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Toxic effects of heavy metals on the immune system

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Toxic effects of heavy metals on the immune system

Evidence from population and in vitro studies

Yu Zhang

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 groningen

Toxic effects of heavy metals on the immune system

Evidence from population and in vitro studies

PhD thesis

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1

GENERAL INTRODUCTION

1. E-waste and Children's health

Electronic waste (e-waste) is defined as discarded electrical and electronic equipment and components. This has become a rapidly increasing global problem [1, 2]. Informal and uncontrolled e-waste recycling processes include acid dissolution, incineration, pyrolysis and gasification, which all result in a large release of heavy metals and organic pollutants into the local environment. With approximately 70% of e-waste import every year, China appears to be the largest e-waste dumping site in the world [3]. Although the Chinese government has implemented policies to control overseas e-waste importation, chemicals in the environment continue to exist due to their long half-lives and persistent accumulation.

Children are most susceptible to threats posed by e-waste exposure. It is believed that unique routes of exposure, including breastfeeding and placental exposures, frequent hand-to-mouth behavior in early years, and immature physical development of children are likely to put them at high risk for being affected by toxicants [2, 4]. Epidemiological investigations have reported that exposure to hazardous substances in e-waste areas poses serious health risks to children, including altered thyroid function, aberrant cellular expression and function, abnormal temperament and behavior, and decreased lung function [2, 5].

As one of the largest e-waste destinations and recycling areas worldwide, Guiyu, nestled in the main manufacturing zone of southeast China, has a 30-year history of unregulated e-waste disposal [3, 6]. High levels of heavy metals are found in the air, soil, water, sediment and plants in Guiyu, as compared with non-polluted areas [7, 8]. Similarly, our previous studies reported high levels of lead, cadmium, mercury, manganese in the blood of Guiyu children [9-11]. Many adverse health problems, including cardiovascular problems, endothelial inflammation, increased prevalence of respiratory symptoms and asthma, aberrant synthesis of hemoglobin and erythrocyte complement receptor 1, and restricted physical growth and development, were reported to be related to high concentrations of heavy metals in this area [9-14]. Furthermore, alterations of the immune system in children from e-waste areas were also previously reported: It has been shown that elevated exposure to heavy metals is associated with increased memory T cell populations, lower NK cell percentage, higher expression of interleukin (IL)-6 and IL-1 β , and decreased titer of antibodies against measles, mumps and rubella [15-17].

2. Heavy metals

Heavy metals are generally defined as metallic elements that possess a high specific density above 5 g/cm^3 with an atomic weight more than 40.04 [18]. They can enter the human body via various routes, such as food intake, water drinking, air inhalation and skin absorption following inadvertent occupational and environmental exposure [19, 20]. Some of these metals, such as zinc, selenium, manganese, cobalt, iron, and copper, are essential trace elements and beneficial for various physiological and biochemical progress in the living organisms when in adequate amounts, however, excessive supplementation of such metals may become noxious to cellular and tissue functions [19]. Other metals, such as lead, cadmium, mercury, arsenic, nickel, and chromium, are deleterious to biological organisms even in minor quantities and are therefore classified as non-essential metals [19, 20].

Those non-essential metals are not easily metabolized by the body and their constant accumulation may cause acute or chronic toxicities on the hematopoietic, renal, reproductive, immune and central nervous system [20, 21]. Lead occurs in different forms, such as metallic lead, lead salts, and tetraethyl lead and organic lead, and has been found in industrial processes, food and smoking, household dust, drinking water and domestic sources [20, 22]. It has no recognized biological function in the body. Once lead enters the bloodstream, more than 95% binds to hemoglobin and is distributed within different organs and tissues of the body. Lead has an approximately 30-day half-life and is excreted mainly in the urine [20, 23]. Cadmium is found in the environment in many forms, including cadmium acetate, cadmium sulfate, cadmium chloride, cadmium oxide and cadmium carbonate [24]. Cadmium is frequently used in batteries, fertilizers, coatings, pigments and alloys [24, 25]. It will remain inside the body permanently due to its strong accumulation competence and long half-life of about 10 to 30 years [26]. Mercury is a transition element and is found in different forms: metallic elements existing as a liquid at room temperature, inorganic salts that exist as ions (Hg^+ or Hg^{2+}) binding to chloride, sulfur or oxygen to form mercuric salts, and organic compounds including phenylmercury, dimethylmercury and methylmercury, with each having its own unique toxicity and bioavailability [20, 27, 28]. Among these forms, methylmercury is found in a large proportion in the environment and is formed as a result of the methylation of Hg^+ or Hg^{2+} by the activities of microorganisms present in soil and water [20, 29]. The half-life of mercury in the human body is around 70 days, after which 90% is excreted [24, 30]. Arsenic is a metalloid

that occurs in an inorganic form including the trivalent arsenite and the pentavalent arsenate, and organic species, such as monomethylarsonic acid, dimethylarsonic acid, and trimethylarsine oxide [19]. All over the world, about 100 million people get exposed to arsenic due to contaminated food and drinking water by arsenical pesticides, natural mineral deposits or inappropriate disposal of arsenical chemicals [31].

