IL-6 [44], local neutrophil and macrophage infiltration and systemic increases in white blood cells [41, 42]. Acute lung injury due to instillations of Cu

resulted in increased levels of proteins measured in the bronchoalveolar lavage fluid [41]. These data suggest that Cu as a part of ambient PM is able to induce acute inflammation resulting in both local and systemic responses.

1.5 Proposed mechanisms of PM health effects

There have been several proposed mechanisms by which PM is able to induce inflammation and subsequent health effects. One of the most extensively investigated is the production of reactive oxygen species (ROS) via several different interactions with cells in the lung. There are a variety of antioxidant mechanisms that exist to maintain the redox homeostasis of the cell. When these systems become overwhelmed and the balance cannot be maintained, ROS are able to induce a cascade of biological effects. Metals that are adsorbed to the surface of ambient PM, such as iron and copper, are able to react within redox cycling reactions such as the Fenton Reaction to produce hydroxyl radicals [15]. PM that is not phagocytosed by resident alveolar macrophages is able to cause oxidative damage to the epithelium of the lung, resulting in the upregulation of proinflammatory cytokines which recruit macrophages and neutrophils [45]. Alternatively, macrophages are also activated and produce ROS by phagocytosing PM. A 3-tier model has been proposed through which cells cope with the oxidative stress induced by exposure to particulates [36]. The first line of defense is antioxidant enzymes, which are activated by the transcription factor Nrf2. These antioxidant enzymes such as HO-1 and superoxide dismutase and are meant to try and establish redox homeostasis within the cell. If the balance cannot be restored, which is detected by ratios of GSH:GSSG, then the second tier would be triggered, in which activation of MAPK and NF-κB pathways would lead to the up-regulation and release of

cytokines and chemokines influencing inflammation. Finally, if these mechanisms fail to mitigate oxidative stress, cell signaling to initiate apoptosis or necrosis is activated, resulting in cell death.

PM has also been shown to induce inflammation via activation of toll-like receptors (TLRs) on innate immune cells. TLRs are conserved pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) which are unique to each of the 10 TLRs currently recognized in humans [46]. The triggering of TLRs on antigen presenting cells (APCs) will result in the upregulation of transcription factors that are responsible for the regulation of proinflammatory cytokine production. Coarse PM has been shown to activate TLR4 in macrophages *in vitro*, while fine PM is involved in the induction of TLR2 in alveolar macrophages, airway epithelial cells and human embryonic kidney (293) cells [19, 47].

Ambient PM activates both the oxidative stress and TLR pathways in the lung and induces the production of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and GM-CSF [48], resulting in the inflammatory process of recruitment and maturation of APCs. Adhesion molecules (ICAM-1, VCAM-1) are upregulated on endothelial cells, leading to the recruitment of leukocytes into the lung [49], resulting in a greater inflammatory response. In addition, these proinflammatory cytokines are able to enter into circulation to produce systemic effects. These cytokines are known to stimulate the bone marrow to produce more leukocytes and platelets [48], a process called leukocytosis, which are then recruited back to the lung leading to increase inflammation and output of inflammatory mediators. This will recruit more phagocytes to clean up the PM that would be the root cause of the inflammation and ROS. There are a variety of Th2 cytokines that are associated with NF-κB signalling such as IL-4, IL-5, IL-13 in addition to other cytokines such as GM-CSF which can act with Th2 cytokines to enhance APC recruitment,

antigen presentation and polarization towards Th2 responses. PM associated oxidative stress leads to the increased expression of CD80/CD86 on APCs which can then more efficiently activate CD4+ T helper cells [36].

Systemically, IL-6 is able to stimulate the liver to produce acute phase response proteins, which is a sign of toxicity. Acute phase proteins such as C-reactive protein (CRP) and fibrinogen are able to increase the ability of the blood to coagulate which can be a risk factor for individuals with heart disease [1, 50]. Furthermore, endothelial cells are impacted by systemic inflammation from PM resulting in their disruption which can dislodge plaque formation [50] in blood vessels resulting in a potential stroke.

The local and systemic inflammatory response stemming from PM exposure can lead to exacerbations of already existing conditions such as chronic obstructive pulmonary disease (COPD), asthma and allergies, where inflammation and immune mediators may affect respiratory physiology and cause difficulties breathing, and may lead to the development of new allergic sensitizations and disease.

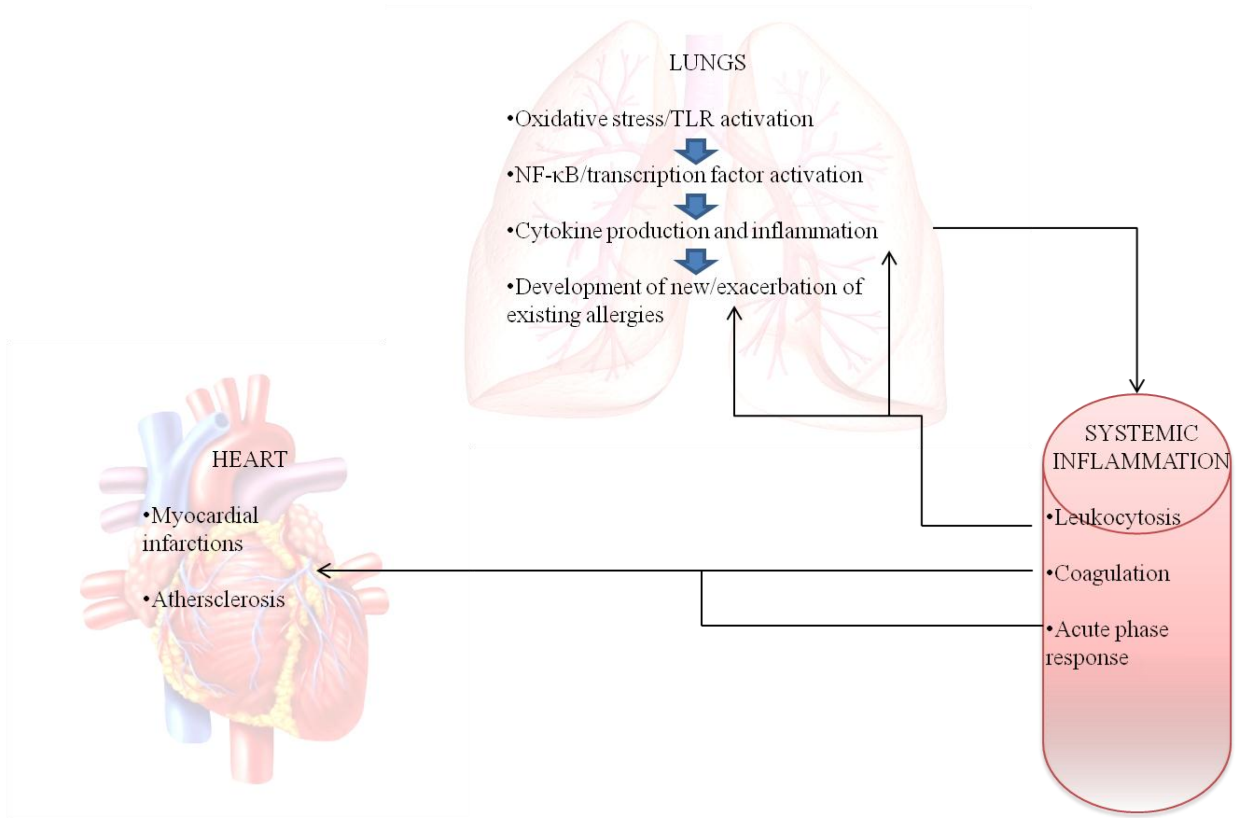

Figure 1.1. Cardiopulmonary PM health effects (modified from [6]).

1.6 Particulate air pollution as an adjuvant for allergic responses

Mechanisms of allergic responses

The respiratory mucosae are continuously exposed to a variety of different antigens which are present in the 10 000-20 000 L of ambient air we breathe in each day [45]. Although there are physiological barriers present which remove the majority of inhaled particles, some still

penetrate these barriers and reach the lower areas of the respiratory tract [51]. The immune system of the respiratory tract must continuously and actively regulate responses towards these antigens, as superfluous immune-inflammatory responses to harmless antigens would impair gas exchange. In the majority of individuals, responses to inert antigens remains highly regulated and under control. However, this process is not infallible, and when tolerance to harmless environmental antigens is not induced, allergic sensitization can result. It is estimated that 15% of the population in industrialized countries suffers from an allergy [52, 53], and the prevalence has increased two-fold over recent decades [54]. Allergic sensitization generates a population of B and T cells specific for the allergen, producing antigen-specific IgE and Th2 memory cells; in sensitized individuals, aeroallergen exposure triggers IgE-mediated mast cell degranulation, Th2-mediated immune-inflammatory responses (characterized by cytokine production, including IL-4, IL-5, and IL-13), blood and tissue eosinophilia [53, 55], leukocyte infiltration into the airways, and airway hyperresponsiveness [55]. This section will focus on the mechanisms of allergic responses and cover the important immune interactions that lead to the development of Th2 responses, IgE and eosinophilia and how they are related to allergy

Immature dendritic cells (iDCs) and alveolar macrophages are antigen presenting cells (APCs), which are able to take up antigens, presenting them and providing signals to naïve CD4+ T helper (nTh) cells, which have not yet encountered an antigen (Figure 1.2). nTh cells circulate through the peripheral blood and return to the draining lymph node where they will encounter and sample self–peptide/MHC. This process is repeated through different lymph nodes until such a time as it encounters an antigen peptide, for which it is specific, presented by an APC such as a dendritic cell or macrophage [56]. T cell activation from a naïve state is dependent on T cell receptor (TCR) stimulation with sufficient antigen from a mature DC. If a naïve T cell

recognizes the displayed peptide during the sampling process that occurs in the lymph node, the nTh will activate and differentiate into its effector subtypes by clonal expansion. Naïve T cells have stringent requirements for activation, and two signals are required for the efficient activation of a nTh cell by an APC [57, 58]:

1. Phagocytosed antigens are processed and displayed at the surface of the dendritic cell as peptides in the binding cleft of major histocompatability complex (MHC) II molecules.
2. Stimulation by co-stimulatory molecules, particularly CD40 on APCs interacting with CD40L on T cells, and CD80 and CD86 (B7.1 and B7.2) on APCs interacting with CD28 on T cells.

With the innate immune system sufficiently stimulated as a result of PM exposure, and DCs bridging the innate and the adaptive immune response in order to create antigen specific responses, there are other signals that serve to drive the differentiation of the naïve T helper (nTh) cells along one of several different possible effector pathways. nTh cells are able to differentiate into at least 4 different effector subtypes: Th1, Th2, Th17, and Th3, depending on the local environment stimulus, APC and cytokines present to polarize differentiation. Their differentiation is an intricate signaling even with cross-regulation of transcription factors, STATs and protein-protein interactions. The strength of the TCR-MHC-peptide binding is believed to be important, and in general weak signals promote a Th2 response (but not so low as to promote tolerance or anergy) [59]. The presence of polarizing cytokines in the microenvironment and/or produced by the APC are also important in driving differentiation. APCs that have interacted with PM through TLRs, phagocytic uptake, or indirectly via the effects of ROS, may have an altered cytokine or costimulatory molecule profile, which could influence nTh cell differentiation and polarization [49].

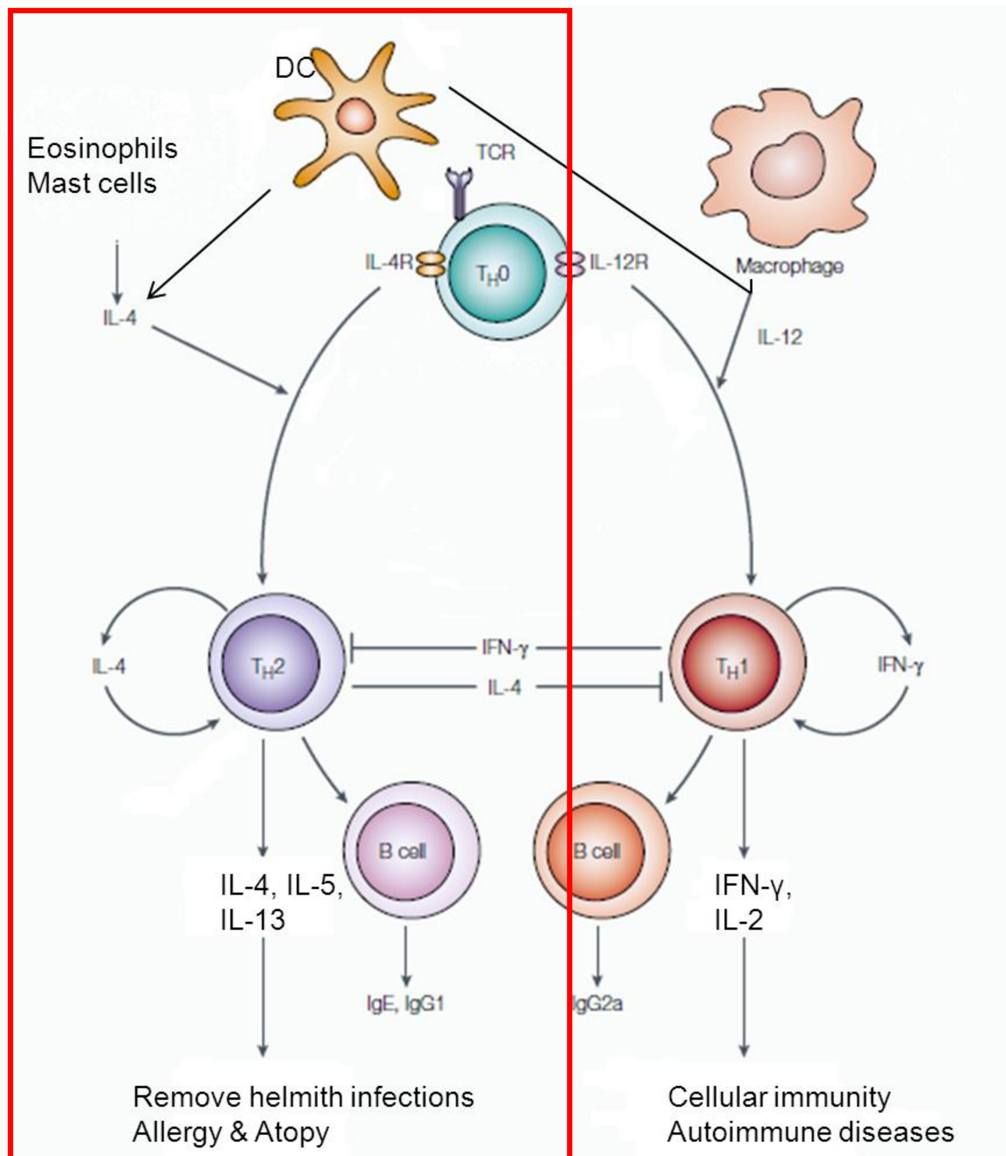

Figure 1.2. Overview of Th1/Th2 differentiation and associated responses (modified from [60]).

Th2

Th2 differentiated cells are critical mediators of allergic responses. Th2 cells are associated with humoral immune responses and are characterized by their production of IL-4, IL-5 and IL-13 among others. From a host defense perspective, Th2 cells are important in the control of

helminth and parasitic infections, which have become less common with modern advances in sanitation. It is hypothesized that with the decreased prevalence of parasitic infections, there is a resulting dysregulation of Th1/Th2 responses, which may contribute to changes in the prevalence of diseases mediated by Th1 and Th2 cells [61, 62]. Not surprisingly, there are multiple cytokines and transcription factors that control the differentiation and function of Th2 cells and their responses. It is worth noting that the differentiation requirement for *in vivo* and *in* vitro are different and due to their dependency on IL-4.

IL-4 influences nTh cells, which can induce Th2 differentiation by activating STAT6 and the transcription factor GATA3 – although the initial source of IL-4 to trigger Th2 responses still has not been identified. After TCR stimulation, IL-2 production and activation of STAT5a, aided by c-maf and Gfi-1, leads to early and low levels of IL-4 and expression of GATA3. IL-4 acting on the IL-4R activates STAT6 which leads to a burst of IL-4 and further expression of GATA3 and STAT5 to polarize and commit to the Th2 lineage (reviewed by [63]).

In vivo differentiation is believed to occur via several different mechanisms; however, the common factor between both *in vivo* and *in vitro* differentiation is the necessary activation of GATA3. GATA3 selectively promotes Th2 differentiation and down-regulates Th1 differentiation [63]. *In vivo* differentiation involves the activation of STAT5a by IL-2 which is an IL-4 independent pathway of differentiation. Investigations with IL-4 k/o mice are still able to induce Th2 mediated responses which would indicate that in the absence of IL-4, Th2 differentiation is able to occur [59, 64]. The cytokines that are important to this investigation and Th2 differentiation will be outlined individually.