Heavy metals exert their toxicities by interactions with cellular constituents. In biological systems, heavy metals are transported and compartmentalized into cells and tissues and bind to cellular organelles and components including cell membrane, mitochondrial membrane, lysosomal membrane, endoplasmic reticulum membrane and nuclear membrane via ionic or coordinating bonds [25, 32]. Moreover, some non-essential heavy metals are capable of replacing essential elements in the body due to the same oxidation state or similar chemical structure. For example, lead and cadmium can substitute calcium, zinc, iron and magnesium to ultimately disrupt the cell metabolism [21, 33, 34]. In addition, heavy metals are responsible for the increased generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as superoxide anion (O_2^-), hydroxyl radical ($HO\cdot$), nitric oxide ($NO\cdot$), nitrogen dioxide (NO_2), and dinitrogen trioxide (N_2O_3). These ROS and RNS are known to induce oxidative stress and contribute to metals associated inflammation, apoptosis and tissue injury in the liver, kidneys, brain or immune organs [32, 35].

3. Overview of the immune system

The immune system is an intricate network of organs, cells and molecules that specializes in maintaining the body's homeostasis and responding to environmental exposures [36, 37]. In vertebrates, the immune system has been conceptually divided into two subsystems: the innate and adaptive immune systems, which are commonly distinguished on the basis of their levels of specificity and immunologic memory [38, 39]. The innate immune system is semi-specific and has the capacity to make a rapid and stereotyped response to offending agents or immunogens, while the adaptive or acquired immune system is thought of as being very specific to a particular antigen and capable of providing long-lasting immunity [39]. In a cooperative way, they effectively form an integrated defense mechanism to provide the host with resistance to antigens and other harmful foreign intruders.

The innate immune system is the first line of defense against a wide range of unwanted invaders [40]. The constituents of the innate immune system can be categorized into external physical and chemical barriers such as the skin and mucosal membranes, cellular components such as monocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, dendritic cells, and natural killer (NK) cells, molecular components such as germ-line-encoded pattern recognition receptors (PRRs), cytokines, lysozyme, antimicrobial peptides, acute phase proteins and complement system [39, 41]. Monocytes are indispensable innate cells with the potential to differentiate into tissue macrophages and dendritic cells, which can produce a series of cytokines to regulate both innate and adaptive immune responses [42]. These cells recognize pathogen-associated molecular patterns (PAMPs) on pathogen's surface or damage-associated molecules patterns (DAMPs) derived from damaged cells via complementary pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) [43]. Upon binding to PAMPs or DAMPs, TLRs signal facilitates interactions with downstream signalling molecules and result in the activation of cytoplasmic transcription factors, such as nuclear factor kappa B (NF- κ B) or interferon regulatory factor 3 (IRF-3), subsequently initiating transcription of pro-inflammatory cytokines [44].

Unlike the innate defense, the adaptive immune system recognizes non-self molecules with specificity for particular antigens, and can also induce a long-term memory. The specificity of adaptive immunity relies on unique antigen receptors on the surfaces of either T or B cells, such as T-cell receptors (TCRs) and immunoglobulins [43, 45]. Both B and T cells are lymphocytes that originate from common lymphoid progenitor cells in the bone marrow. However, they are responsible for different immune responses based on cell engagement and antibody production. B cells contribute to the humoral response of the adaptive immune system, whereas T cells govern cell-mediated immune responses [39]. Upon encountering invaded antigens, T and B cells are brought into the play, in which they proliferate largely to maximize the fighting capacity and differentiate into several specialized subsets to further facilitate their responsiveness to antigens [43]. When B cells are stimulated by antigens, they can transform into either effector B cells (antibody-producing plasma cells) that have the ability to produce high-affinity immunoglobulins to eliminate the triggering antigens or into memory B cells that closely resemble the original B cells and respond quickly to a second invasion by the same antigens [39, 45]. T cells develop in the thymus, where they differentiate into discrete subtypes,

including helper T cells and cytotoxic T cells [43, 45]. Helper T cells have CD4 markers on their surface and can develop into effector T cells, such as Th1 and Th2 cells, which secrete different cytokines for different kinds of immune responses. Cytotoxic T cells express CD8 and have the capacity to directly kill virus-infected cells and tumor cells [46].

Inflammation is an essential immune response that eliminates injury-inducing agents, clears out necrotic cells and tissue damage from the original insult, and initiates repair processes and restores the physiological function of the tissue or organ affected [47, 48]. This process inevitably requires the participation of both the innate and adaptive immune systems. For example, large numbers of neutrophils are the first to reach the site of injury or infection within a short time. After inflammation begins, monocytes in the bloodstream migrate to the site and differentiate into macrophages and dendritic cells, both of which are capable of phagocytosis, antigen presentation and cytokine releasing. Lymphocytes occur somewhat later during the inflammatory process and work in conjunction with antigen presenting cells (APCs) to process antigens, thereby coordinating a suitable inflammatory response [49, 50]. In addition, overexpression of inflammatory mediators, such as pro-inflammatory cytokine IL-1 β , IL-6, IL-8 and TNF- α , have been implicated as regulators of cellular proliferation and host inflammatory events [51].

4. Toxicity of heavy metals on the immune system

The toxicity of heavy metals on immune homeostasis has been increasingly recognized [52, 53]. Heavy metals are known to either stimulate or suppress the immune response, altering the number and function of innate immune cells as well as B, T lymphocytes and immunological memory cells of the body at different stages [54, 55]. Regarding the effects of heavy metals on the immune system, studies were identified with conflicting results due to the dose, route of exposure, and chemical species, as well as the age, gender, genetics, and nutritional status of exposed individuals [19, 55].

4.1 Heavy metals and innate immune cells

With regard to neutrophils, researchers observed short-term exposure to low dose mercuric may inhibit spontaneous apoptosis of human neutrophils in vitro, whereas impaired neutrophil

chemotaxis and enhanced generation of ROS were found in mercury-exposed workers [56, 57]. Also, NK cells are sensitive to mercury: NK cells exposed to methylmercury showed an inhibition of the tumoricidal activity of NK cells in blood and spleen of mice and rats [58, 59]. The functions of neutrophils, such as cell movement, the generation of ROS and the killing of *Candida albicans* in workers occupationally exposed to lead were observed to be depressed [60, 61]. Cadmium exposure has been reported to be associated with the increase in neutrophil counts in occupationally exposed workers [62, 63]. Macrophages displayed an impairment of phagocytic and migratory activity due to increased ROS production by cells exposed to methylmercury [64, 65]. Inhibitory effects of lead exposure on phagocytosis and migratory responsiveness of macrophages have also been reported [60, 66, 67]. Arsenic-exposed human blood monocytes were found to undergo marked apoptosis due to inhibition NF- κ B-related survival pathways; further, differentiation of human monocytes into macrophages was inhibited but LPS-induced macrophage TNF- α and IL-8 mRNA and secretion were enhanced due to arsenic stimulation [68, 69].

4.2 Heavy metals and lymphocytes

The toxicity of heavy metals on B and T lymphocytes has been well recognized. Epidemiological and experimental studies have demonstrated that exposure to heavy metals may alter the numbers of lymphocytes and immunoglobulin levels [70-74]. Cadmium can suppress antigen-specific T cell responses and can induce abnormalities in antibody-production [75-77]. Cadmium has also been reported to be an effective apoptosis inducer in B cells [77]. Lead may promote the proliferation of B cells, enhance major histocompatibility complex activity and antibody production [78, 79]. It has also been suggested that lead is able to favor the activation and development of Th2 cells but suppresses the proliferation and responses of Th1 cells in vitro, since in animal studies, it has been shown that lead inhibits IFN- γ production of Th1 cells and enhances the production of IL-4 by Th2 cells [78, 80]. Exposure to mercuric chloride and methylmercury has been reported to inhibit the function and development of lymphocytes in animals and humans, including proliferation, expression of cell surface markers and cytokines [81, 82]. It also has been well documented that inorganic mercury may induce autoimmunity by inducing excessive activation of B cells and Th cells, with manifestations like elevations in immunoglobulin (IgG1 and IgE) and specific antibodies and formation of renal IgG deposits [82-84]. Studies in Hg-induced autoimmunity mouse models revealed a reduction

of CD8⁺ T cells and the resistance of T cells to apoptosis after mercury treatment [85-87]. It has been demonstrated that mercury not only disturbs B-cell receptor (BCR) signalling cascade pathways but also depresses its downstream activated extracellular signal-regulated kinase (ERK) signalling pathways in B-cell lymphoma cell line [88, 89].

4.3 Heavy metals and inflammation

A growing body of evidence has shown the contribution of heavy metals to inflammation. Epidemiological data suggest that exposure to arsenic may exacerbate the severity of inflammatory reactions based on elevated IL-6, IL-10 and TNF- α levels [54]. As evidenced by occupational studies, plasma levels of TNF and IL-10 were found higher in lead-exposed workers relative to those of non-exposed control subjects [90]. A positive association between lead exposure and the numbers of circulating neutrophils was observed in occupationally-exposed workers, particularly in those who smoked [67]. Cadmium exposure was demonstrated to cause pulmonary inflammation, which is characterized by the elevation in the number of neutrophils and macrophages and pro-inflammatory cytokines TNF and IL-6 in both blood and lung tissue of mice and rats [91, 92]. Exposure of human peripheral blood mononuclear leukocytes to mercury and cadmium showed increased pro-inflammatory IL-1 β and TNF levels but decreased anti-inflammatory IL-10 and IL-1 receptor antagonist production [93]. In addition, the production of TNF- α , IL-6, IL-12 by macrophages exposed to lead and lipopolysaccharide (LPS) was found augmented, while production of anti-inflammatory IL-10 was decreased [94, 95]. Studies carried out in isolated human neutrophils, murine macrophages, macrophage cell lines and animal models of experimental pulmonary inflammation have demonstrated that cadmium possesses pro-oxidative, pro-inflammatory activities, which was characterized by increases in the production of ROS, inducible NO synthase, and TNF- α , IL-6 and IL-8 [96-98].