Table 1.2. Cytokines involved with Th2 responses

IL-4	IL-4 is the classic cytokine associated with Th2 mediated responses. *In vivo*, studies have shown that IL-4 is not necessary for Th2 differentiation [64], while *in vitro* studies have demonstrated a more prominent role for IL-4 in Th2 differentiation. Innate immune cells both within tissues and recruited to the site of exposure such as mast cells, basophils, and eosinophils among others can be sources of IL-4 [58]. IL-4 is the primary influence for B cells to undergo isotype switching to IgE, a hallmark of allergic inflammation.
IL-5	IL-5 is another classic Th2 cytokine that is responsible for the survival and proliferation of eosinophils [65]. In addition IL-5 secreted by Th2 cells is able to augment the effector abilities of eosinophils.
IL-13	IL-13 is strongly associated with Th2 cells and is a key cytokine in the development of airway hyprresponsiveness. IL-13 has overlapping functions with IL-4 and can also induce degranulation of mast cells, and influence B cells to undergo isotype switching [66].
IL-10	IL-10 at one point in time was considered to be a cytokine involved in the Th2 mediated response. Now, it is considered to be more immunosuppressive in nature, and the signature cytokine of T regulatory cells, Tr1 (see Tolerance section). In addition to being able to suppress immune responses it is also able to inhibit DCs and inhibit Th1 responses by suppressing IL-12.
IL-25	IL-25 is a relatively new cytokine to be added to the Th2 family and is thought to be involved with the expansion of Th2 memory cells and sustaining Th2 responses [67]. IL-25 is involved in promoting Th2 cytokines and antibody responses and is expressed early by the lung epithelium [68]. Eosinophils and mast cells, which are hallmark cells of the Th2 mediated allergic response, are the primary producers of IL-25 [68].

Features of Th2 Responses

IgE and Mast Cell Activation

The adaptive immune system generates antigen-specific responses by highly specialized cells, the B lymphocytes and T lymphocytes. B cells are responsible for the production of

immunoglobulins, and also have the ability to act as APCs. Upon activation, T helper cells interact with B cells, triggering them to undergo isotype switching and change the immunoglobulin (Ig) class produced. In allergic responses, Th2 associated cytokines such as IL-4 and IL-13 mediate istotype switching to IgE, which is the immunoglobulin hallmark of allergic diseases. IgE will bind to the $Fc_{\varepsilon}RI$ receptor with high affinity, and crosslinking by antigen triggers activation of the cell [69]. While the high affinity $Fc_{\varepsilon}RI$ receptor is found on other innate immune cells, mast cells are a very significant contributor to immediate allergic symptoms [70] due to rapid degranulation and the release of histamines.

Mast cells are important in the pathology of allergies. Mast cells are distributed throughout the body and are often found in areas where there is extensive contact with antigens such as the respiratory tract [71]. Once primed by IgE and cross-linked by antigen, mast cells rapidly release mediators such as histamines and proinflammatory cytokines that are principally responsible for the immediate symptoms of allergy, and facilitate recruitment of other leukocytes to the site [72]. Mast cell degranulation products can help consolidate the Th2 phenotype, as histamines are able to suppress IL-12 from DCs, suppressing Th1 polarization and favouring Th2 responses [73]. Mast cell degranulation and the release of histamines results in a variety of symptoms including, but not limited to, runny nose, sneezing, congestion, bronchoconstriction and shortness of breath [74].

Eosinophilic Responses

Eosinophils are important in host defense against parasites, but are often involved in Th2 mediated immune responses, and eosinophilic inflammation is frequently considered a cellular hallmark of allergic respiratory diseases. Activated eosinophils produce ROS and release

proteolytic enzymes, resulting in tissue damage during allergic inflammatory responses [75]. IL-5, secreted by Th2 lymphocytes, is the primary cytokine involved in the differentiation of eosinophils from the bone marrow, while eotaxins are the main chemoattractants that recruit eosinophils to the site of infection [58, 65]. Until recently, eosinophils were regarded solely as an effector leukocyte, however, it is now recognized that they have antigen presenting capabilities and function to enhance Th2 responses during innate and adaptive immunity. In addition to influencing T lymphocytes, eosinophils may polarize resident DCs to a Th2 phenotype by secreting IL-4 [58]. Eosinophils themselves can express MHC class II and costimulatory molecules, and migrate to the lymph nodes, which may allow them to act as APCs promoting Th2 polarization [58, 76, 77].

Tolerance

The immune system has evolved such that it can protect us from a wide variety of pathogens by mounting an appropriate and specific response towards harmful antigens; at the same time, it is equally important that this same system is able to suppress and regulate immune responses towards inert and ubiquitous antigens that it is exposed to on a daily basis. There are multiple body locations at a direct interface with the external environment where immunological tolerance is normally induced against harmless, ubiquitous antigens: the gastrointestinal tract, the respiratory tract, and the oral cavity are particularly notable.

Although inhalation tolerance has been actively studied since the 1980s, the underlying mechanisms are still not entirely elaborated. However, it is clear that tolerance is not simply a passive process in which antigens are ignored; it is an active suppression of specific immune responses towards harmless antigens by a variety of mechanisms. Currently, it is understood that

DCs are a key player in the establishment of tolerance. When immature DCs interact with naïve T cells, and the level of antigen presented is not enough to adequately signal activation and differentiation, the result is T cell neglect and the inactivation of the T cell, anergy [78]. Anergy results in the T cell being unable to respond either partially or completely to stimuli that normally would result in proliferation [79]. This active suppression would result in the inability to mount a response towards the presented antigen, as seen in tolerance [78].

More recently, another class of T cells, called T regulatory cells (Tregs), have been implicated in the establishment and maintenance of immunological tolerance. Tregs are able to regulate immune response by several different methods, including the downregulation of MHC class II molecules on APCs, suppression of the effector functions of CD4+ T cells, suppression of B cell immunoglobulin secretion, and the inhibition of mast cells, eosinophils and basophils [reviewed by 80]. Several types of Tregs have been identified, including CD4+CD25+ FoxP3+ "natural Tregs", and IL-10 producing Tr1 cells [80, 81]. The Tregs that are associated with tolerance to allergens are primarily the CD4+FoxP3+ 'natural' Tregs; they are considered to be "natural" (nTregs) in that they originate in the thymus and are considered to be functionally mature, as they do not need to differentiate to gain effector function. The transcription factor FOXP3 is the major transcriptional regulator responsible for nTregs [56] as a lack of this transcription factor resulted in the loss of immunosuppressive effects. They are able to actively suppress immune function by interacting with APCs or effector T cells and inhibiting their proliferation and/or cytokine production [80, 81], including the immunosuppressive cytokines IL-10 and TGF-β [56, 81, 82].

The Tr1 subset of T regulatory cells differentiates in the presence of IL-10 secreted from a DC [82]. This adaptive Treg secretes high levels of IL-10 and moderate levels of TGF-β, suppressing

both Th1 and Th2 [82]. Interestingly, Tr1 secrete no detectable levels of IL-2 or IL-4 and are poor proliferators [83]. A study by Cottrez et al. in 2000 investigated the ability of Tr1 cells to regulate allergic immune responses *in vivo*, and they observed that Tr1 cells are able to inhibit IgE and Th2 responses by secreting high levels of IL-10. These immunoregulatory systems described result in tolerance.

An experimental mouse model of inhalation tolerance has been established; in the initial description, ovalbumin (OVA) was passively administered as an aerosol for 8 weeks, followed by IP challenges with OVA, resulting in blunted OVA-specific IgE responses [84]. Thirty years later the principle of this model is still employed; however, the details of the experimental protocol have been altered by various groups, with most using a 10-day exposure protocol. As previously mentioned, tolerance induction is what would normally be expected as a response to ubiquitous harmless antigens; although this is not always the case. To experimentally determine how tolerance is subverted, tolerance models are generally used in conjunction with protocols that induce allergic airway inflammation to determine the difference in induction and development of either tolerance or allergies and asthma. Although protocols vary, in general the results are similar: in mice previously exposed to aerosolized OVA, sensitization to OVA is blunted, and there is a reduction of immune-inflammatory responses in the lung after OVA challenge and a decrease in OVA-specific IgE.

In general, what all of these models of inhalation tolerance have in common is that respiratory exposure to an aerosol of OVA is able to prevent the development of allergic sensitization when the mice are later subjected to protocols which would normally induce sensitization.

PM as an adjuvant for allergies

Immunologically speaking, an adjuvant is a substance that enhances the immune response to an antigen. Most often we think of adjuvants as substances that are used to deliberately stimulate immune responses, for example the use of alum as an adjuvant in some vaccines. In these cases, the adjuvant is used to successfully stimulate a strong innate immune response with less antigen present, resulting in a stronger adaptive immune response with memory for subsequent exposures [85], which is advantageous in a case like vaccination. However, some substances can serve as adjuvants to trigger undesirable immune responses as well.

The idea that particulate air pollution might serve as an adjuvant for allergic disease is supported by both epidemiological and experimental evidence. Children who live in rural farming area have a significantly lower prevalence of outdoor allergies than children from urban environments [88]. Studies have also shown a significant positive correlation between allergic sensitization and proximity to roadways and traffic pollution [13, 89]. The connection between PM exposure and the development of allergy has been repeatedly demonstrated experimentally using murine models of OVA exposure, in which exposure to PM was able to act as an adjuvant for responses to OVA, including, DEP [90], and ROFA [91-93] ambient PM [91, 94, 95-97]. DEP is comprised mostly of carbon which is different than other particulates such as EHC-93, SPaM and ROFA which have extensive levels of metals present. Part of the adjuvanticity of PM is believed to be related to the ability of the PM to induce ROS [21].

1.7 Hypothesis and Objectives

While the majority of the focus with respect to previous research on allergy and PM has been on the whole particulate, in our investigation, we were interested in examining the immunomodulatory effects of the metal constituents of SPaM, using doses of these metals that were relevant for real-life exposures. We specifically chose Ni and Cu because of their high content in SPaM and relevance to local concerns, but note that they are also present in other types of PM, such as ROFA, that have been shown to have adjuvant potential. We used both *in vivo* and *in vitro* experiments in order to examine different aspects of these effects. We had 2 main objectives:

1. In an *in vivo* murine model, to examine the ability of metals present in SPaM to subvert tolerance and act as an adjuvant for allergic respiratory sensitization.
2. Using primary human naïve CD4+ T cells *in vitro,* to examine whether Ni would alter the profile of cytokines produced by the T cells after activation.

In addition, I had the opportunity to examine an aspect of sex in an experimental design thanks to a scholarship from the CIHR Team in Gender, Environment and Health. We chose to incorporate a hormone, estrogen, to represent one aspect of the hormonal milieu, adding this new component to the second objective (which is further discussed in Chapter 3). Examining an aspect of sex extended the objective of the *in vitro* work to include the following:

3. Examine whether the presence of estrogen will alter the inflammatory profile of the primary human naïve CD4+ T cells *in* vitro.

We hypothesized that metals present in SPaM would have the ability to alter immune responses in ways that would be conducive to the development of allergic respiratory disease.

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CHAPTER 2: INHALATION OF NICKEL AND COPPER INDUCE INFLAMMATION AND DISTINCT CYTOKINE PROFILES AFTER CONCURRENT EXPOSURE TO A HARMLESS ANTIGEN IN A MOUSE MODEL OF INHALATION TOLERANCE

The following manuscript investigates how the development of inhalation tolerance can be subtly altered by low levels of nickel and copper. We investigated both local and systemic immune responses by looking at bronchoalveolar lavages, leukocyte profiles in the lung, OVA-specific immunoglobulin levels and cytokines present after splenic challenge with OVA.

From these investigations we determined that: (i) nickel is able to subtly influence the inflammatory profile of OVA from a normal tolerance response to a Th1-like profile; (ii) Cu induced mild eosinophilia infiltration and mild changes in inflammation , although not to the same extent as if tolerance had been totally subverted; (iii) metals present in SPaM are able to induce mild changes in inflammation, cell infiltrate and cytokine response that could lay the ground work for potential allergic sensitization.

This manuscript has been prepared for submission to *Inhalation Toxicology*. I am responsible for the design, execution, analysis and interpretation of all experiments with consultation and input from Dr. Ritz. I also wrote and prepared the manuscript with edits from Dr. Ritz. My co-authors were responsible for the preparation of splenocyte cultures, provided assistance during tissue collection and during the administration of the treatments to the mice (Dr. Sandhya Khurana and Sebastien Lefebvre). I acknowledge that the OVA-specific ELISAs were developed and performed at the Jordana Lab at McMaster University.

Inhalation of nickel and copper induce inflammation and distinct cytokine profiles after concurrent exposure to a harmless antigen in a mouse model of inhalation tolerance.

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List of Abbreviations

AM – alveolar macrophage

ANOVA – analysis of variance

APC – antigen presenting cell

BAL – bronchoalveolar lavage

Cu – copper

DEP – diesel exhaust particulate

ELISA – enzyme-linked immunosorbent assay

GM-CSF – granulocyte macrophage colony-stimulating factor

IFN – interferon

Ig – immunoglobulin

IL – interleukin

IP – intraperitoneal

JAK/STAT – Janus kinase/signal transducer and activator or transcriptor

NF-κB – nuclear factor

Ni - nickel

OVA – ovalbumin

PBS – phosphate buffered saline

PM – particular matter

PMN – polymorphonuclear cell

ROFA – residual oil fly ash

ROS – reactive oxygen species

SEM – standard error of the mean

SPaM – Sudbury particulate matter

TLR – toll-like receptor

TGF – transforming growth factor

TNF – tumor necrosis factor

Th – T helper

2.1 Abstract

Background/Objective: Airborne particulate matter (PM) has been implicated as one factor in the increasing prevalence of allergic respiratory disease. PM is heterogeneous in nature with its chemical composition depending on its origin, and some constituents of PM have been identified as having adjuvant capabilities. Nickel (Ni) and copper (Cu) are present at relatively high levels in Sudbury Particulate Matter (SPaM), which is reflective of local environmental factors. In this study we investigated the adjuvant capabilities of Ni and Cu in a mouse model of inhalation tolerance.

Materials and Methods: Balb/c mice were exposed to a 1% ovalbumin (OVA) aerosolization for 10 days, with concurrent intranasal instillation of Ni_3S_2 (250 ng), $CuSO_4$ (266 ng), or saline as control. Airway inflammation, cytokine production, OVA-specific immunoglobulins, and splenocyte recall responses were evaluated immediately after the tolerance protocol (on day 13), and also after recall challenge.

Results: Mice that were exposed to Ni or Cu and OVA had increased levels of leukocytes in the BAL, including a mild eosinophilia. *In vitro* splenocyte cultures showed significant differences ($P < 0.05$) in a variety of cytokines associated with both Th1 and Th2 mediated inflammatory responses. However, levels of serum IgE were barely detectable in all groups measured.

Conclusion: Mice exposed to OVA in the context of concurrent Ni or Cu exposure developed unique profiles of inflammation that were distinct from one another and also from the OVA only group. While exposure to these doses of Ni or Cu did not appear to act as an adjuvant *per* se, it is clear that exposure to low doses of these metals was able to induce modest inflammation and cytokine production. We speculate that this may potentially lead to skewing of immune

responses and susceptibility to the development of lung inflammation, which could have relevance for allergic disease.

2.2 Introduction

The prevalence of allergic disease has been increasing over the last several decades (Robinson, Larché & Durham, 2004), and in 1997 an estimated 15% of the population in developed countries were affected (Farber, Wattigney & Berenson, 1997). In 2011, it was estimated that upwards of 40% of the world's population have some form of an allergy (Pawankar et al., 2011). In spite of ubiquitous exposure to aeroallergens, the majority of the population does not develop allergy. This is thought to be largely due to the induction of inhalation tolerance, in which the immune system actively develops mechanisms to suppress responses to harmless antigens to which it is chronically exposed. Experimental models of inhalation tolerance have demonstrated the suppression of inflammation, Th2 cytokine production, and antigen-specific IgE production in tolerized animals, and the induction of subsets of regulatory T cells.

Although there is a genetic susceptibility to allergy, the rise in the prevalence of allergies is partially attributable to environmental factors, and epidemiological studies suggest that exposure to ambient air pollution is among the likely contributors. Higher levels of ambient air pollution have been clearly associated with a rise in hospital admissions and exacerbations of previously existing health conditions, including cardiovascular and respiratory disease (Pope, 2004). In addition, children who live in rural and farming communities have a lower incidence of allergies and atopy than those that live in urban areas, which has been suggested to be related to a reduced exposure to traffic emissions (Braun-Fahrländer et al., 1999; Foster et al., 2001; Morgenstern et al., 2008). Finally, children living in areas of metal-rich PM are also more prone to allergic sensitization (Heinrich et al., 1999). PM has been shown to enhance allergic respiratory disease and act as an adjuvant for the development of allergic respiratory disease in humans (D Diaz-

Sanchez, 1997) and animal experimental models (Gavett et al., 2003; Lambert et al., 2000; Steerenberg et al., 2003; Steerenberg et al., 2004).

Airborne particulate matter (PM) is a heterogeneous mixture of metals, carbon, and other materials from both natural and anthropogenic sources. PM contains both solid and liquid components and is often categorized by its size into coarse (particles <10 μm, PM_{10}) and fine (particles <2.5 μm, $PM_{2.5}$) respectively. Reactive oxygen species (ROS)-induced mechanisms are partially responsible for the PM-induced inflammatory responses, with both coarse and fine PM able to deposit within the lung and induce ROS, leading to the activation of Jak/STAT and NF-κB pathways (Li et al., 2003). Activation of these pathways upregulates cytokine production, resulting in inflammatory responses from epithelial cells, resident and recruited leukocytes, and the activation of antigen presenting cells (APCs). In addition, PM is able to activate immune pathways via TLR2 and TLR4 on alveolar macrophages (Becker et al. 2002; Shoenfelt 2009) which can also activate inflammatory pathways.

PM can differ substantially in chemical composition due to local factors and can be influenced by primary sources such as natural, industrial and vehicular sources and also by seasonal effects (reviewed by Kelly & Fussell, 2012). Metal-rich PM from industrial areas and residual oil fly ash (ROFA) has been shown to have adjuvant capabilities in murine models of OVA sensitization and challenge (Steerenberg et al., 2003, Steerenberg et al., 2005). PM containing Ni and Cu were of particular interest in our inquiries, due to their relatively high concentrations in PM from the Sudbury region (McCartney 2009). There are a number of reasons to think that Ni and Cu could act as immunological adjuvants. Previous studies have established the ability of Ni(II) to act as an immunogen and induce delayed type hypersensitivities directed against itself in humans through TLR4 and NF-κB pathways (Rothenberg 2010; Schmidt et al., 2010), leading

to inflamamation and increased levels of proinflammatory cytokines (Salnikow, Li, & Lippmann, 2004). Low levels of inhaled Ni_3S_2 are able to induce persistent increases of alveolar macrophages in addition to decreased viability of macrophages in rats (Benson et al., 1995; Cohen 2004). Ni(II) is able to indirectly induce ROS by causing inflammation and activation of macrophages (Kawanishi et al., 2002) in addition to altering the expression of co-stimulatory molecules on the surface of murine macrophages resulting in more efficient antigen presentation and activation of T helper cells (D'Antò et al., 2009), which could facilitate allergic sensitization.

Cu is a common component of PM and is often found in areas of traffic because of its presence in brake pads (Wallenborn et al., 2009). Cu is theoretically able to participate in Fenton reactions because of its ability to change oxidation states, resulting in the release of superoxide and hydrogen peroxide (Kelly & Fussell, 2012). High concentrations of $CuSO_4$ instilled into the trachea of mice resulted in an inflammatory response with cell infiltrate of PMNs and AMs in addition to protein in the BAL (Prieditis & Adamson, 2002). $CuSO_4$ instilled in rats lead to early pulmonary inflammation and infiltration with increases in leukocytes after 4 hours as compared to other metals that are commonly present in PM (Wallenborn et al., 2009). The presence of Cu in PM and its ability to readily induce inflammation makes it a potential candidate for facilitating allergic sensitization.

Our goal was to establish whether physiologically-relevant doses of nickel subsulfide (Ni_3S_2) and copper sulfate ($CuSO_4$) could act as adjuvants for allergic sensitization towards an otherwise harmless antigen. In order to test the adjuvanticity of these metals, we used a previously established model of inhalation tolerance in which aerosolized OVA is administered for 10 days with simultaneously exposure to one of the metals by intranasal instillation. Our findings suggest

that exposure to Ni or Cu via the respiratory tract leads to unique profiles of inflammation and cytokine production, which may affect the outcome of concurrent exposure to harmless antigens.

2.3 Materials and Methods

Animals - 6 week old female Balb/c mice were purchased from Charles River (Montreal, Quebec). They were housed at the Animal Care Facility at Laurentian University in a HEPA-filtered "Innorack" caging system (Fisher Scientific, Whitby, ON), with a 12 hour light-dark cycle, and had access to irradiated food (PicoLab 5058, LabDiet, Aberfoyle, ON) and sterile water *ad libitum*. To further minimize potential microbiological contamination, protocols were in place requiring all personnel to don booties, gloves, mask, hairnet and an autoclaved lab coat before entering the handling room or handling the mice. All experiments were approved by the Animal Care Committee at Laurentian University.

Intranasal Instillations - Nickel subsulfide (Ni_3S_2) (Sigma, St. Louis, MO) was sonicated into suspension in sterile PBS at a concentration of 250 ng/30 uL. Copper sulfate pentahydrate ($Cu(II)SO_4 \cdot 5H_2O$) (Sigma, St. Louis, MO) was dissolved in sterile PBS at a concentration of 266 ng/30 μL. These concentrations roughly approximate the amount of Ni and Cu that would be inhaled in a 24h period of breathing SPaM at ambient concentrations on a mass/kg basis.

For intranasal instillations, mice were lightly anaesthetized with isoflurane, and metal suspensions were applied drop wise, 15 μL per nare, as described by Southam *et al* (2002). Instillations were given for 11 days, starting 1 day prior to the inhalation protocol, and administered immediately prior to the exposure to aerosolized OVA each day.

Inhalation Tolerance Protocol. Mice were exposed to an aerosol containing OVA (Grade V, Sigma, St Louis, MO) (1% w/v in 0.9% saline) for 20 minutes daily for 10 consecutive days, as has been previously described by others (Holt, Batty, & Turner, 1981; Swirski et al., 2002). Control mice were exposed to sterile 0.9% saline alone. The aerosolizations were performed in

custom-made plexi-glass chambers (15x20x30 cm) using De Vibliss 800C nebulizers and the PulmoMate 4650D compressor that produced a flow rate of 6L/min.

OVA Re-challenge – In order to evaluate recall responses to OVA in the airways, mice were subjected to OVA rechallenge three weeks after the completion of the inhalation tolerance protocol. For challenge, aerosolized OVA was delivered for one hour (1% w/v in 0.9% saline).

Tissue and specimen collection – Mice were anaesthetized with isoflurane and euthanized via exsanguination. Peripheral blood was collected by cardiac puncture, transferred to Capiject® Gel/Clot Activator tubes (Terumo Medical Corporation), and centrifuged to prepare serum, which was stored at -20˚C until analysis.

Broncholveolar lavage (BAL) was performed as previously described (Ohkawara et al., 1997); briefly, the heart and lungs were removed and the trachea cannulated with polyethylene tubing (Becton Dickson, Sparks, MD) and secured with surgical thread (LIGAPAK™ Silk, Ethicon, Markham, ON). Two aliquots of PBS (0.25 mL and 0.2 mL) were instilled via the polyethylene tube, the lungs gently agitated, and the instilled fluid recovered. The BAL samples were centrifuged, and the supernatant collected and stored at -20˚C for cytokine analysis; the cell pellet was resuspended in 100 mL PBS and smears prepared by cytocentrifugation using the Cytopsin 4 (Thermo Scientific) for 5 minutes at 300 RPM. The slides were stained with Kwik™-Diff (Thermo Scientific, Pittsburgh, PA). Differential white blood cells counts were made from 500 leukocytes, with cells classified as either mononuclear cells, neutrophils or eosinophils using standard histological criteria.

For histological examination, lungs were inflated with 10% formalin, stored in formalin and later transferred to 70% (v/v) ethanol. Tissues were embedded in paraffin, sectioned at 3 μm

thickness, and stained with haematoxylin and eosin (H&E). Spleens were removed and immediately placed in cold (4˚C) HBSS (Sigma St Louis, MO).

Cytokine and Immunoglobulin Analysis - OVA-specific IgE, IgG2a and IgG1 ELISAs were generously performed by the Jordana lab at McMaster University, using their previously published protocols (Ohkawara et al., 1997; Swirski et al., 2002). Cytokine measures in the BAL supernatants, serum, and splenocyte culture supernatants were conducted using commercially available ELISA kits for, IL-4, IL-10, IL-5, IL-13, IL-25, IFN-γ, TNF-α (eBioscience, San Diego, CA), and GM-CSF (BD, San Diego, CA). The assay sensitivity levels were: 15 pg/mL for IFN-γ ; 4pg/mL for IL-4, IL-13, and IL-5; 30 pg/mL for IL-10; 16 pg/mL for IL-25; 8 pg/mL for TNF-α; and 2 pg/mL for GM-CSF.

Splenocyte Isolation and Culture - Under sterile conditions, spleens were cut into pieces, ground using the frosted ends of sterile slides into HBSS (Sigma, St Louis, MO), and filtered using a mesh filter (BD Falcon, Franklin Lakes, NJ). The suspension was centrifuged at 1500 RPM, for 10 minutes at 4˚C and then resuspended in ACK lysis buffer (0.15 M NH_4Cl, 1 mM $NaHCO_3$, 0.1 nM Na_2EDTA, pH adjusted to 7.2-7.4) to remove red blood cells. The cells were washed with HBSS and centrifuged, and resuspended in culture medium RPMI1640 (Mediatech Inc, Manassas, VA) supplemented with 5% FBS, 1% L-Glutamine (Mediatech Inc, Manassas VA) and 1% penicillin/streptomycin (Mediatech Inc, Manassas VA). Splenocytes were cultured with media alone or 40 μg OVA at a density of $8x10^5$ cells/well in a flat-bottomed 96 well plate at 37˚C for 5 days. Supernatants were stored at -20˚C for cytokine analysis.

Data Analysis Section - Data are expressed as mean ± SEM and Sigma Stat v. 3.5 was used for statistical analysis. After a normality test was performed, the data were analyzed using ANOVA

with a Holm-Sidak test or ANOVA on Ranks if the normality test failed with a Dunn's test. Differences were considered statistically significant if $p < 0.05$ as compared to another treatment group.

2.4 Results

Airway inflammatory responses.

The cellular profile of the BAL was assessed on day 13, 2 days after the final exposure to aerosolized OVA with concurrent metal intranasal instillations. Mice exposed to OVA alone had a modest, but statistically insignificant increase in total leukocytes compared to naïve mice (Fig 2.1A) with minimal eosinophils present (Figure 2.1B). Similarly, no significant increase in total cell number was observed in mice exposed to Ni alone or concurrently with OVA compared to naïve mice, however there was a trend towards increased eosinophils when exposed to Ni+OVA as opposed to Ni alone. Administration of Cu alone resulted in a modest increase in total leukocytes with a trend towards increased eosinophilia which was enhanced when exposed concurrently with OVA. Mice exposed to Cu+OVA had a significant increase in eosinophils present in the BAL.

To examine whether immunological memory for OVA was generated by these exposures, additional groups of mice were exposed to OVA and either Ni or Cu, and were challenged with OVA 3 weeks after the original exposure protocol; the 3 week wash-out period allowed any initial inflammatory response to OVA, Ni, or Cu to dissipate. After challenge there was a modest recall response to OVA as seen in increases in total leukocytes in the BAL of all groups compared to the naive control group (Fig 2.2A). In mice that had been exposed to OVA only there was a very modest recall response with little eosinophil infiltration into the lung (Fig 2.2B). There was a significant recall response to OVA in mice that had been originally exposed to Ni+OVA, with increases in total leukocyte infiltration into the BAL. There was a modest recall response to OVA in mice that were originally exposed to Cu+OVA although this was not statistically significant. There was an overall trend towards increased eosinophilia after challenge

with OVA from mice exposed to either Ni+OVA or Cu+OVA, although this did not reach statistical significance.

BAL cytokine production

Cytokines were measured in the BAL on day 9. There were no detectable levels of IL-4, IFN-γ, TNF-α, GM-CSF or IL-10 in any of the treatment groups (data not shown). Low levels of IL-5, IL-13, and IL-25 were measured, although there were no statistically significant changes in cytokine levels between treatment groups (Fig 2.3A-C).

Serum levels of immunoglobulins.

The OVA-specific B cell response was evaluated by measuring OVA-specific IgE, IgG1, and IgG2a in the serum. OVA-specific IgE, which is one of the hallmarks of allergic responses, was barely detectable in all groups measured, with no significant changes between treatment groups (Fig 2.4A). We also measured levels of OVA-specific IgG1, which are also associated with Th2 responses in mice, (Fig 2.4B) and overall found no significant changes between treatment groups. The OVA exposed group had levels of OVA-specific IgG1 that were higher than the naive group and there was a trend towards a modest increase with respect to the Ni+OVA recall group, however, it was not significant. There were no detectable levels of OVA-specific IgG2a (associated with Th1 responses in mice) in any group measured (data not shown).

In vitro cytokine production in cultured splenocytes

Splenocytes were cultured and stimulated with OVA *in vitro* to evaluate antigen-specific recall responses. We examined cytokine levels associated with inflammation and both Th1 and Th2 responses: IL-4, IL-5, IL-13, IFN-γ, TNF-α, IL-10, IL-25 and GM-CSF. No detectable levels of

IL-4 were present in any of the groups (data not shown). Low levels of each of the other cytokines were detected in supernatants of the splenocyte cultures from naïve mice (Fig. 2.5-11). There were no significant changes in the amount of TNF-α produced from splenocytes cultured from mice exposed to OVA, OVA+Ni or Cu *in vivo* and challenged with OVA *ex vivo* (Fig 2.5A). There was a significant increase in IFN-γ detected from splenocytes from mice that had been exposed to Ni alone (Fig 2.6B). Splenocytes from mice that were exposed to Ni+OVA and cultured with OVA had a significant increase in IFN-γ (Fig 2.6A). Splenocytes from mice that were exposed to Cu had a significant increase in levels of GM-CSF produced (Fig 2.7B). There was a significant increase of GM-CSF from splenocytes of mice that had been exposed to Cu+OVA and recalled with OVA *in vitro* (Fig 2.7A). Surprisingly, we saw increased levels of Il-5 from splenocytes cultured with OVA that had been previously exposed to OVA *in vivo* (Fig. 2.8A) although these results were not significant between treatment groups or to the control. We saw a significant increase in levels of IL-13 from splenocytes exposed to Cu alone (Fig 2.9B), but not in splenocytes from mice exposed to Cu+OVA. We also saw a significant increase of IL-13 from splenocytes of mice exposed to OVA and cultured with OVA (Fig. 2.9A). There was also a significant increase in levels of IL-10 from splenocytes from mice that had been exposed to OVA and cultured with OVA *in vitro* and there was a significant decrease of IL-10 seen in splenocytes from mice exposed to Ni+OVA and challenged with OVA *in vitro* (Fig. 2.10). Finally, we examined levels of IL-25 from splenocytes of mice exposed to OVA+/-Ni and Cu and cultured with OVA *ex vivo* and there were no significant changes in any groups tested (Fig 2.11A).

2.5 Discussion

Experiments in mouse models have previously established that an immunological tolerance to OVA can be induced by passively administering a 1% OVA aerosol for 10 days (Holt et al., 1981; Stämpfli et al., 1998; Swirski et al., 2002; Ostroukhova et al., 2004). Responses towards the otherwise harmless antigen are actively suppressed, with dampened inflammatory responses and lower OVA-specific IgE levels in response to subsequent attempts to sensitize and challenge with OVA (Holt et al., 1981; Holt & McMenamin, 1989; Hurst et al., 2001; Swirski et al., 2002; Ostroukhova et al., 2004; Alvarez et al., 2006). This inhalation tolerance is thought to be an evolutionary mechanism to prevent the generation of immune responses against harmless ubiquitous airborne antigens. Pathological immune responses to such harmless antigens, such as those seen in allergic respiratory diseases, may be the result of the subversion of tolerance by concurrent exposure to exogenous agents which act as immunological adjuvants. PM and its constituents may be examples of such agents with adjuvant capacity. In this study, our aim was to determine whether the metals Ni and Cu have adjuvant activity which can disrupt the development of tolerance towards OVA. These two metals are present in ambient urban particulate air pollution from many geographical locations, but are of particular interest to us because they are especially abundant in the particulates present in Sudbury ON, a city with a major nickel mining and refining industry.

To the best of our knowledge, our findings are the first to investigate the ability of specific metals present in PM to act as adjuvants in a murine model of inhalation tolerance. Our results suggest that while short-term exposure to low levels of these metals may not be able to completely subvert tolerance, they lead to subtle alterations in airway leukocyte profiles which differ from animals exposed to OVA alone. Furthermore, our restimulated splenocyte culture

data indicate that systemic immune responses induced against OVA in the presence of metals has an altered profile of cytokine production. We speculate that these kinds of modest changes could have a cumulative, reinforcing effect that could promote the development of pathologic immune responses to otherwise harmless antigens.

It has been established that PM with different chemical compositions can have different immunological effects. Metal-rich PM such as ROFA and those from industrially active areas are able to act as adjuvants for allergic respiratory disease (Gavett et al., 2003; P. Steerenberg et al., 2003). Previous investigations have demonstrated that metal components of PM, including Ni and Cu, are able to cause pulmonary inflammation, injury and systemic responses (Adamson et al., 1999; Prieditis & Adamson, 2002; Wallenborn et al., 2009). The metals used by others have generally been soluble (Wallenborn et al., 2009); however, we elected to use a partially soluble form of Ni, nickel subsulfide (Ni_3S_2), because the ambient particulate matter in Sudbury contains a large proportion of insoluble nickel subsulfide (Ni_3S_2) resulting from smelting and refining activities (Denkhaus & Salnikow, 2002). Although of considerable local concern, effects of Ni and Cu-containing particulates have broader implications as well, in part because SPaM is dispersed across a large geographical area and has purportedly been detected in areas of New York (Lippmann et al., 2006), but also because Ni and Cu are present in PM from other urban locations (Adamson et al., 2000), and in other types of PM relevant to occupational exposure, including ROFA (Carter et al., 1997; Lambert et al., 2000; Wallenborn et al., 2009). We expected that Ni would act as an adjuvant for Th2 responses, as Ni has been shown to act as a sensitizer for the house dust mite (HDM) allergen, increasing HDM-specific IgE levels and increased mRNA levels of IL-5 and IL-13 in lung tissue which are reflective of a Th2 response (Lambert et al., 2000). However, our results did not appear to show a distinctly Th2-like

response to OVA in the presence of Ni. It is possible that the minimal level of eosinophilia we observed may be explained by the observation of Ishihara & Ja (2009), who showed that Ni decreased eosinophil viability by enhancing apoptosis (Ishihara et al., 2009). However, given that there was a lack of IL-5 and IL-13 present in the BAL and the splenocytes cultured *ex vivo* from the nickel alone and Ni+OVA treated groups, it seems more likely that the low dose of Ni used in our study did not induce sufficiently high levels of the cytokines required for the differentiation and recruitment of eosinophils, which would explain the lack of eosinophilia. We suspect that the dose of Ni we used, which was selected because it reflects real-life levels of exposure, was simply too low to induce a strong Th2 polarizing response. It may be of interest to conduct further studies with higher doses of Ni, or a longer duration of exposure (which would more faithfully model the human condition), to more fully examine the hypothesis.