5. Molecular Mechanisms of heavy metal toxicity

Each heavy metal has its unique physicochemical characteristics that relate to its specific toxic actions. Although many mechanistic aspects involved in heavy metal-induced immunotoxicity have been proposed, the underlying mechanism has not yet been fully elucidated. Possible mechanisms (*Figure 1*) regarding heavy metal toxicity include oxidative damage,

mitochondrial dysfunction, cellular signalling modification, induction of apoptosis, and inhibition of DNA repair and DNA binding activity of transcription factors [19, 69, 99].

5.1 Heavy metals enter cells via ionic mechanism

Some heavy metals are able to substitute bivalent cations such as calcium, zinc, magnesium and iron in cells [22, 25]. For example, lead and cadmium mimic calcium and zinc at their specific sites and bind to calmodulin, protein kinase C, and synaptic proteins [60]. Cadmium can replace zinc present in metallothionein, thereby inhibiting its ability to scavenge free radicals [25]. Although heavy metals have the capacity to replace or mimic essential metals in the early transport and metabolism, they are incapable of mediating subsequent vital cellular functions, ultimately disrupting various biological processes, such as intra and intercellular signalling, cell adhesion, protein folding and maturation, ionic transportation and apoptosis [22, 25].

In addition, heavy metals impair Ca^{2+} homeostasis as a result of their entry route into mammalian cells by voltage-dependent and receptor-operated calcium channels [60]. As a second messenger, Ca^{2+} concentrations contribute to intracellular signalling by protein phosphorylation, such as phosphorylation of p38 mitogen-activated protein kinase (MAPK) [20, 100-102]. This indicates that disrupting Ca^{2+} homeostasis may have large cellular effects. Lead and cadmium were reported to interrupt intracellular Ca^{2+} homeostasis generating an uncontrolled release of Ca^{2+} [100]. Hg^{2+} may stimulate Ca^{2+} influx through voltage-dependent channels [101]. The divalent cations of lead, cadmium and mercury have an extremely high affinity for sulfhydryl groups, which impair Ca^{2+} translocases present in cellular membranes, resulting in uncontrolled rises in cytosolic Ca^{2+} stores [101]. Calcium-mediated activation of c-Jun NH2-terminal kinase (JNK) intracellular signalling pathway in a murine macrophage cell line was found to be altered by cadmium exposure, which promotes phosphorylation/dephosphorylation of JNK and p38 MAPK, modulating mitochondrial activity and cell apoptosis [102].

5.2 Heavy metals interact with TLRs

Recognition of microbial pathogens relies on PRRs. As the first identified PRRs, the essential role of TLRs in the innate immune response against microbial pathogens, as well as in the subsequent activation of immune responses, is widely accepted [103]. With a different location to either the cell surface or to endosomal vesicles, TLRs recognize diverse DAMPs generated by the host itself, and PAMPs from bacteria, fungi, parasites and viruses [104-106]. Upon

PAMPs and DAMPs recognition, TLRs interact with TIR domain-containing adaptor proteins such as MyD88 and TRIF, which drive the activation of downstream NF- κ B, interferon regulatory factors (IRFs) and MAPK, leading to inflammatory cytokine release and cell apoptosis [104, 107].

Various studies have indicated that heavy metals may bind to TLRs affecting their function. Evidence suggests that TLR4 is a target of lead [108]. Mercury elicited NF- κ B activation and pro-inflammatory cytokine expression via endosomal TLR7 and TLR9 binding and signalling [109]. Ionized gold is able to mimic viral dsRNA in triggering TLR3 in keratinocytes. Cobalt, nickel and zinc ions are involved in TLR4 signalling, contributing to the release of the pro-inflammatory mediators NO, IL-6 and IL-8 [110-114]. Due to its ligating capacity with two histidine residues of the TLR4 molecule, the nickel ion can activate human TLR4 to promote the inflammatory response through NF- κ B-, p38- and IRF3-dependent signal pathways [115]. An in vivo study revealed that zinc supplementation is beneficial to protect intestinal tissue from inflammation via inhibiting the TLR4-MyD88 signalling pathways [116]. Unfortunately, systematic studies showing which TLRs are activated or inhibited by heavy metals have not been performed yet.

5.3 Oxidative damage and mitochondrial dysfunction

Heavy metals are able to dysregulate the catalytic activity of the defense enzymes involved in oxidative stress because of their high affinity for sulfhydryl groups in the active sites [20]. In vivo studies demonstrated that exposure to lead, mercury and arsenic suppress activities of delta-aminolevulinic acid synthase (δ -ALAS), δ -aminolevulinic acid dehydratase (δ -ALAD), and glutathione peroxidase (GPx) [22, 117, 118]. These enzymes are responsible for protecting intracellular components against ROS and RNS, which induce oxidative stress and cause mitochondrial dysfunction and cell apoptosis by eliminating them and maintaining the balance of their generation [20, 119]. On the other hand, lead, cadmium, mercury and arsenic are also known to increase oxidative stress by inducing the generation of ROS and RNS in the mitochondria [20, 25].