Cu inhalation also appears to be able to modify the response to OVA. In our experiments, mice treated with Cu alone had some eosinophilic infiltration in the BAL, which was significantly enhanced when exposed to OVA in the presence of Cu. This is consistent with our observation that Cu is able to induce modest levels of IL-13 by splenocytes from mice treated with Cu only. Cu appeared to be a stimulus for GM-CSF production, as splenocytes from Cu only and Cu+OVA treated groups had increased levels of GM-CSF. There were no significant changes in other cytokine levels measured, suggesting that Cu may act to promote antigen presentation through the induction of GM-CSF, and support the development of nascent Th2 responses through the induction of IL-13. It is important to note that the levels of eosinophilia we observed in the Cu and Cu+OVA mice were far lower than what is seen generally in murine models of asthma and allergic respiratory diseases, where eosinophil numbers can sometimes reach upwards of 80% of total BAL leukocytes (Ritz et al., 2002; Epstein, 2004; Ostroukhova et al.,

2004). Thus, it would be a stretch to suggest that what we have observed here is similar to allergic respiratory disease or that tolerance was clearly subverted.

However, despite the fact that many of our observations were of relatively subtle changes in inflammation, eosinophilia, and cytokine production, we believe that these findings are notable because they reflect outcomes after exposure to physiologically relevant levels of PM-associated metals, unlike many other studies which use much higher doses that are unlikely to be achieved under real-world conditions. For example, Steerenberg et al. in 2005 looked at the response of PM and its adjuvanticity at a dose that worked out to be 450 µg/mouse, which is approximately 22.5 mg/kg, and much higher than what would be expected for real-world exposures in humans (Diaz-Sanchez et al., 1994). While these data are useful in interpreting the burden of PM in large doses, it is difficult to extrapolate these findings to the likely effects of chronic exposure to lower doses, as these models do not imitate the duration and chronicity of exposure that humans experience, with ubiquitous and ongoing exposure to PM on a daily basis from birth. We designed the experiments to use modest and physiologically relevant levels of Ni and Cu to determine what would happen after short-term but repeated exposures that more accurately mimic real doses. However, our approach still falls short of modelling the chronicity of exposure that occurs in humans.

The rechallenge experiments allowed us to discern the extent to which OVA-specific immunological memory was affected by the presence of metal during the initial exposure to OVA, as opposed to the mix of responses to the metals themselves along with OVA that would have been present at day 13. A significant increase of leukocytes into the BAL in the Ni+OVA group after recall challenge with OVA suggests that Ni did in fact act as a mild adjuvant for responses to OVA, resulting in a stronger recall response than mice exposed to OVA only, long

after the metal was cleared from the lung. In both the Ni+OVA and Cu+OVA groups, recall with OVA elicited a small but non-significant eosinophila in the airways; although it did not reach statistical significance, the presence of eosinphils in these groups further suggests a subtle alteration in the immune response to OVA as a result of the presence of metal during the initial exposure protocol.

Ex vivo restimulation of splenocyte cultures with OVA demonstrated that the absence of detectable cytokines in the BAL did not mean that an immune response was not generated. GM-CSF and IFN-γ levels were increased and IL-10 levels decreased in cultures of splenocytes from Ni-treated animals with media only, suggesting that Ni itself promoted the production of cytokines associated with inflammatory and Th1 responses while downregulating immunosuppressive cytokines. Similar results were observed from the splenocytes from Ni+OVA treated mice restimulated with OVA. Together these results suggest that the presence of Ni may stimulate the production of cytokines which increase the efficiency of antigen presentation, particularly GM-CSF, which is a proinflammatory cytokine and a strong inducer of dendritic cells and other antigen presenting cells. The depression of IL-10 would suggest that there is deviation from Th2 polarization and immunoregulation; along with the increased levels of IFN-γ, the decreased levels of IL-10 could facilitate the predominance of a Th1 mediated response, since IL-10 is known to suppress Th1 through IL-12 (reviewed by Holt & Stumbles, 2000), which is consistent with our observation of increased IFN-γ seen in the Ni groups. Thus, while previous work had led us to predict that Ni would promote Th2 responses, and we see a small percentage of eosinophils infiltrate into the lung, we also see some evidence that Ni may stimulate Th1 responses. This would suggest that the situation may be more complex than the simple Th1/Th2 dichotomy would suggest.

Although we did not conduct flow cytometric analysis to directly evaluate the presence of Tr1 cells, our findings are consistent with the possibility that Tr1 cells are among those contributing to the IL-10 production in the OVA-only treated mice, as we would expect in this model of inhalation tolerance. We saw significant increases in IL-10 in cultures of splenocytes from OVA-treated mice that were restimulated with OVA *ex vivo,* consistent with the known role of IL-10 in mediating inhalation tolerance, produced by the Tr1 subset of T regulatory cells (Wakkach et al., 2003). IL-10 is able to decrease MHC class II and co-stimulatory molecules on APCs (reviewed by Braga et al., 2012), resulting in less antigen presentation and a decreased ability to activate Th cells. IL-10-producing Tr1 are able to suppress Th2 and IgE production, and induce isotype switching of B cells to IgG1 (Cottrez et al., 2000). This is consistent with our findings as well, where OVA-specific IgG1 levels were modestly increased in the OVA-only treated mice. The OVA treated splenocytes restimulated with OVA showed modest levels of IL-5, no detectable IL-4 and significant levels of IL-10 which is a previously confirmed cytokine profile of Tr1 (Groux et al., 1997).

In conclusion, our results suggest that while Ni and Cu at these doses did not act as strong adjuvants for responses to OVA, they were able to cause modest changes in the local inflammatory response and the systemic immune response. By enhancing antigen presentation and inducing cytokines that can influence Th differentiation, metals in PM may modify the normal tolerance response towards innocuous antigens, leading towards a greater probability of sensitization and atopy. Although the changes we observed are relatively subtle, these findings suggest that even at low and physiologically relevant levels, these metals may have the ability to cause alterations in the immune responses towards otherwise harmless protein antigens, the cumulative effects of which could potentially promote hypersensitivity disorders.

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Fig 2.1. Leukocyte infiltration into the BAL after aerosolization period. Mice were exposed to metals for 11 days and a 1% (w/v) OVA aerosolization for 10 days. Total leukocytes (panel A) and eosinophils (panel B) were evaluated on day 13. Data are shown as mean +SEM, with n=3-9. * indicates a statistically significant difference ($p < 0.05$) compared to naïve.

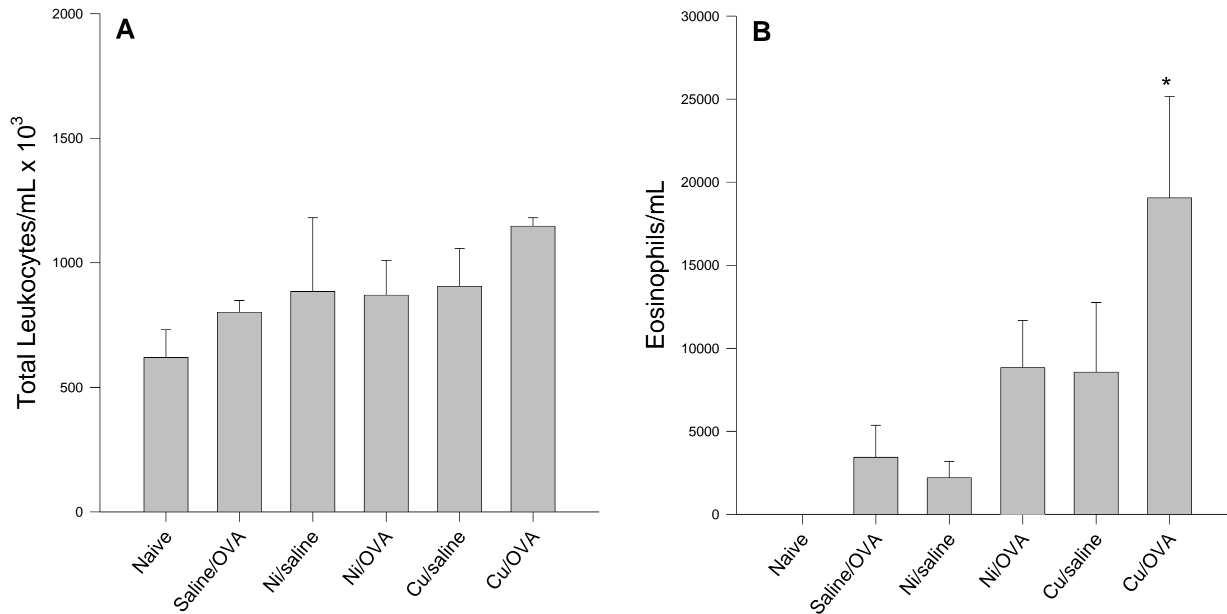


Fig 2.2. Leukocyte infiltration into the BAL after challenge with OVA. Mice were exposed to metals for 11 days and a 1% (w/v) OVA aerosolization for 10 days. Mice were rechallenged with OVA 3 weeks later, and total leukocytes (panel A) and eosinophils (panel B) were evaluated 72 hours after challenge. Data are shown as mean +SEM, with n=3-8. * indicates a statistically significant difference ($p < 0.05$) compared to naïve.

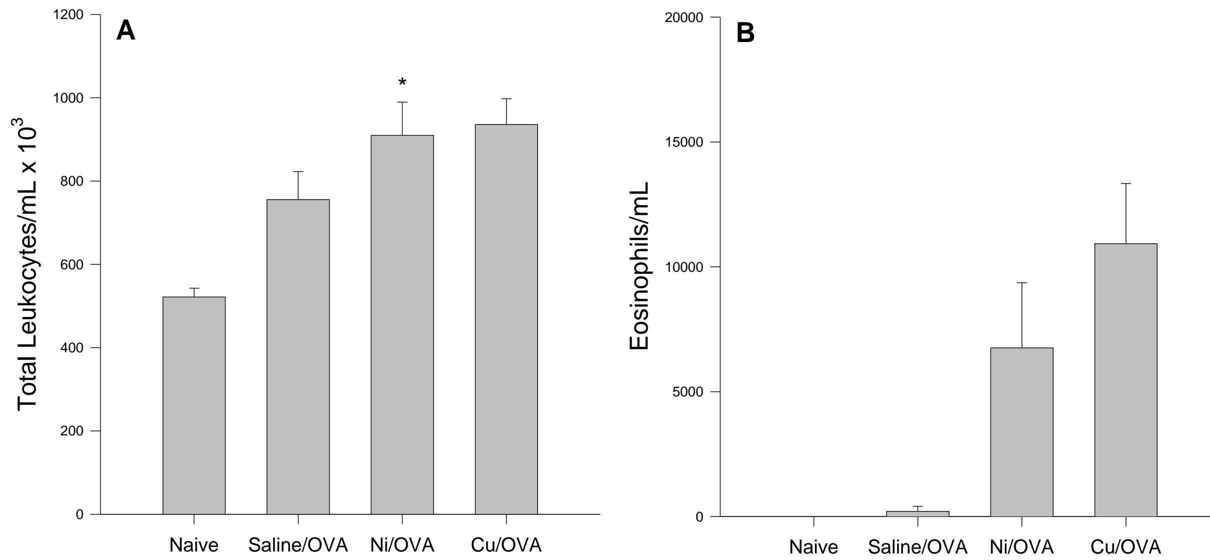


Fig 2.3. Cytokines in BAL on day 9. IL-5 (panel A), IL-13 (panel B), and IL-25 (panel C) levels were measured in the BAL on day 9 of the exposure protocol. Data are shown as mean +SEM, with n=3.

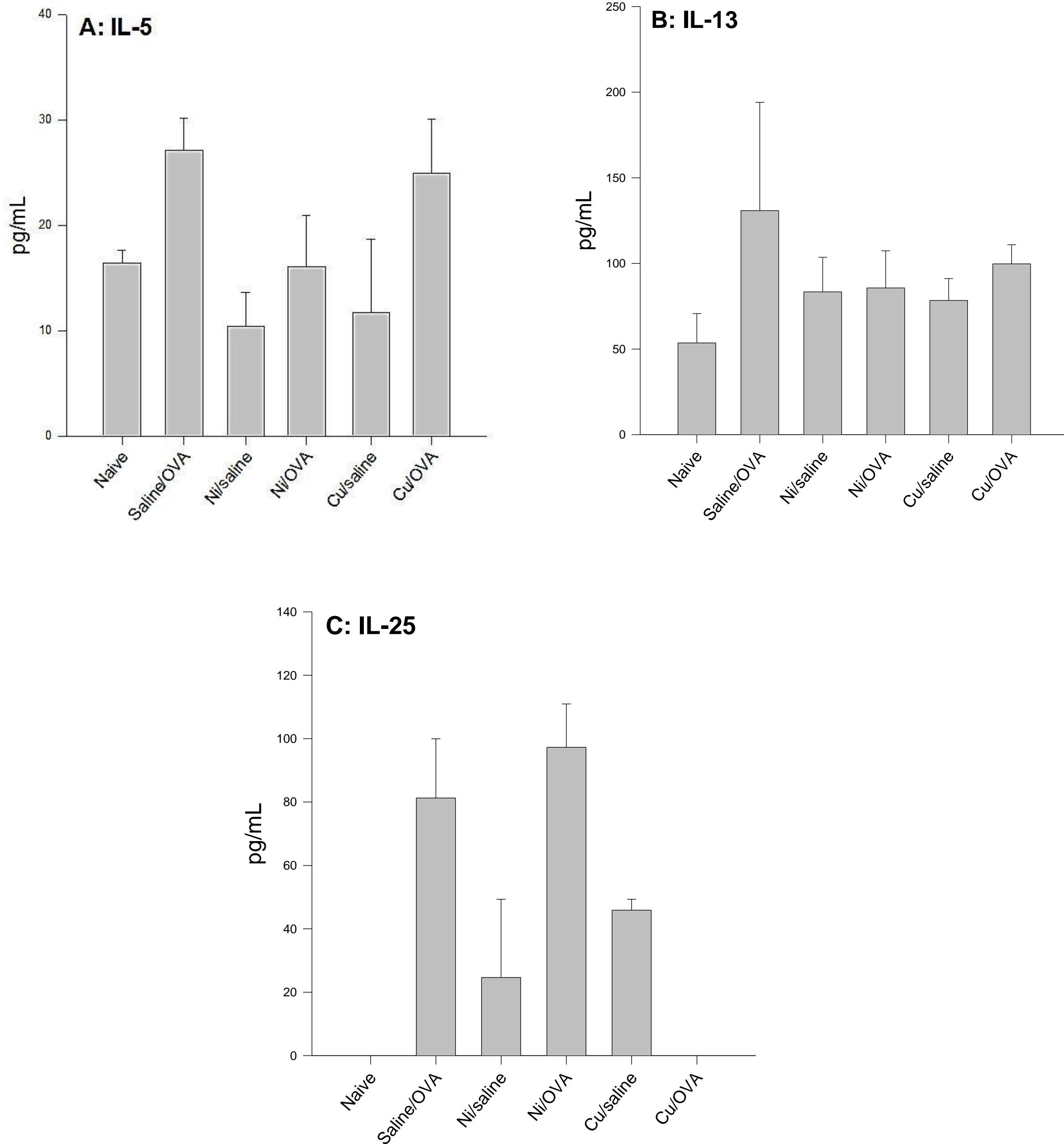


Fig 2.4. OVA-specific immunoglobins in serum of challenged mice. Mice were exposed to metals for 11 days and a 1% (w/v) OVA aerosolization for 10 days, and rechallenged with OVA 3 weeks later. Serum levels of OVA-specific IgE (panel A) and IgG1 (panel B) were determined by ELISA. Data are shown as mean +SEM, with n=3-5.

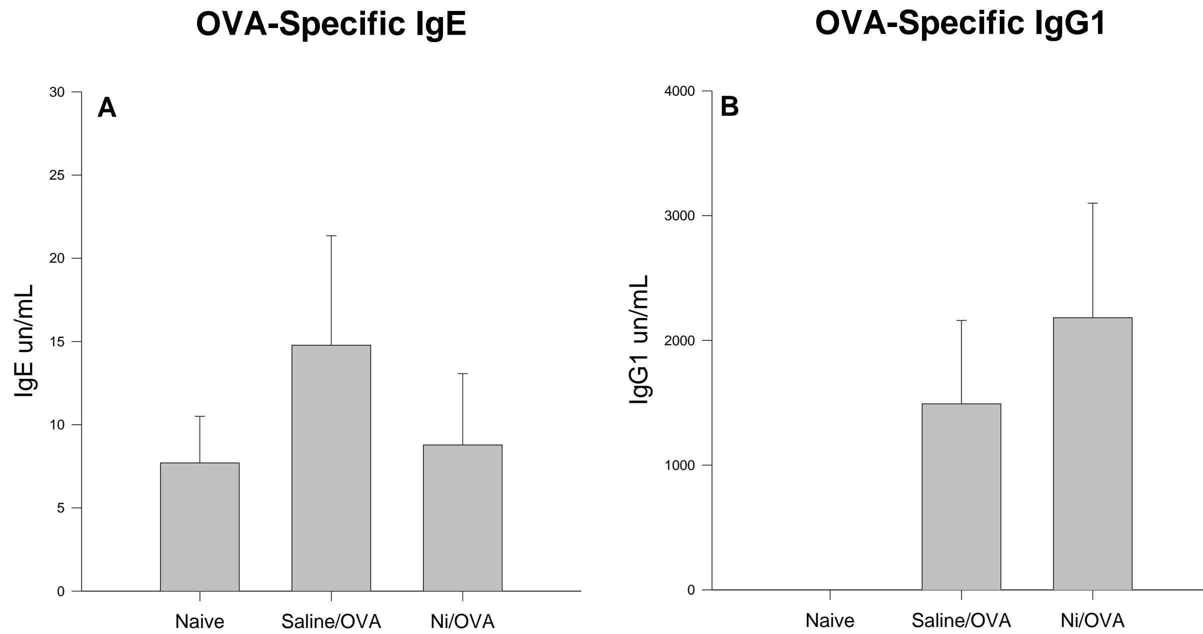


Fig 2.5. Levels of TNF-α in splenocytes cultured with OVA *in vitro*. Mice were exposed to metals for 11 days and a 1% (w/v) OVA aerosolization for 10 days (panel A) or with 0.9% saline as a control (panel B). On day 13 splenocytes were isolated and cultured alone or with 40 μg/well OVA. Data are shown as mean +SEM with n=3-6.

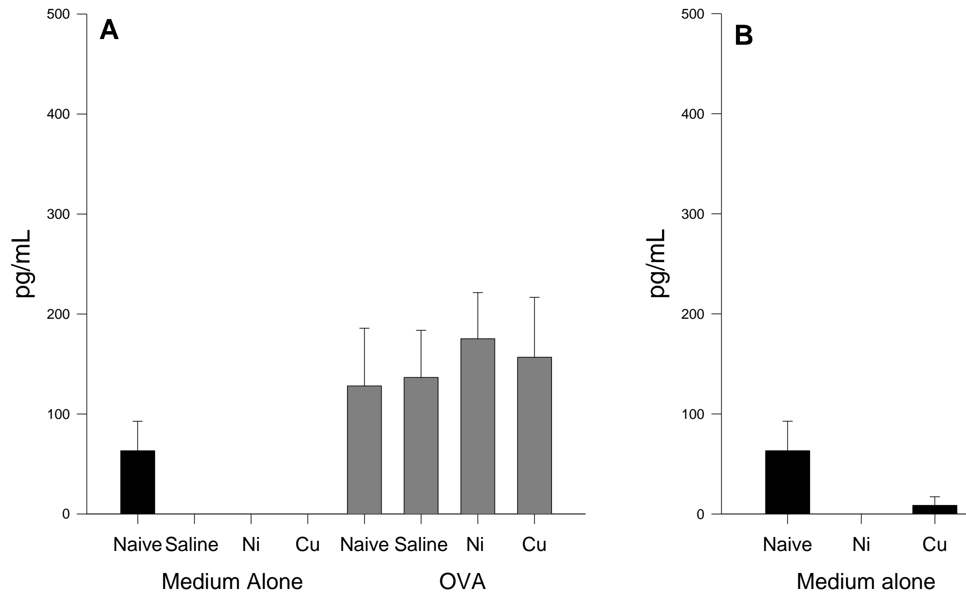


Fig 2.6. Levels of IFN-γ in splenocytes cultured with OVA *in vitro*. Mice were exposed to metals for 11 days and a 1% (w/v) OVA aerosolization for 10 days (panel A) or with 0.9% saline as a control (panel B). On day 13 splenocytes were isolated and cultured alone or with 40 µg/well OVA. Data are shown as mean +SEM with n=3-6. *indicates a statistically significant difference ($p < 0.05$) as compared to another treatment group.

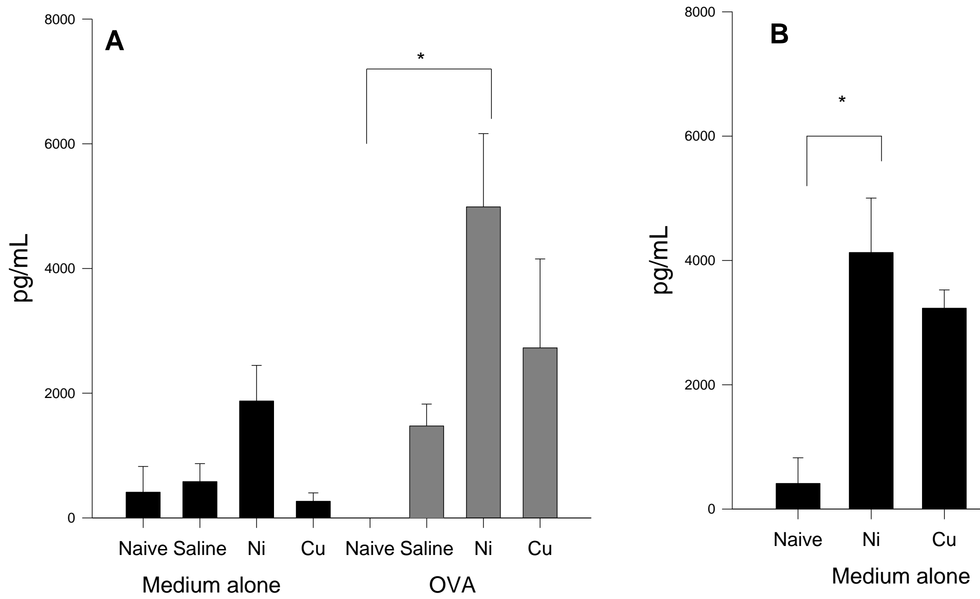


Fig 2.7. Levels of GM-CSF in splenocytes cultured with OVA *in vitro*. Mice were exposed to metals for 11 days and a 1% OVA (w/v) aerosolization for 10 days (panel A) or with 0.9% saline as a control (panel B). On day 13 splenocytes were isolated and cultured alone or with 40 µg/well OVA. Data are shown as mean +SEM with n=3-6. *indicates a statistically significant difference ($p < 0.05$) as compared to another treatment group.

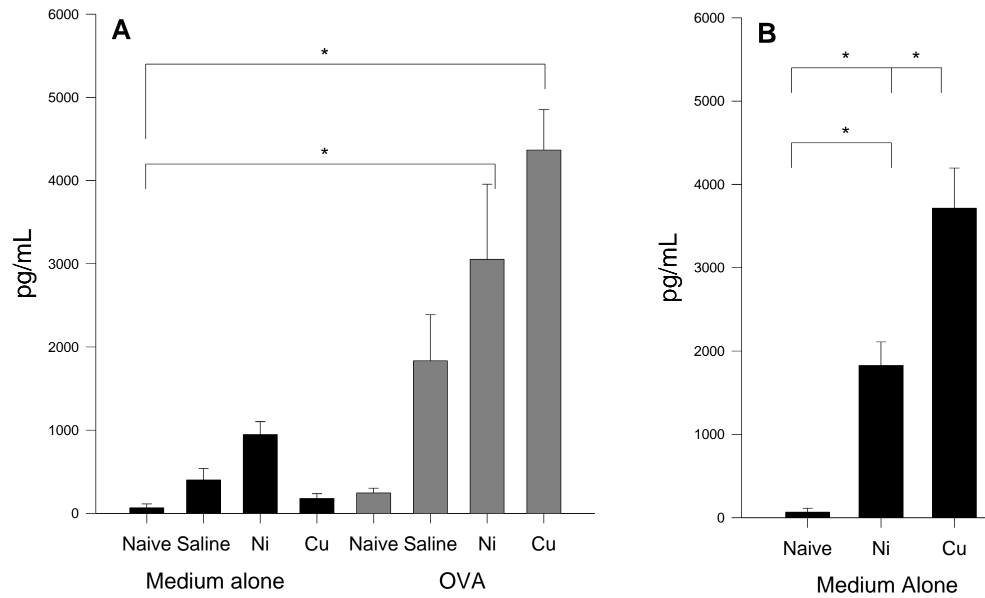


Fig 2.8. Levels of IL-5 in splenocytes cultured with OVA *in vitro*. Mice were exposed to metals for 11 days and a 1% (w/v) OVA aerosolization for 10 days (panel A) or with 0.9% saline as a control (panel B). On splenocytes were isolated and cultured alone or with 40 μg/well OVA. Data are shown as mean +SEM with n=3-6.

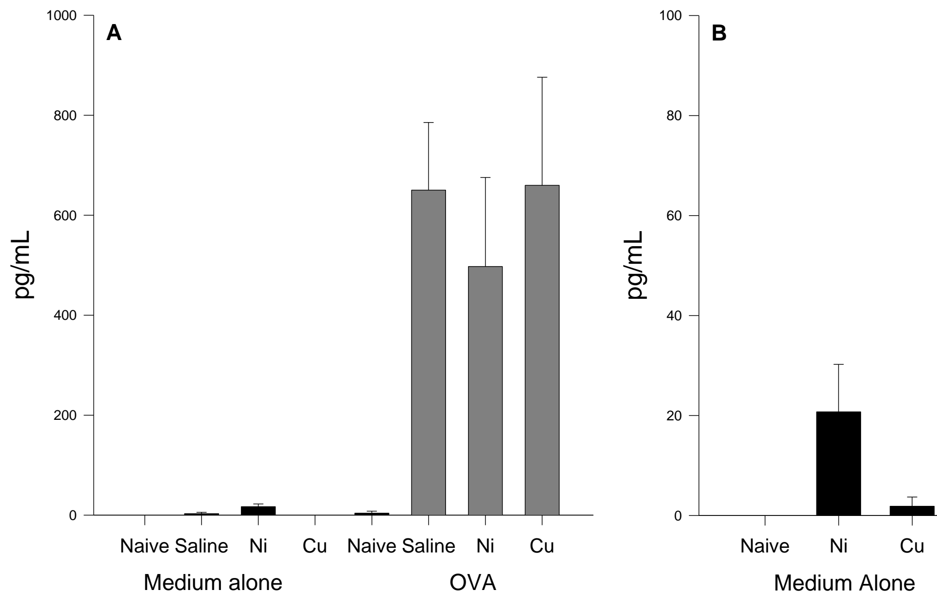


Fig 2.9. Levels of IL-13 in splenocytes cultured with OVA *in vitro*. Mice were exposed to metals for 11 days and a 1% (w/v) OVA aerosolization for 10 days (panel A) or with 0.9% saline as a control (panel B). On day splenocytes were isolated and cultured alone or with 40 μg/well OVA. Data are shown as mean +SEM with n=3-6. *indicates a statistically significant difference ($p < 0.05$) as compared to another treatment group.

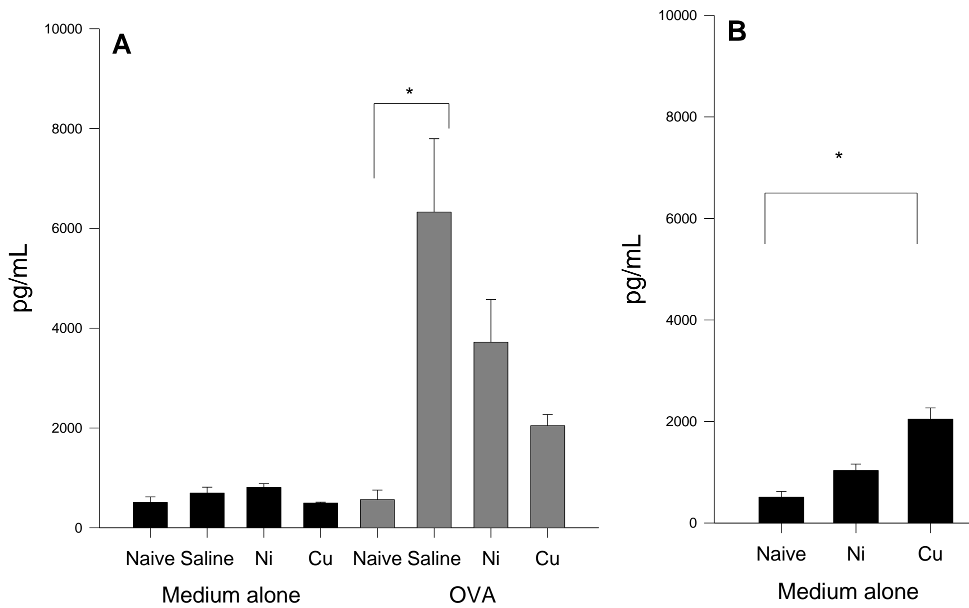


Fig 2.10. Levels of IL-10 in splenocytes cultured with OVA *in vitro*. Mice were exposed to metals for 11 days and a 1% (w/v) OVA aerosolization for 10 days (panel A) or with 0.9% saline as a control (panel B). On day 13 splenocytes were isolated and cultured alone or with 40 µg/well OVA. Data are shown as mean +SEM with n=3-6. *indicates a statistically significant difference ($p < 0.05$) as compared to another treatment group.

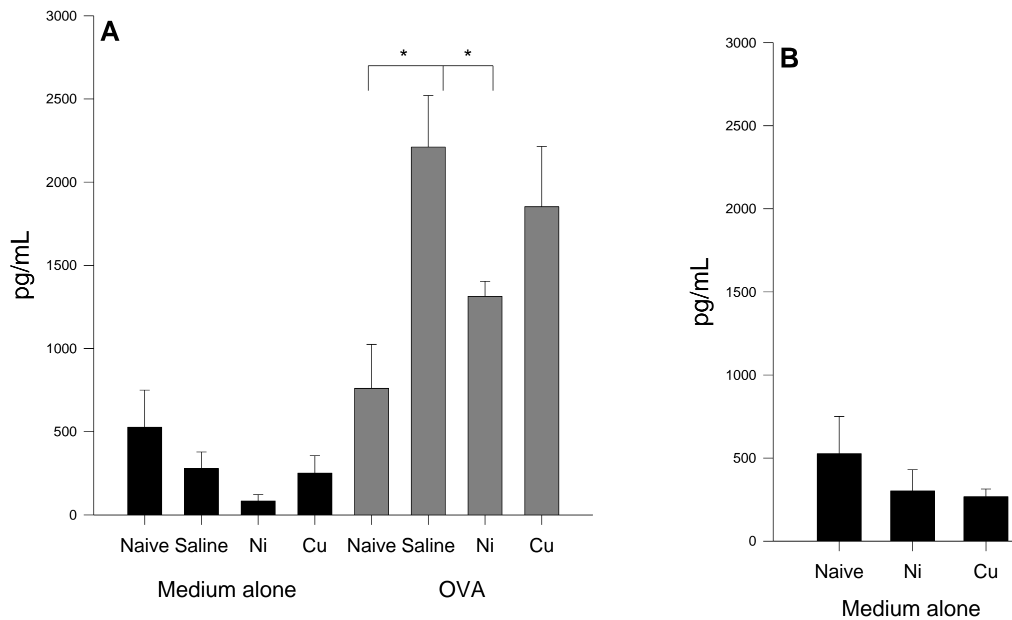
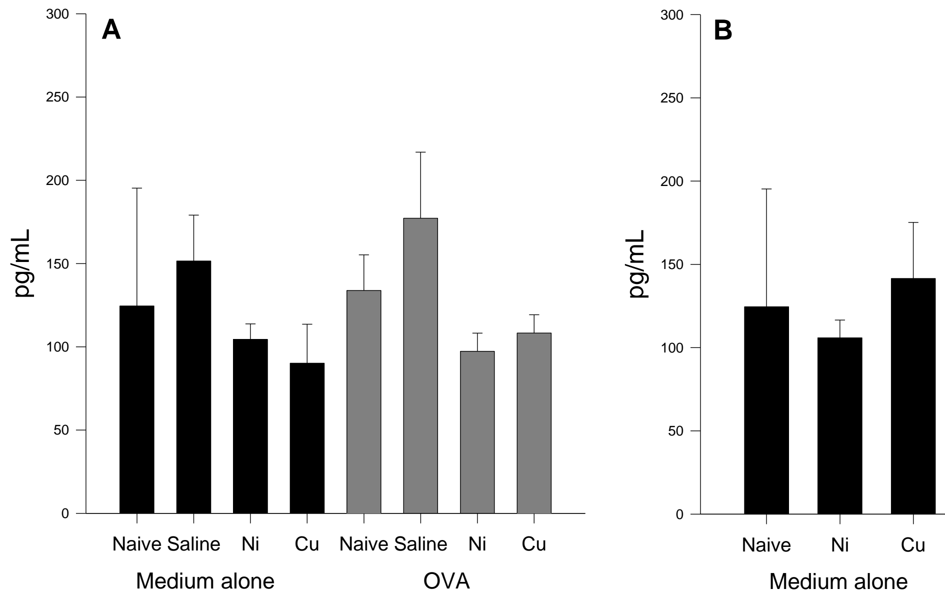


Fig 2.11. Levels of IL-25 in splenocytes cultured with OVA *in vitro*. Mice were exposed to metals for 11 days and a 1% (w/v) OVA aerosolization for 10 days (panel A) or with 0.9% saline as a control (panel B). On day 13 splenocytes were isolated and cultured alone or with 40 µg/well OVA. Data are shown as mean +SEM with n=3-6.



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CHAPTER 3: ESTROGEN MODULATES THE INFLUENCE OF NICKEL ON CYTOKINE PRODUCTION BY T HELPER CELLS

The following manuscript investigates how estrogen in able to influence the cytokine production in the presence of nickel from activated T helper cells. We isolated naïve CD4+ T cells from human PBMCs and activated them prior to stimulating them with estrogen and nickel. We used ELISAs to analyze Th1 and Th2 associated cytokines including IL-2, IFN-γ, IL-4 and IL-10.

From these investigations we determined that: (i) nickel is able to alter the cytokine production from activated T helper cells after 4 hours; (ii) estrogen is able to alter the inflammatory profile from activated T helper cells after 4 and 24 hours; (iii) nickel in the presence of estrogen led to a different inflammatory profile than nickel alone from activated T helper cells suggesting that oversimplifications of data in regards to particulate matter could be false.