Heavy metals have the capacity to induce lipid peroxidation, a process of oxidative degradation of lipids, causing membrane structure damage, for instance in mitochondria [20, 117, 120]. Existing evidence has shown that lead, mercury and arsenic interrupt mitochondrial function

by inducing mitochondrial membrane damage, interfering with calcium ion (Ca^{2+})-dependent reactions, and inhibiting the mitochondrial electron transport chain and the phosphorylation of mitochondrial enzymes [20, 121-123].

Figure 1. Possible mechanism of heavy metal-induced immunotoxicity. Ionic characteristics of heavy metals allow them to enter cells through voltage-dependent and receptor-operated ion channels. In the cell they can replace or mimic essential metals in transport and metabolism process. Some heavy metals, such as lead (Pb), cadmium (Cd), mercury (Hg) and arsenic (As), are known to bind to proteins largely due to their affinity for sulfhydryl groups in the active sites. They also induce oxidative damage and mitochondrial dysfunction by facilitating ROS and RNS generation and suppressing antioxidant activity. In addition, the TLR signalling pathway is also a potential mechanism that can explain heavy metal-mediated aberrant immune and inflammatory response. Created with BioRender.com.

Aim and outline of this thesis

The overall objective of this thesis is to investigate immune alterations in preschool children in the e-waste area Guiyu, who suffer from heavy metal exposure. Moreover, we aim to explore the potential effects and mechanisms of heavy metals on monocytes and lymphocytes *in vitro*. We focus on the heavy metals lead, cadmium, mercury and arsenic as they are closely associated with e-waste as components of electrical and electronic equipment and largely generated during the dismantling process. Thus, they have been the most studied and essential concern on environmental issues and human health. This thesis contains two parts: **Part 1** focuses on reporting the alterations in immune cells among children exposed to heavy metals in Guiyu, while **Part 2** comprises two *in vitro* studies on the effects and mechanisms of the heavy metals lead and cadmium on monocytes and lymphocytes.

The immune system of children is not completely matured and particularly sensitive to environmental toxicants. In **Part 1**, we investigated the immune function of preschool children from Guiyu (an e-waste recycling area which has been introduced above) and Haojiang (the reference area). Haojiang is about 31.6 km to the east of Guiyu, without e-waste pollution. The two regions both belong to Shantou, Guangdong province, and have similar characteristics in latitude, demography, life-styles, eating habits, traffic conditions, cultural background and socioeconomic status. We evaluated the exposure of children to heavy metals, especially lead and cadmium. This part starts with **Chapter 2**, which gives insight into the number and percentage of innate immune cells, such as monocytes, neutrophils, eosinophils and basophils in preschool children from an e-waste recycling area, as well as the association between alterations in these cells and heavy metal lead and cadmium. In **Chapter 3**, lead exposure and its adverse effects on the percentage and development of NK lymphocytes were assessed in preschool children from this e-waste area. In **Chapter 4**, alterations in apoptosis and development of B lymphocytes were investigated in children from this area; in addition, caspase-9 activation and NF- κ B transcription factor c-rel and p65 were explored in order to link heavy metal exposure to B cell apoptosis. In **Chapter 5**, immune cells (monocytes, neutrophils and lymphocytes) and cytokines (IL-1 β , IL-6, IL-8, TNF- α , IL-1RA, IL-4, IL-10, IL-13) were analyzed to explore the states of immune regulation and inflammatory activation in children exposed to lead, cadmium, mercury and arsenic.

In **Part 2**, in vitro studies were conducted. In **Chapter 6**, we systematically studied TLR activation and inhibition by lead and cadmium using TLR reported cell lines and measured the downstream effects on NF- κ B and p38 MAPK pathways and cell apoptosis, activation and inflammatory cytokine production in a monocytic cell line (MonoMac 6 cells). In **Chapter 7**, mitochondrial function and NF- κ B activation in a human T cell line (Jurkat cells) exposed to lead and cadmium were explored.

In **Chapter 8**, the findings in this thesis are summarily discussed, and a perspective of future research into heavy metal-induced immunotoxicity is proposed.