This manuscript has been prepared for submission to *Biology of Sex Difference*. I am responsible for the design, execution, analysis and interpretation of all experiments with consultation and input from Dr. Ritz. I also wrote and prepared the manuscript with edits from Dr. Ritz.

ESTROGEN MODULATES THE INFLUENCE OF NICKEL ON CYTOKINE PRODUCTION BY T HELPER CELLS

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3.1 Abstract

Background: Previous research has demonstrated that ambient particulate air pollution has the capacity to act as an immunological adjuvant. Although the last 20 years have been very fruitful in establishing immunological effects of exposure to airborne particulate matter and dissecting the mechanistic pathways involved, much of the research has not taken sex or gender into consideration as factors potentially influencing these responses. In this study, we investigated the effect of metals present in particulate matter on the differentiation of helper T cells *in vitro*, and whether this is influenced by the presence of estrogen.

Methods: Naive CD4+ T cells were isolated from human peripheral blood mononuclear cells (PBMCs) and exposed to $NiCl_2$ *in vitro* in the presence or absence of 17β-estradiol for 4 or 24 hours. Supernatants were collected and cytokine levels analyzed by ELISA.

Results: Exposure to Ni alone enhanced IL-2 production by activated CD4+ T cells, while depressing IFN-γ after 4 hours. In the presence of both estrogen and Ni, IL-2 levels were suppressed while IFN-γ production was enhanced. There was a time dependent response with respect to IFN-γ, since after 24 hours there was a decrease in the levels of IFN-γ elicited from activated CD4+ T cells as compared to the 4 hour treatment.

Conclusion: Our results suggest that the presence of Ni is indeed able to alter the profile of cytokines produced by Th cells upon activation. Furthermore, this study provides evidence that estrogen modulates the effects of environmental chemicals on the immune response, which may help to explain sex/gender differences in the natural history and prevalence of environmentally-triggered immunological disease. More broadly, these findings affirm that we ought to be

cautious about over-generalizing the impact of PM on the immune system if sex and gender factors have not been addressed.

KEY WORDS: Nickel, estrogen, particulate matter, sex

ABBREVIATIONS:

ANOVA – analysis of variation

BAL – bronchoalveolar lavage

Cu – copper

DEP – diesel exhaust particulate

E2 – 17-β estradiol

FBS – fetal bovine serum

IFN-γ – interferon gamma

IL – interleukin

OVA - ovalbumin

Ni – nickel

PBMC – peripheral blood mononuclear cell

PBS – phosphate buffered saline

PM – particulate matter

SEM – standard error of the mean

SPaM – Sudbury particulate matter

Th – T helper cell

3.2 Introduction

Particulate matter (PM) is a heterogeneous component of ambient air pollution consisting of both liquid and solid particles. PM is of considerable interest because it has been well established that PM exposure is associated with a variety of adverse health effects [1–3], including increased cardiovascular morbidity and mortality [4, 5] exacerbations of respiratory disorders [4], and immunological disorders such as autoimmunity [6, 7] and allergy [3, 8–10].

There is an increasing amount of evidence demonstrating that exposure to airborne PM may promote the development of allergic respiratory disease. Children close to high density traffic, where PM levels are higher, showed increased incidence of allergic respiratory diseases, with the negative effects decreasing with distance [11]. Furthermore, children who live in areas with metal-rich PM from industry and mining have been shown to have an increased incidence of allergic respiratory disease as opposed to children from agricultural regions [8]. It has been speculated that children from farming communities are exposed to less PM than their counterparts who live in urbanized areas with high levels of traffic, resulting in a lower incidence of wheezing, allergies and atopy among rural children [9, 12, 13]. These epidemiological findings are supported by experimental studies which showed that PM is able to act as an adjuvant in murine models using ovalbumin [14–16]. Furthermore, human investigations have revealed that diesel exhaust particulate (DEP), which is a large component of PM in many urban settings, is able to act as an adjuvant for allergic sensitization [17].

PM is not a homogeneous entity: the physical properties and chemical constituents of PM vary as a result of local environmental, vehicular, and industrial influences. Others have demonstrated that the physical and chemical properties of PM have an influence on the outcome of exposure to PM [18]. Experimental studies using murine models have investigated the adjuvanticity of PM

from different cities, and found that the course fraction and the insoluble fraction have adjuvant capabilities [15, 16]. Metal-rich PM have been shown to increase total leukocytes in the BAL of healthy individuals [3]. We are especially interested in the immunological effects of nickel (Ni), since the local ambient PM in Sudbury ON has been shown to have relatively high levels of Ni compared to particulates from other sources [19].

T helper (Th) cells play a vital role in allergic disease, with Th2 cells promoting allergy, and Th1 and T regulatory (Treg) cells playing potentially protective roles [20–24]. Interactions between naive Th cells and antigen presenting cells are an important determinant of the differentiation of Th cells towards one of these Th subtypes. IL-12 is able to drive the Th1 response along with IFN-γ, while IL-4 is one of the primary drivers of the Th2 response, while Tregs require IL-10 and/or TGF-β for differentiation [20].

It is notable that although the endocrine system is known to be intimately associated with the immune system, very few studies of the effects of PM on the immune system acknowledge the possible influence of sex hormones [25]. Many cells of the immune system express either the α- and β-estrogen receptor resulting in the possible modulation of the immune response at different points [26]. Estrogens are known to affect Th function, and it is generally accepted that *in vivo* high levels of estrogen tend to induce a Th2 bias [27, 28]. This is consistent with the observation that allergic respiratory disease is more prevalent in women [29, 30]. In contrast, high *in vitro* doses of estrogen have been able to induce significant production of IFN-γ from peripheral blood mononuclear cells (PBMCs), the prototypical cytokine of Th1 responses [31]. Thus, the effect of estrogen on T helper cell responses is most likely dependent on several different parameters such as duration of exposure, dose, and cell type.

In this study, we have examined cytokine production by T helper cells activated in the presence of Ni. Since the immune system is influenced by estrogen, it is likely that these responses are influenced by hormone levels as well; as such, we also examined the ability of estrogen to alter cytokine production by T helper cells activated in the presence of Ni. Our findings indicate that estrogen is indeed able to modulate the immune response in the presence of Ni, suggesting the need to address sex considerations when investigating the immunological effects of PM.

3.3 Methods

Subjects – Peripheral blood was obtained from human subjects 18 years of age or older, in good general health, and free of recent illness or active allergies. Subjects were excluded if they had made a blood donation within the previous 2 weeks, were using immunosuppressive medications, or with current illness. Subjects were asked to refrain from the use of anti-inflammatory medications for at least 3 days prior to participation. These experiments were approved by the Research Ethics Board at Laurentian University (Sudbury, ON) and subjects provided written consent prior to participating.

PBMC Isolation – Approximately 60-70mL of peripheral blood was obtained by venipuncture using a 22g PrecisionGlide® vacutainer blood collection needle (Becton Dickinson) and 10 mL Vacutainer® sodium heparin coated tubes. Blood was diluted with PBS supplemented with 2% FBS (Sigma, St-Louis MO), divided into 30 mL aliquots and slowly overlaid with 15 mL of Ficoll-Paque Plus® (StemCell Technologies, Vancouver, BC, Canada). The suspensions were then centrifuged at 400 x*g* for 30 minutes at 20˚C with the brake off. The plasma was removed, and the buffy coat was then carefully isolated, placed into a new conical tube, suspended in PBS, and centrifuged at 400 x*g* for 10 minutes to remove any residual Ficoll-Paque. This was repeated for a total of 3 washes.

*Naï*ve *CD4+ T cell Isolation and Culture* – Total cell count was performed using a haemocytometer, and cells were resuspended at $5x10^7$ cells. Naïve T helper cells were isolated by negative selection using the EasySep® Negative Selection Human Naïve CD4+ T cell enrichment kit (StemCell Technologies) using a RoboSep® magnetic cell sorter (StemCell Technologies, BC). The resulting naive helper T cells were resuspended with RPMI 1640

supplemented with 10% FBS, 5% L-Glutamine and 5% Penicillin/Streptomycin at a density of $1x10^6$ cells per mL.

Incubation with nickel and estrogen – 250 μM nickel chloride ($NiCl_2$) (Sigma) and 5000 pg/mL 17β-estradiol (E2) (Sigma) were added to $1x10^5$ naïve T cells seeded in a sterile 96 well flat-bottomed plate, with RPMI 1640 supplemented with 10% FBS, 1% L-Glutamine and 1% Penicillin/Streptomycin. T cells were incubated with no treatment, with Ni for 4 h, with E2 for 4 h, with Ni and E2 concurrently for 4 h, or with E2 for 24 h and Ni added in the last 4 h. The cells during the exposure period were incubated at 37˚C, 5% CO_2

Naïve CD4+ T Cell Activation – Sterile 96 well flat bottom plates were coated with 50 µL of 1 µg/mL anti-CD3 (eBioscience) for 2 hours at 37˚C, and then washed to remove excess anti-CD3. After incubation with Ni and/or E2, cells were washed with PBS and resuspended in 50 µL of fresh RPMI 1640 media supplemented with 10% FBS, 1% L-Glutamine and 1% Penicillin/Streptomycin. For activation, the resuspended cells were then added to the CD3-coated plate along with 50 µL of 10 µg/mL anti-CD28, and incubated at 37˚C, 5% CO_2. After 96 hours, cells and supernatants were recovered and stored at -20˚C for future analysis

Th1/Th2 Cytokine Analysis – Supernatant was analyzed using the commercially available Human Th1/Th2 ELISA Panel Ready-Set-Go! kit (eBioscience) to measure levels of IL-2, IFN-γ, IL-4 and IL-10. The assay sensitivity level was 2 pg/mL (IL-4 and IL-10) and 4 pg/mL (IFN-γ and IL-2).

Statistical Analysis - Data are expressed as mean ± SEM. SigmaStat v. 3.5 was used for statistical analysis. After a normality test was performed, the data were analyzed using ANOVA with a Holm-Sidak test or ANOVA on ranks if the normality test failed with a Dunn's test.

Differences were considered statistically significant if $p < 0.05$ as compared to another treatment group.

3.4 Results

Effect of Ni on naive T cells after activation

To examine the effect of Ni on Th cell differentiation, naive CD4+ T cells were isolated from human PBMCs and treated for 4 hours with 250 μM $NiCl_2$. This concentration of $NiCl_2$ was selected after a series of preliminary experiments to identify the highest dose that would have a minimal effect on viability (data not shown). After incubation with Ni, cells were non-specifically activated with anti-CD3/anti-CD28 antibodies, and the supernatant analyzed for cytokine content 96 hours later. In untreated naïve CD4+ T cells, activation alone elicited the production of IL-2 and IFN- γ as expected (Fig. 3.1), with no detectable levels of IL-4 of IL-10 (data not shown). In naïve CD4+ T cells treated with $NiCl_2$ for 4 hours prior to activation, levels of IL-2 were unchanged as compared to untreated cells, with a decrease in IFN-γ levels; no detectable levels of IL-4 and IL-10 were present (data not shown).

Effect of estrogen on naive T cells after activation

Next, we examined whether the presence of estrogen would influence the pattern of cytokine secretion by T cells. For our investigations, we selected a dose of 5000 pg/mL 17β-estradiol (E2), which is reflective of physiological levels in the serum in a female during the first trimester of pregnancy [32]. Naïve CD4+ T cells were treated with E2 for 4 or 24 hours prior to activation with anti-CD3/anti-CD28 antibodies, and the supernatant analyzed for IL-4, IL-10, IL-2 and IFN- γ. There were no detectable levels of IL-4 or IL-10 for either time point (data not shown). After the 4 hour treatment with E2 and subsequent activation, levels of IL-2 were equivalent to those produced by T cells that were activated alone (Fig 3.1A). In contrast, cells pre-incubated with E2 showed a trend toward an increase in IFN-γ levels produced after activation as compared

to the activated control, but this did not reach statistical significance (Fig 3.1B). When treatment with E2 was extended to 24 hours, there was a depression of IL-2 (Fig 3.2A) and IFN-γ secretion (Fig 3.2B) as compared to controls.

Combined effect of Ni and E2 on cytokines produced by CD4+ T cells

Finally, we investigated whether the presence of E2 would alter the response of naïve CD4+ T cells activated after incubation with $NiCl_2$, and whether such effects were time-dependent. When cells were incubated with both E2 and $NiCl_2$ for 4 hours prior to activation, similar levels of IL-2 were produced compared to those treated with Ni alone (Fig 3.1A); in contrast, the presence of E2 increased the production of IFN-γ in the presence of Ni (Fig 3.2B). When cells were incubated with E2 for 24 hours and Ni added to the cultures for the last 4 hours prior to activation, we observed a suppression of IL-2 (Fig 3.2A) as well as IFN-γ (Fig 3.2B) compared to the T cells that were activated alone and to those that were treated with Ni for 4 hours prior to activation. Levels of IL-4 were consistently non-detectable while IL-10 levels were inconsistent (data not shown).

3.5 Discussion

This pilot study elaborates on the ability of Ni to exert effects on health via its influence on the activities of T helper cells. This is of interest due to the presence of Ni in airborne PM generally, but especially in contexts where exposure to Ni may be higher due to occupational or local industrial factors. Others have shown that metal-rich PM from a variety of sources is able to elicit various forms of pulmonary injury [1, 4, 10]. We are particularly interested in Ni-containing particulates, because we have analyzed ambient PM from our local environment, Sudbury ON, and found high levels of nickel, likely due to the presence of local nickel smelting and refining industries [19]. Ni has been shown to elicit the production of various types of cytokines *in vitro* using human PBMCs, including IFN-γ [33, 34] but was able to suppress IFN-γ secretion from T lymphocytes from rats [35]. However, the cells used and their source appear to influence whether or not Ni is able to enhance or suppress IFN-γ levels. Our data suggest that Ni is able to dampen levels of IFN-γ and enhance Th cell proliferation. Taken together, these findings suggest that Ni may be able to skew polarization towards Th2 through the dampening of IFN-γ [36].

It has been very well established that the endocrine system interacts with and influences the functioning of the immune system, and sex hormones are known to affect leukocytes in a variety of ways. In spite of this knowledge, there has been little investigation pertaining to how the health effects of air pollution exposure might be influenced by sex, both in the epidemiological and experimental literature. In this study, we wanted to introduce an aspect of sex into the experimental design, but recognized that this is not a simplistic endeavour. We decided to focus on the effects of E2 because of the existing literature on its ability to modulate many cells of the immune system. E2 is a potent estrogen that is able to influence many aspects of the immune

response. E2 is found in both males and females, but in females it fluctuates throughout the month and steadily increases during pregnancy [27, 32, 37]. This preliminary investigation suggests that while more data are needed, it is indeed warranted to incorporate aspects of sex into the experimental design, as it can alter the outcomes in fairly dramatic ways.

We treated naïve CD4+ T cells with E2 prior to activation, and while it did not alter the levels of IL-2 there was an increase in the levels of IFN-γ. We were surprised by the observation that cells pre-treated with E2 produced higher levels of IFN-γ, since the literature suggests that high levels of estrogen promote a Th2 bias, as seen during pre-ovulation and pregnancy [27, 28]. However, there is indeed literature to support the finding that estrogen alone is able to promote IFN-γ production [22]; although the cell types are different, our findings are consistent with those of Grasso & Muscettola (1990), in that the presence of estrogen promoted an IFN-γ response from leukocytes *in vitro*. This is not surprising given that estrogen, once bound to its receptor, is able to bind to an estrogen response element on the IFN-γ gene and promote IFN-γ expression [38]. High levels of E2 alone have been shown to suppress IL-2 in peripheral blood lymphocytes *in vitro* [37]. We did not see IL-2 suppression after 4 hours, however there was no change as compared to the T cells that had been activated alone. Similarly to IFN-γ, this may be a time dependent response as after 24 hours E2 was able to suppress levels of IL-2. Our data suggest that E2 at high doses is indeed immunosuppressive, blunting T cell growth and proliferation through suppression of IL-2, and the inability to polarize Th1 responses as there was a suppression of IFN-γ after pre-treatment with E2 for 24h. This may help to explain why high levels of E2 are associated with a Th2 bias, in that it may not promote Th2 responses directly, but rather indirectly through the suppression of Th1 responses which could otherwise cross-regulate the polarization of Th2 responses.

In our experiments, the presence of E2 promoted the production of IFN-γ, a Th1-associated-cytokine, in the presence of Ni after 4 hours. This may have implications with respect to allergic respiratory diseases and PM. PM is able to exacerbate pre-existing cardiopulmonary conditions, and can act as the adjuvant for the sensitization towards otherwise harmless antigens. Traditionally, IFN-γ is associated with the suppression of Th2 responses since it can suppress IL-4 which is important in polarizing Th2 responses [22, 24], which has implications for the development of allergic sensitization in the first place. However, in cases of established disease, it has been recently suggested that IFN-γ from CD4+ T may act in concert with Th2 cytokines to enhance the severity symptoms [39]. Interestingly, after the 24 hour treatment with E2 there was a suppression of both IL-2 and IFN-γ in the presence of Ni. This may suggest that estrogen's ability to alter the immune response towards a Th1 polarization in the presence of Ni is time dependent as the 4 hour pre-treatment resulted in IFN-γ enhancement while the 24 hr pre-treatment resulted in the suppression of IFN-γ. This would suggest that we should exercise caution in making generalizations about the effect of E2 on immune responses since, depending on the length of exposure and the timing with respect to the development of allergy, it could either suppress or exacerbate allergic responses.

This work has potential implications for our understanding of how exposure to ambient air pollution might affect immune responses differently in boys and girls and in men and women. It has been established that as children, boys have a greater prevalence of allergic diseases than girls [30, 40]. However, there is a shift in the disease post-adolescence that has been attributed to changes in hormone levels, including estrogen [40]. As our data suggest, estrogen is able to both enhance and dampen IFN-γ levels, depending on duration of exposure, which could potentially suppress or exacerbate symptoms. Interestingly, although asthma is understood as a

predominantly Th2-mediated disease, the Th1-associated cytokine IFN-γ is also present [39] and may contribute to the development of airway hyperresponsiveness [41]. Our findings suggest that acute exposure to Ni-containing PM may cause more severe exacerbations of asthma as a result of inflammation in females because of the ability of E2 to enhance levels of IFN-γ in the presence of Ni.

It is clear that sex is an important influence on immune function, and needs to be considered more often in experimental research. In this study, we found that the profile of cytokines produced by T cells activated in the presence of Ni differs when E2 is also present, demonstrating that even the presence of a single sex hormone can profoundly alter the outcome. The real-life situation will be quite a bit more complex, given the presence of multiple hormones at fluctuating doses which will likely interact with one another and modulate immunity. For instance, lower levels of Ni are needed for sensitivities in females than males [42] and there is research that suggests that nickel sensitivity may change with estrogen levels during the menstrual cycle [43]. Although the aforementioned study did not yield significant results, it is still important to consider nonetheless. Things will become even more complicated when we consider gender in addition to sex, as exposure to PM may vary considerably depending on social factors such as cooking, smoking, or occupational exposures that are typically gendered [33, 34]. We suggest that studies that investigate PM exposure both *in vivo* and *in vitro* should take this into consideration in both the design of experiments and interpretation of findings.

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Fig 3.1. T cell cytokine production after 4h incubation with Ni and/or E2. Naïve T cells were activated alone or pretreated with either 250 μM Ni, 5000 pg/mL E2 or both for 4 hours. 96 hours after activation the supernatants were collected and analyzed for IL-2 (panel A) or IFN-γ (panel B). Data are shown as mean +SEM, with n=3. * indicates a statistically significant difference ($p < 0.05$).

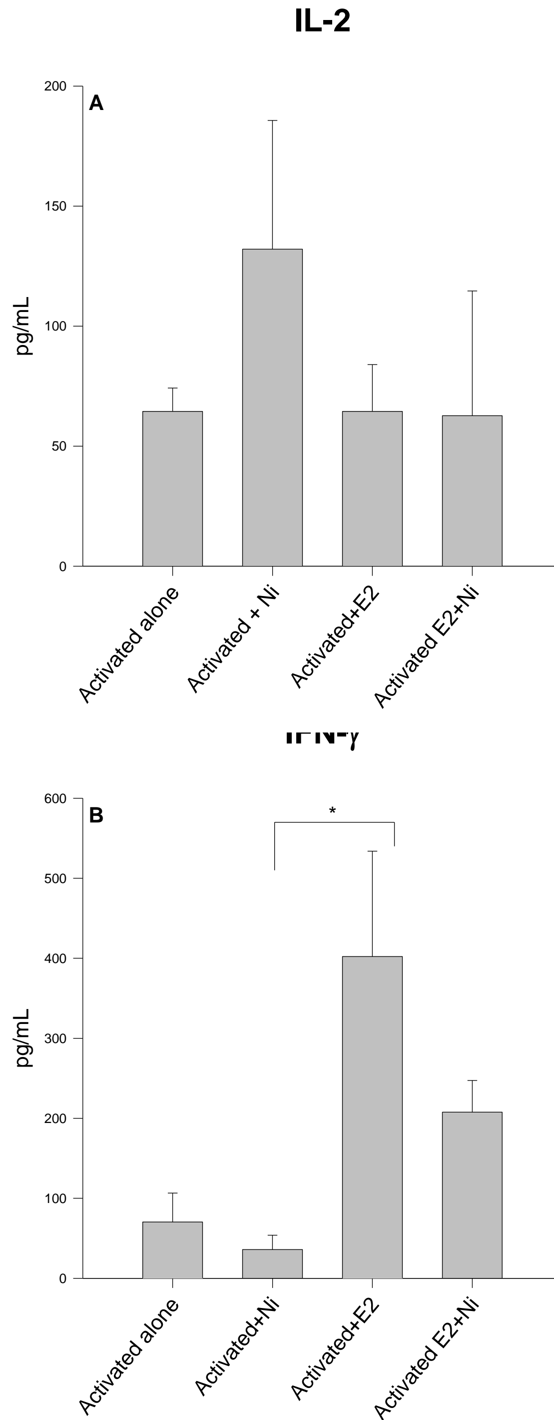
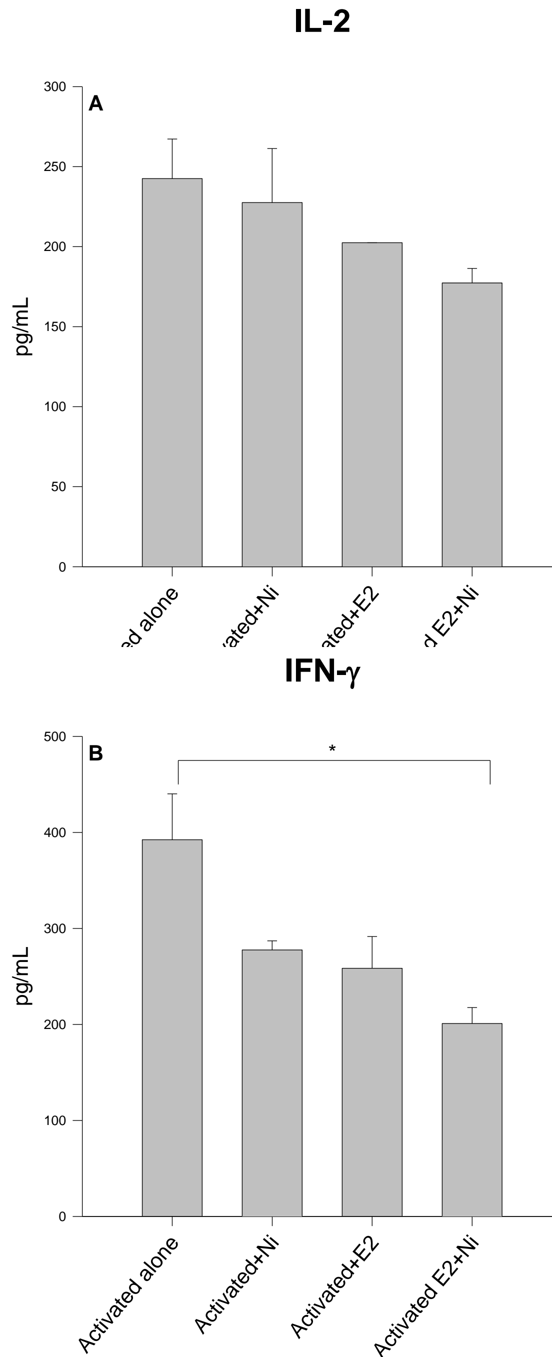


Fig 3.2. T cell cytokine production after 4h incubation with Ni and/or E2 for 24h. Naïve T cells were activated alone or pretreated with either 250 μM Ni for 4 hours, 5000 pg/mL E2 or both for 24 hours. 96 hours after activation the supernatants were collected and analyzed for IL-2 (panel A) or IFN-γ (panel B). Data is representative of 1 experiment, shown as mean +SEM, with n=3. * indicates a statistically significant difference *(p* < 0.05).



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CHAPTER 4: UNIFYING DISCUSSION

The presence of major smelting and refining activities in the Greater Sudbury area creates concern in the community about the possible health effects of airborne emissions. Although there is an extensive literature regarding the impact of PM in general on health, there is little research that focuses on the individual constituents of PM, including metals, using physiologically relevant doses. The research described in this thesis attempts to answer some of these concerns by investigating how Ni and Cu influence immune-inflammatory responses.

Although quite different in terms of methodology, the work in Chapters 2 and 3 are part of a common project to test the hypothesis that metals present in PM influence immune responses and potentially promote the development of allergic disease or other hypersensitivity disorders. Additionally, as part of the *in vitro* work described in Chapter 3, we added estrogen to the cell cultures to examine how aspects of sex might play a role in modulating the effects of metals on immune responses.

While the adjuvant capacities of PM have been previously investigated by others, our studies are unique in a number of regards. First, the experiments were designed using low and physiologically relevant levels of Ni and Cu, for two main reasons: first, Ni is toxic in all chemical forms except as the metal itself [1], and we did not want to induce acute metal toxicity; and second (and more importantly), we wanted to use a dose that was relevant for real-life human exposures. Other studies have used higher doses of metals that are present in PM or in single instillations [2–5]; while these concentrations induce a response, it is not a reflection of the daily exposure that individuals exposed to PM are subjected to daily. Our investigations demonstrate that low-dose acute exposures can alter antigen-specific immune responses to harmless antigens, which could potentially result in pathogenic immune responses.

Each of the manuscripts that are included in this thesis contains its own discussion of the results as they pertain to that individual chapter. As such, I will refrain from reiterating previously discussed material, instead focusing on aspects that have not previously been discussed, or which are unifying themes in the entire work.

4.1 What counts as 'tolerance'?

Inhalation tolerance has been extensively studied, and there has been some demonstration of the ability of air pollution to affect the development of airway responses, resulting in allergic sensitization [6, 7]; here, we contribute to this literature by investigating whether physiologically relevant levels of metals present in PM that are of particular interest in Northern Ontario may influence the development of allergy or tolerance. The experiments described in Chapter 2 were intended to investigate the ability of metals that are present in SPaM to alter an OVA-specific immune response, with the hypothesis that concurrent exposure to metal and OVA would disrupt the processes of inhalation tolerance to OVA and result in immunological sensitization to OVA. We found that low levels of metals were able to subtly alter immunological responses to OVA. Interpretation of these results was challenging as the responses were subtle, and did not necessarily conform to the classic Th1/Th2 paradigm, especially with respect to Cu.

An experimental protocol for inhalation tolerance was first introduced by Holt *et al.* in 1981, where mice were repeatedly exposed to aerosolized OVA; when attempts were made to sensitize these mice to OVA using intraperitoneal injections with adjuvant, mice that had previously been exposed to aerosolized OVA were largely protected from the development of OVA-specific IgE and Th2 responses after challenge. Adaptations of the original protocol have resulted in a

number of variant tolerance models, with the main parameters defining tolerance being a reduction in OVA-specific IgE, reduced infiltration of leukocytes into the BAL and a decrease in Th2 associated cytokines [8]. We used a murine model of inhalation tolerance involving 10 consecutive days of exposure to aerosolized OVA, followed by challenge with aerosolized OVA 3 weeks later; the ability of metal exposure to influence tolerance was tested by administration of saline, Ni, or Cu intranasally concurrent with the initial 10-day aerosolization protocol. To evaluate the induction of tolerance to OVA, we examined a number of biological outcomes indicative of immune reactivity to OVA: leukocyte infiltration and cytokine production in the airways immediately following the tolerance protocol and also after rechallenge, OVA-specific immunoglobulin levels in the serum, and cytokine production by splenocytes co-cultured *in vitro*.

In analyzing the results, it became apparent to us that certain assumptions about the inhalation tolerance protocol itself may warrant additional investigation. Implicit in the literature is the assumption that the induction of tolerance is established by the 10-day inhalation protocol, and that the purpose of the intraperitoneal injections with OVA and adjuvant in this model is to 'test' the establishment of that tolerance. When we originally planned this project we shared this implicit understanding, and designed the experiments to test whether Ni or Cu could act as adjuvants, by disrupting the induction of tolerance to OVA and triggering active immune responses. However, our findings here have led us to consider that these assumptions about how the inhalation tolerance protocol works might be mistaken. This speculation arises from our observations looking at the control group only: while it initially appeared that tolerance was induced (since there was a lack of inflammation in the airways and very low levels of OVA-

specific serum IgE), the results of the splenocyte cultures revealed that there were still cells that have the ability to produce Th2 cytokines in response to OVA stimulation.

In our original design of this project, we did not believe that the intraperitoneal injections were relevant to the question we were asking. However, in reviewing our data, we wonder if the intraperitoneal injections, rather than acting as a 'test' of tolerance, may in fact serve a tolerogenic function; that is, perhaps the initial 10-day aerosolization protocol induces a nascent OVA-specific T regulatory cell response, and subsequent intraperitoneal injection of OVA serves as a strong stimulus which consolidates and strengthens the immunologic memory for tolerance to OVA. This is speculative at this point, but it might be of interest to examine this possibility in future studies.

There is a body of literature that attempts to explain the development of inhalation tolerance, which may be due to several different immune phenomena such as a deviation from a Th2 to a Th1 response [9], the involvement of regulatory cytokines and T regulatory cells [10, 11], or perhaps some combination of the two. In our study, there appears to be immune deviation and immunosuppression that is not seen in models that use aerosolization, IP sensitization and challenge, based on the cytokine profile that was present in splenocytes from mice treated with OVA and challenged with OVA *in vitro*. A subtype of adaptive T regulatory cells, Tr1, have been shown to secrete IL-5, IFN-γ and IL-10 with an absence of IL-4 [12], which is consistent with the cytokine profile we measured from the pooled splenocytes of mice exposed to OVA and challenged *in vitro* with OVA. The increase in IL-10 and a modest increase of IFN-γ we observed in our splenocyte culture may suggest that the response is dependent on polarization towards a Th1 phenotype, in addition to IL-10 producing Tregs. However, we did not specifically isolate subsets of T cells to determine their phenotype and cannot definitively say

that we had this specific subset of Tregs; further experimentation would be required to delineate this. Tolerance has been suggested to require Tregs that rely on TGF-β and FOXP3 [13]; these are also known as natural Tregs (nTregs). We did not measure levels of TGF- β so we cannot comment further in regards to whether or not they were present.

Taking all of these factors into account, we suspect that the exposure to OVA only did indeed induce a degree of tolerance, albeit perhaps not to the same extent as might have been induced had the i.p. injections been given to consolidate the response. Future studies would be desirable to further enhance our understanding of the tolerance protocol and the role of the secondary priming in consolidating the tolerance phenotype.

4.2 Immunomodulatory effects of metals present in SPaM

Given that we cannot be entirely certain that the administration of 10 OVA aerosolizations is sufficient to induce inhalation tolerance, we are cautious about suggesting that our data indicate that concurrent exposure to a metal may 'subvert' tolerance. However, our findings – both in Chapters 2 and 3 – quite clearly indicate that the presence of metals can influence the development of immune responses.

Research into the health effects of PM is complicated by its enormous heterogeneity, which is dependent upon local environmental factors. Using any single model of PM is too simplistic for understanding the potential for health effects, as chemical or physical differences between two different types of PM could endow them with radically different potential for causing biological effects. This has been demonstrated by studies that have compared metal rich PM from an industrial area with an urban PM from a different location, resulting in greater levels of

inflammation and allergic sensitization in mice exposed to the metal-rich PM [14]. Metal-rich PM from Sudbury may therefore have different effects than other ambient urban PM. Population-based investigations of PM that do not account for the heterogeneity of PM may be underestimating the risk for adverse health effects by treating all PM as equal. The approach that we have adopted, in which we investigate individual constituents of PM, will enhance our understanding by determining the relative contribution of specific components, and thereby hone in on those components with the greatest potential for causing adverse health effects. Different components of PM, as we have demonstrated, are able to induce different subtle changes in the nature of the ensuing immune-inflammatory response. We argue that it may be more effective to start developing regulation policies that target particularly harmful components of PM rather than PM in general. This focus on ameliorating the more problematic components of PM may have a greater impact on improving the general health of populations and reduce the number of PM-associated health risks and diseases.

Both animal [6, 7, 15] and human [16] studies have demonstrated that PM is able to act as an adjuvant for allergic sensitization, but most of these focus on the adjuvant abilities of whole PM rather than on their individual constituents. Previous studies using acute and chronic exposures of SPaM in our lab have demonstrated the ability of SPaM (metal-rich) to induce an inflammatory response that is different from other types of PM, such as DEP [17], a PM with very little metal content, highlighting that the differences in composition will impact the overall response. Since Sudbury PM has a unique profile of metal constituents [18], it is of interest to determine its adjuvanticity as compared to other types of PM used in experimental studies, such as DEP, ROFA and EHC-93 [7, 16]. Rather than investigate SPaM as a whole particulate, in this work we focussed on Ni and Cu because although they are found in most urban PM samples,

they are present at substantially higher levels in SPaM [5]. In addition, we aimed to use relatively low doses of the metals, reflecting concentrations that might be achievable under real-life circumstances.

Although it is clear that allergic diseases are predominantly driven by Th2 responses, there is still a lack of information pertaining to how these responses are triggered in the first place. Furthermore, there are still questions regarding why the prevalence is increasing [11]. In addition, contrary to the predominant dogma, Th1 cells and their respective cytokines may not always counterbalance allergy, but may contribute to the development of allergic respiratory diseases in some circumstances [19, 20]. Our findings suggest that metals present in PM may be able to influence the development of allergic sensitization to innocuous protein antigens, since both Ni and Cu were able to alter the inflammatory profile towards OVA resulting in increased cell infiltrate, inflammation, and alterations in cytokine production.