REFERENCES

- [1] Huo, X., et al., Elevated blood lead levels of children in Guiyu, an electronic waste recycling town in China. *Environ Health Perspect*, 2007. 115(7): p. 1113-1117.
- [2] Grant, K., et al., Health consequences of exposure to e-waste: a systematic review. *Lancet Glob Health*, 2013. 1(6): p. e350-361.
- [3] Wong, N.W.M., Electronic Waste Governance under "One Country, Two Systems": Hong Kong and Mainland China. *Int J Environ Res Public Health*, 2018. 15(11).
- [4] Suk, W.A., et al., Environmental threats to children's health in Southeast Asia and the Western Pacific. *Environ Health Perspect*, 2003. 111(10): p. 1340-1347.
- [5] Heacock, M., et al., E-Waste and Harm to Vulnerable Populations: A Growing Global Problem. *Environ Health Perspect*, 2016. 124(5): p. 550-555.
- [6] Chen, A., et al., Developmental neurotoxicants in e-waste: an emerging health concern. *Environ Health Perspect*, 2011. 119(4): p. 431-438.
- [7] Leung, A.O., et al., Heavy metals concentrations of surface dust from e-waste recycling and its human health implications in southeast China. *Environ Sci Technol*, 2008. 42(7): p. 2674-2680.
- [8] Yekeen, T.A., et al., Assessment of health risk of trace metal pollution in surface soil and road dust from e-waste recycling area in China. *Environ Sci Pollut Res Int*, 2016. 23(17): p. 17511-17524.
- [9] Yang, H., et al., Effects of lead and cadmium exposure from electronic waste on child physical growth. *Environ Sci Pollut Res Int*, 2013. 20(7): p. 4441-4447.
- [10] Liu, W., X et al., S100 β in heavy metal-related child attention-deficit hyperactivity disorder in an informal e-waste recycling area. *Neurotoxicology*, 2014. 45: p. 185-191.
- [11] Xu, X., et al., Blood concentrations of lead, cadmium, mercury and their association with biomarkers of DNA oxidative damage in preschool children living in an e-waste recycling area. *Environ Geochem Health*, 2018. 40(4): p. 1481-1494.
- [12] Dai, Y., et al., Elevated lead levels and changes in blood morphology and erythrocyte CR1 in preschool children from an e-waste area. *Sci Total Environ*, 2017. 592: p. 51-59.
- [13] Zeng, X., et al., Heavy metals in PM2.5 and in blood, and children's respiratory symptoms and asthma from an e-waste recycling area. *Environ Pollut*, 2016. 210: p. 346-353.
- [14] Zeng, X., et al., Heavy metal exposure has adverse effects on the growth and development of preschool children. *Environ Geochem Health*, 2019. 41(1): p. 309-321.
- [15] Cao, J., et al., Increased memory T cell populations in Pb-exposed children from an e-waste-recycling area. *Sci Total Environ*, 2018. 616-617: p. 988-995.
- [16] Lu, X., et al., Elevated inflammatory Lp-PLA2 and IL-6 link e-waste Pb toxicity to cardiovascular risk factors in preschool children. *Environ Pollut*, 2018. 234: p. 601-609.
- [17] Zhang, Y., et al., Elevated lead levels and adverse effects on natural killer cells in children from an electronic waste recycling area. *Environ Pollut*, 2016. 213: p. 143-150.
- [18] Järup, L., Hazards of heavy metal contamination. *Br Med Bull*, 2003. 68: p. 167-82.
- [19] Tchounwou, P.B., et al., Heavy metal toxicity and the environment. *Exp Suppl*, 2012. 101: p. 133-164.
- [20] Rehman, K., et al., Prevalence of exposure of heavy metals and their impact on health consequences. *J Cell Biochem*, 2018. 119(1): p. 157-184.
- [21] Fu, Z., et al., The effects of heavy metals on human metabolism. *Toxicol Mech Methods*, 2020. 30(3): p. 167-176.
- [22] Assi, M.A., et al., The detrimental effects of lead on human and animal health. *Vet World*, 2016. 9(6): p. 660-671.
- [23] Kasperczyk, A., et al., Gene expression and activity of antioxidant enzymes in the blood cells of workers who were occupationally exposed to lead. *Toxicology*, 2012. 301(1-3): p. 79-84.
- [24] Rodríguez, J., et al., A Review of Metal Exposure and Its Effects on Bone Health. *J Toxicol*, 2018. 2018: p. 4854152.
- [25] Jaishankar, M., et al., Toxicity, mechanism and health effects of some heavy metals. *Interdiscip Toxicol*, 2014. 7(2): p. 60-72.
- [26] Järup, L., et al., Current status of cadmium as an environmental health problem. *Toxicol Appl Pharmacol*, 2009. 238(3): p. 201-208.
- [27] Clarkson, T.W., et al., The toxicology of mercury--current exposures and clinical manifestations. *N Engl J Med*, 2003. 349(18): p. 1731-1737.
- [28] Risher, J.F., et al., Mercury exposure: evaluation and intervention the inappropriate use of chelating agents in the diagnosis and treatment of putative mercury poisoning. *Neurotoxicology*, 2005. 26(4): p. 691-699.
- [29] Dopp, E., et al., Environmental distribution, analysis, and toxicity of organometal(loid) compounds. *Crit Rev Toxicol*, 2004. 34(3): p. 301-333.
- [30] Bernhoft, R.A., Mercury toxicity and treatment: a review of the literature. *J Environ Public Health*, 2012. 2012: p. 460508.
- [31] Zarei, M.H., et al., Toxicity of arsenic on isolated human lymphocytes: The key role of cytokines and intracellular calcium enhancement in arsenic-