Exposure to Cu and Ni is widespread, and is known to trigger proinflammatory cytokine expression and inflammation [2, 5]. We have evidence that both Cu and Ni were able to alter the development of OVA-specific immune responses in these studies. The induction of GM-CSF by both Cu and Ni in murine splenocytes *ex vivo* suggests that antigen presentation would be enhanced, as GM-CSF is a strong stimulus for dendritic cell maturation; such enhancement of APC activity would be expected to promote the development of active immune responses, consistent with our hypothesis that Ni and Cu might act as adjuvants. *Ex vivo*, Ni significantly enhanced OVA-specific production of IFN-γ while depressing levels of IL-10, suggesting that Ni may promote Th1 polarization and undermine the immunosuppressive mechanisms of inhalation tolerance. In a different context, *in vitro* stimulation of human T helper cells with Ni was able to depress levels of IFN-γ, suggesting an ability to polarize responses from naïve T cells towards a

Th2 phenotype. It is clear that both Cu and Ni are able to alter the inflammatory profile and Ni has the ability to not only act as an adjuvant as seen *in vivo* but is also able to affect activated T cells as seen *in vitro*.

Although IFN-γ is conventionally believed to downregulate Th2 responses, under some circumstances it can actually play a co-operative role with Th2 cells. This is an important idea, as even though the predominant dogma about Th1 or Th2 responses is that they suppress and counterbalance each other, it may not be that simple. Randolph *et al*. have shown that OVA-specific Th1 and Th2 cells differentiated *in vitro* and transferred to naive BALB/c mice were together able to induce eosinophilic inflammation after challenge with OVA [21]. Eosinophilic inflammation, which is a hallmark of Th2 and allergy, may not simply be due to Th2 responses, and may require additional signals such as Th1 mediated upregulation of adhesion molecules [21]. This may be partly why even though our responses in mice concurrently exposed to Ni+OVA induced significant levels of IFN-γ, there were eosinophils present at levels above OVA treated mice. This may be important as Ni could promote Th1 polarizing responses towards otherwise innocuous antigens and upon re-exposure (challenge) there is a Th1 inflammatory response that can act synergistically to upregulate adhesion molecules and recruit eosinophils resulting in exacerbation of existing allergic respiratory disease. However, this is purely speculation since we did not examine adhesion molecules; it is possible that the mild eosinophilia is just an artifact and not necessarily due to a cooperative Th1/Th2 response.

There are conflicting reports in the literature regarding whether Ni is able to suppress or enhance levels of IFN-γ. Previous studies have established that Ni is able to induce a variety of cytokines from human PBMCs [22, 23], but was able to suppress IFN-γ secretion from T lymphocytes from rats [24]. Despite the source and cell type being different, it would appear that there are

different factors influencing whether Ni can enhance or suppress IFN-γ levels, and subsequently promote Th1 or Th2 respectively. Our studies also suggest that the relationship between Ni exposure and IFN-γ production is a complex one. We can see that Ni was able to alter the nature of the response to OVA, resulting in increased levels of inflammation, OVA-specific IFN-γ, and suppressed levels of OVA-specific IL-10 after challenge. In our mouse studies it appears that Ni was not acting as an adjuvant for a Th2 response, but may have subtly promoted Th1 polarization.

Although Cu elicited the greatest recruitment of cells into the lung, it did not induce either a strong Th1 or Th2 response. Splenocytes stimulated *ex vivo* produced IL-10, IL-5 and IFN-γ at levels greater than the OVA/saline and naive groups, with eosinophilia observed in the lungs of these mice (Table 1). There is literature that suggests that there could be concomitant Th1/Th2 responses that are similar to the responses generated by Cu: investigators using a model of airway eosinophil inflammation have evidence to suggest that the adoptive transfer of Th2 cells were not enough to promote eosinophil recruitment, and both Th1+Th2 were able to promote eosinophilia in the airways [21]. We did not see evidence of Th2 associated cytokines or significant eosinophil recruitment into the BAL either before or after challenge with respect to Ni (Table 1). In contrast, exposure to Cu concurrently with OVA resulted in significant eosinophil infiltration after the aerosolization period (Table 1). While these results were significant it is important to emphasize that these are very modest levels of eosinophilia that are much lower than in conventional experimental models of allergic asthma, where there may be as much as 80% eosinophils in the BAL [25–27]; in contrast, the levels we observed were on the order of 3%. Notably, this low level of eosinophil infiltration is consistent with that seen in a model of airway inflammation where mice are exposed to OVA inhalations after the adoptive transfer of

both Th1+Th2 cells [28], and our splenocyte cultures of Cu+OVA treated mice after stimulation with OVA *ex vivo*, produced levels of GM-CSF, IFN-γ, IL-5, and IL-13 (Table 1). However, in order to accurately confirm the inflammatory profile of Cu as an adjuvant, we would need to investigate Cu further by isolating T cells and stimulating them *ex vivo*, and using flow cytometry to determine whether the cytokines being produced are by Th1, Th2 cells or both. Together our data suggest that perhaps Cu is able to induce both local and systemic responses reflecting both pro-APC and Th1 and Th2 profiles.

Table 4.1. Summary of inflammatory profiles seen *in vivo* and *ex vivo* based on data from Chapter 2.

	OVA only	Ni only	Ni+OVA	Cu only	Cu+OVA
Total airway inflammation (after aerosolization period)	-	-	-	-	+
Total airway inflammation (after challenge)	+	n/a	++	n/a	++
Airway eosinophilia	+	+	+	+	++
OVA-specific IgE	-	n/a	-	n/a	n/a
Cytokines (splenocyte cultures):					
GM-CSF (Th1/Th2)	+	++	++	+++	+++
IFN-γ (Th1)	+	++	+++	++	+
IL-5 (Th2)	++	+	++	+	++
IL-13 (Th2)	+++	++	++	++	+
IL-10 (immunoregulatory)	+++	+	+	+	++
Inflammatory Profile	Minimal inflammation	Majority macrophages	Majority macrophages	Mild eosinophilia	Mild eosinophilia

Legend: - = absent, + = low, ++ = moderate, +++ = strong

4.3 The importance of sex and gender considerations in experimental research

Over the past 30 years, it has become increasingly apparent that we need to take sex and gender into account when conducting biomedical research. In spite of the clear interactions between the immune system and the endocrine system, there is a still a lack of epidemiological and experimental literature pertaining to how immune-inflammatory responses are influenced by sex, including the literature on the immunological effects of air pollution exposure. In fact, a 2011 study by Beery & Zucker found that immunological studies remain the least likely to address sex considerations compared to several other areas of research [29].

There is no question that sex is relevant for studies of allergic disease, as a clear sexual dimorphism between boys and girls in the prevalence of allergic respiratory disease has been documented [30, 31]. Prior to puberty, allergic disease is more prevalent in boys than in girls; this changes around puberty, when boys tend to outgrow their allergies, while girls are more likely to develop new ones [31]. This has often been attributed, at least in part, to changes in hormone levels that emerge at puberty. This is not surprising as just estrogen alone is known to influence a variety of immune cells as many of them express either one or both of the estrogen receptors (ER), ERα and ERβ. In addition, most autoimmune diseases are significantly more prevalent in women than men [32] and these autoimmune diseases are impacted when there are alterations in the endocrine system. For example, during pregnancy there is a maternal immune shift in order to accommodate a favorable fetal implantation and environment, which is the result of increased levels of estrogen, progesterone [33] and human chorionic gonadotropin [34]. During this time, autoimmune diseases that are principally Th2-based, such as systemic lupus erythematosus (SLE) [34, 35], can have potentially exacerbated symptoms [36]; this is similarly the case in asthma [37]. In contrast, people who have a Th1 mediated autoimmune diseases such

as rheumatoid arthritis usually have an alleviation of symptoms during pregnancy [38]. This can be at least partially attributed to the changes in hormones although the exact mechanisms are not entirely known.

Although most studies of the immunological effects of PM ignore sex entirely, the literature also provides us with many examples where sex has been handled in less than optimal ways including inappropriate uses of vocabulary. In a missed opportunity, an investigation by Schaumann *et al* used male (4) and female (8) human subjects, assessing airway inflammation after instillations with PM from 2 different cities [39]. Disappointingly, the results were not analyzed by sex, nor were the figures constructed in a way that allowed for the reader to discern between male and female subjects. Furthermore, when the authors were discussing future studies based on the data they failed to mention any aspect of sex and how that could influence the immune response. By failing to consider sex, this study may have missed valuable information pertaining to sex and PM exposure. Morgenstern *et al* also used male and female subjects, but instead of ignoring sex as a factor, they opted to treat sex as a "confounder", and statistically removed it from the data [40]. While this study at least recognizes that sex is indeed a factor to be considered, it is not discussed beyond its removal. In both cases, valuable information may be lost by ignoring sex or removing any sex effects using statistical methods, rather than reporting the data in a way that would allow any potential sex difference to be evaluated. In other cases, although sex factors are being considered, the vocabulary around sex is misused. For example, in a study investigating the impact of estrogen on female mice that had been ovariectomized, the authors used the term 'castration' interchangeably in reference to the ovariectomy; they also used the terms 'sex' and 'gender' interchangeably where the term 'sex' should have been used in all instances [41]. While some investigators are attempting to incorporate sex into their experimental designs and

data interpretation, there is a clear lack of continuity regarding terminology and appropriate relevance resulting in over-generalized data and a lack of nuance in interpretation.

Even though it may have been ideal to use both males and female mice to properly address sex within our experimental designs, it was not feasible in this case. In addition to doubling the resources necessary to conduct the study, female mice are easier to house in larger groups, are generally less aggressive, and other investigations in related fields have also used female mice. Since we only used female mice, it is important to bear in mind a number of additional considerations. When female mice are housed together, they develop estrous suppression either through pseudopregnancy, anestrous, or an extended period of diestrus [42]. The murine estrous cycle has similarities to the human menstrual cycle, although it is much shorter (4-6 days [43] compared to 28 days). During the diestrus phase there is a slow increase in the level of estrogen present [44], which is relatively equivalent to the levels of estrogen present at the late luteal phase (diestrus-1) when levels of estrogen dip after ovulation and the menstruation phase in females (diestus-2) [45]. We housed female BALB/c mice in groups of 3-5, and at this density, there may have been extended periods of diestrus [46] and suppression of cyclical estrus cycles. We did not confirm this with vaginal swabs or test levels of estrogen, but based on the considerable amount of literature that exists in regard to estrus cycle suppression it would be reasonable to assume that the mice in our studies experienced extended periods of low levels of estrogen. In general, low levels of estrogen *in vivo* tend to promote a Th1 bias [32, 47]. Thus, low levels of estrogen that would be present during prolonged diestrus could have created a Th1-promoting environment in our studies. However, it is important to recognize that within the *in vivo* model there are also other hormones that can influence the immune response: female mice also have progesterone, testosterone, FSH, and LH which are also able to influence the immune

response [48]; progesterone is generally considered immunosuppressive, as it downregulates proinflammatory cytokines and MHC class II expression by DCs *in vitro* [49]. As there are varying hormones and concentrations depending on estrus suppression in mice, at this point we can only speculate as to how these hormones would impact our results without having specifically measured their levels. However, we feel it is important to acknowledge the potential impact of diestrus on these studies.

Although we did not incorporate sex considerations into the experimental design of our animal studies, with the *in vitro* experiments we examined the influence of one aspect of sex by adding estrogen to the cultures. In general, the addition of estrogen appeared to suppress cytokine production, and did not induce a consistent Th1 or Th2 polarization as indicated by the hallmark cytokines examined. The presence of estrogen did indeed influence the effects of Ni exposure, as cells exposed to Ni with short term exposure to E2 had an enhanced Th1 polarization while a longer exposure to E2 resulted in lower cytokine production. As previously discussed, although IFN-γ is typically thought of as a classic Th1 cytokine that is generally not associated with Th2 responses such as allergic respiratory disease, recently it has been suggested that IFN-γ may be present during asthmatic flare ups in addition to Th2 cytokines such as IL-4 and IL-5 [19]. If correct and substantiated by others, these findings may suggest that high levels of estrogen may make individuals susceptible to exacerbations of allergic respiratory disease upon exposure to PM. These data demonstrate the importance of incorporating sex into experimental design as sex hormones are able to influence the cytokine response from T cells which is further evidence as to why it is not sufficient to make broad assumptions of data for both sexes.

One key observation we made is that the effects of estrogen on cytokine production in this model appeared to be time sensitive. After 4 hours, estrogen appeared to promote IFN-γ production

while IL-2 remained unchanged, but after 24 hours suppressed both cytokines. Based on the data that we obtained, high doses of E2 appear to suppress both Th1 and Th2 polarization. While this is somewhat consistent with a previous investigation regarding inhibition of IL-2 by estrogen [50], we would have expected that the levels of E2 used for our experimental design would have promoted an allergic phenotype, since the concentration of E2 used mimicked the high levels present during pregnancy, and it is generally thought that high E2 levels bias the immune system towards a Th2 phenotype. One possible explanation for this contradiction is that the experimental design used only one specific cell type, whereas in *in vivo* or *ex vivo* situations there would be other cellular interactions, such as cytokines from epithelial cells and APCs that could influence T cell activation and polarization. *In vitro* such dynamic interactions are absent, and so we are only able to examine the interaction between the metal, the hormone and the T helper cell itself. Furthermore, these cells can be influenced by other circulating hormones, which may interact with the effects of estrogen exposure. Thus, as with any *in vitro* study, we must be cognizant that our *in vitro* models are simplified representations of the complexity of the *in vivo* context.

In fact, the effects of estrogen may help to explain some of the conflicting data between our *in vivo* and *in vitro* experiments with respect to the effect of Ni on nascent immune responses. The *in vitro* study examined the influence of estrogen on naive Th cells prior to nonspecific activation, where the dose of estrogen used was high, 5000 pg/mL, reflective of serum levels in pregnant women; in this context, we found that Ni promoted enhanced levels of IFN-γ after 4 hours and IFN-γ suppression after 24 hours. In the *in vivo* investigations, estrogen levels were likely low, as they were not pregnant and more than likely in estrous suppression; in this context, we found that Ni promoted antigen presentation and Th1 polarization with significant levels of GM-CSF and IFN-γ respectively. Thus, the effects of estrogen may in part account for the

disparities seen between the murine model and the naive Th cell model. This can serve as an example of the way that recognizing the possible effects of sex on experimental outcomes can help shed light on the often conflicting results within different experimental designs.

Incorporating estrogen into our *in vitro* study was a way of addressing one aspect of sex, but it is important to emphasize that this does not fully speak to the complexity of sex. The fluctuations of other sex hormones, such as progesterone or testosterone, are also going to impact the immune response generated towards PM in ways that may be unpredictable depending on age, hormone levels, receptors present and in what ratios; they may interact in additive, synergistic, or antagonistic ways. While addressing this would have been too cumbersome to incorporate in this small study, it would be interesting to investigate the influences of a number of different hormones together to help us to further tease out the influences of sex on the immunomodulatory effects of Ni. The main point here, however, is to recognize that the presence of sex hormones can have rather dramatic effects on the outcome of exposure to environmental agents.

While we recognize that gender is also important, it was not explicitly taken into consideration as it is difficult to address the complexities of human gender in the setting of experimental biomedical science. In general, we should avoid using the term 'gender' to refer to non-human animals, as 'gender' is a human construct that is dependent upon social and cultural norms [51]. For work with cells *in vitro*, although 'gender' would be appropriate to describe the human subjects from whom the cells were derived, the socio-cultural aspects of gender cannot be replicated within a petri-dish, and thus it would be a stretch to say the cells themselves had a 'gender'. In both instances, we must be cautious about our interpretation around sex/gender, as stereotypes about men and women may insidiously inform our theorizing. However, as we recommended in our paper, we have taken 'baby steps' to at least consider and acknowledge the

importance of sex and gender [51]. With respect to this study, gender is relevant as a complex socio-cultural construct that influences exposure to Ni through socio-cultural mechanisms such as occupational roles and use of jewelry. Occupational exposure to Ni spans many occupations, and Ni hypersensitivities are more prevalent in women [52]. There may be interacting effects here between exposure to Ni in occupational settings and other gendered forms of Ni exposures, for example though the use of Ni-containing jewelry. Another article, reviewing nickel sensitivity in relation to sex was in fact discussing gender in terms of environment, occupation and jewelry adornment [53]. At this point, there is not enough research to definitively associate nickel hypersensitivities to sex or gender alone; it is likely an intricate interplay of both biological and social factors.

4.4 Concluding Remarks

These studies have demonstrated that metals present in PM in Sudbury are able to alter immune responses, both in *in vitro* and *in vivo* experimental models. The studies described in Chapter 2 examined the ability of Ni and Cu at physiologically-relevant levels to act as an adjuvant for OVA in a murine model of exposure. In Chapter 3, we studied the influence of Ni on T helper cell differentiation *in vitro,* while simultaneously examining an aspect of the influence of sex on these responses, by testing whether the presence of estrogen would modulate the effects of Ni on cytokine production by T cells We demonstrated that exposure to low levels of Ni and Cu can modestly influence immune responses in ways that may promote allergy or other forms of hypersensitivity. In addition, we demonstrated that aspects of sex, specifically the presence of steroid hormones, may shape the character of such responses in important ways.

Overall, this research contributes to our understanding of the influence of particulate air pollution on the development of allergic disease, and also helps us to understand sex disparities in the prevalence and manifestations of allergic disease, and suggests that low, physiologically relevant doses of metals found in SPaM can significantly alter inflammatory responses towards OVA. As government regulations continue to monitor and regulate levels of PM, our data suggest that it may in fact be pertinent to address specific levels of constituents found in PM rather than treating all PM the same, as even small doses within the limits of daily exposure can have immunomodulatory effects.

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