- induced cell death. *Main Group Metal Chemistry*, 2019. 42(1): p. 125.
- [32] Leonard, S.S., et al., Metal-induced toxicity, carcinogenesis, mechanisms and cellular responses. *Mol Cell Biochem*, 2004. 255(1-2): p. 3-10.
- [33] Genestra, M., Oxyl radicals, redox-sensitive signalling cascades and antioxidants. *Cell Signal*, 2007. 19(9): p. 1807-1819.
- [34] Flora, S.J., et al., Heavy metal induced oxidative stress & its possible reversal by chelation therapy. *Indian J Med Res*, 2008. 128(4): p. 501-523.
- [35] Rahman, S., et al., Chemopreventive activity of glycyrrhizin on lead acetate mediated hepatic oxidative stress and its hyperproliferative activity in Wistar rats. *Chem Biol Interact*, 2006. 160(1): p. 61-69.
- [36] Cruvinel Wde, M., et al., Immune system - part I. Fundamentals of innate immunity with emphasis on molecular and cellular mechanisms of inflammatory response. *Rev Bras Reumatol*, 2010. 50(4): p. 434-461.
- [37] MacGillivray, D.M., et al., The role of environmental factors in modulating immune responses in early life. *Front Immunol*, 2014. 5: p. 434.
- [38] Vivier, E., et al., Innate and adaptive immunity: specificities and signalling hierarchies revisited. *Nat Immunol*, 2005. 6(1): p. 17-21.
- [39] Smith, N.C., et al., A Comparison of the Innate and Adaptive Immune Systems in Cartilaginous Fish, Ray-Finned Fish, and Lobe-Finned Fish. *Front Immunol*, 2019. 10: p. 2292.
- [40] Simon, A.K., et al., Evolution of the immune system in humans from infancy to old age. *Proc Biol Sci*, 2015. 282(1821): p. 20143085.
- [41] Riera Romo, et al., Innate immunity in vertebrates: an overview. *Immunology*, 2016. 148(2): p. 125-139.
- [42] Karlmark, K.R., et al., Monocytes in health and disease - Minireview. *Eur J Microbiol Immunol (Bp)*, 2012. 2(2): p. 97-102.
- [43] Yatim, K.M., et al., A brief journey through the immune system. *Clin J Am Soc Nephrol*, 2015. 10(7): p. 1274-1281.
- [44] Kumar, H., et al., Toll-like receptors and innate immunity. *Biochem Biophys Res Commun*, 2009. 388(4): p. 621-625.
- [45] Chaplin, D.D., Overview of the immune response. *J Allergy Clin Immunol*, 2003. 111(2 Suppl): p. S442-459.
- [46] Kumar, B.V., et al., Human T Cell Development, Localization, and Function throughout Life. *Immunity*, 2018. 48(2): p. 202-213.
- [47] Olszowski, T., et al., Pro-inflammatory properties of cadmium. *Acta Biochim Pol*, 2012. 59(4): p. 475-482.
- [48] Wessling-Resnick, M., Iron homeostasis and the inflammatory response. *Annu Rev Nutr*, 2010. 30: p. 105-122.
- [49] Sakai, Y., et al., Lymphocyte 'homing' and chronic inflammation. *Pathol Int*, 2015. 65(7): p. 344-354.
- [50] Silva, M.T., et al., Neutrophils and macrophages: the main partners of phagocyte cell systems. *Front Immunol*, 2012. 3: p. 174.
- [51] Chen, L., et al., Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget*, 2017. 9(6): p. 7204-7218.
- [52] Marth, E., et al., The effect of heavy metals on the immune system at low concentrations. *Int J Occup Med Environ Health*, 2001. 14(4): p. 375-386.
- [53] Jin, Y., et al., Cadmium exposure to murine macrophages decreases their inflammatory responses and increases their oxidative stress. *Chemosphere*, 2016. 144: p. 168-175.
- [54] Carey, J.B., et al., Immune modulation by cadmium and lead in the acute reporter antigen-popliteal lymph node assay. *Toxicol Sci*, 2006. 91(1): p. 113-122.
- [55] Tutkun, L., et al., Arsenic-induced inflammation in workers. *Mol Biol Rep*, 2019. 46(2): p. 2371-2378.
- [56] Moisan, E., et al., Prolongation of human neutrophil survival by low-level mercury via inhibition of spontaneous apoptosis. *J Toxicol Environ Health A*, 2002. 65(2): p. 183-203.
- [57] Perlingeiro, R.C., et al., Measurement of the respiratory burst and chemotaxis in polymorphonuclear leukocytes from mercury-exposed workers. *Hum Exp Toxicol*, 1995. 14(3): p. 281-286.
- [58] Wild, L.G., et al., Immune system alteration in the rat after indirect exposure to methyl mercury chloride or methyl mercury sulfide. *Environ Res*, 1997. 74(1): p. 34-42.
- [59] Ilbäck, N.G., Effects of methyl mercury exposure on spleen and blood natural killer (NK) cell activity in the mouse. *Toxicology*, 1991. 67(1): p. 117-124.
- [60] Gr, T.A.J., Harmful Interactions of Non-Essential Heavy Metals with Cells of the Innate Immune System. *Journal of Clinical Toxicology*, 2011. s3.
- [61] Undeğer, U., et al., Effects of lead on neutrophil functions in occupationally exposed workers. *Environ Toxicol Pharmacol*, 1998. 5(2): p. 113-117.
- [62] Karakaya, A., et al., An immunological study on workers occupationally exposed to cadmium. *Hum Exp Toxicol*, 1994. 13(2): p. 73-75.
- [63] Moitra, S., et al., Impact of occupational cadmium exposure on spirometry, sputum leukocyte count, and lung cell DNA damage among Indian goldsmiths. *Am J Ind Med*, 2015. 58(6): p. 617-624.
- [64] InSug, O., et al., Mercuric compounds inhibit human monocyte function by inducing apoptosis: evidence for formation of reactive oxygen species, development of mitochondrial membrane permeability transition and loss of reductive reserve. *Toxicology*, 1997. 124(3): p. 211-224.

- [65] Christensen, M.M., et al., Comparison of the interaction of methyl mercury and mercuric chloride with murine macrophages. *Arch Toxicol*, 1993. 67(3): p. 205-211.
- [66] Mitra, P., et al., Clinical and molecular aspects of lead toxicity: An update. *Crit Rev Clin Lab Sci*, 2017. 54(7-8): p. 506-528.
- [67] Di Lorenzo, L., et al., Evaluation of peripheral blood neutrophil leucocytes in lead-exposed workers. *Int Arch Occup Environ Health*, 2006. 79(6): p. 491-498.
- [68] Lemarie, A., et al., Human macrophages constitute targets for immunotoxic inorganic arsenic. *J Immunol*, 2006. 177(5): p. 3019-3027.
- [69] Dangleben, N.L., et al., Arsenic immunotoxicity: a review. *Environ Health*, 2013. 12(1): p. 73.
- [70] Chou, C., et al., Tissue-Resident Lymphocytes Across Innate and Adaptive Lineages. *Front Immunol*, 2018. 9: p. 2104-2104.
- [71] Dvorožňáková, E., et al., Heavy metal intoxication compromises the host cytokine response in *Ascaris* Suum model infection. 2016. 53(1): p. 14.
- [72] Mishra, K.P., et al., Effect of lead exposure on the immune response of some occupationally exposed individuals. *Toxicology*, 2003. 188(2-3): p. 251-259.
- [73] Hsiao, C.L., et al., Effects of environmental lead exposure on T-helper cell-specific cytokines in children. *J Immunotoxicol*, 2011. 8(4): p. 284-287.
- [74] Başaran, N., et al., Effects of lead on immune parameters in occupationally exposed workers. *Am J Ind Med*, 2000. 38(3): p. 349-354.
- [75] Simonet, M., et al., Impaired resistance to *Listeria* monocytogenes in mice chronically exposed to cadmium. *Immunology*, 1984. 53(1): p. 155-163.
- [76] Hurtenbach, U., et al., Modulation of murine T and B cell reactivity after short-term cadmium exposure in vivo. *Arch Toxicol*, 1988. 62(1): p. 22-28.
- [77] de la Fuente, H., et al., Effect of arsenic, cadmium and lead on the induction of apoptosis of normal human mononuclear cells. *Clin Exp Immunol*, 2002. 129(1): p. 69-77.
- [78] Kasten-Jolly, J., et al., Impact of developmental lead exposure on splenic factors. *Toxicol Appl Pharmacol*, 2010. 247(2): p. 105-115.
- [79] Razani-Boroujerdi, S., et al., Lead stimulates lymphocyte proliferation through enhanced T cell-B cell interaction. *J Pharmacol Exp Ther*, 1999. 288(2): p. 714-719.
- [80] Heo, Y., et al., Lead differentially modifies cytokine production in vitro and in vivo. *Toxicol Appl Pharmacol*, 1996. 138(1): p. 149-157.
- [81] Gump, B.B., et al., Low-level mercury in children: associations with sleep duration and cytokines TNF- α and IL-6. *Environ Res*, 2014. 134: p. 228-232.
- [82] Maqbool, F., et al., Immunotoxicity of mercury: Pathological and toxicological effects. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev*, 2017. 35(1): p. 29-46.
- [83] Suzuki, Y., et al., Autoimmunity-inducing metals (Hg, Au and Ag) modulate mast cell signalling, function and survival. *Curr Pharm Des*, 2011. 17(34): p. 3805-3814.
- [84] Via, C.S., et al., Low-dose exposure to inorganic mercury accelerates disease and mortality in acquired murine lupus. *Environ Health Perspect*, 2003. 111(10): p. 1273-1277.
- [85] Field, A.C., et al., Regulatory CD8+ T cells control neonatal tolerance to a Th2-mediated autoimmunity. *J Immunol*, 2003. 170(5): p. 2508-2515.
- [86] McCabe, M.J., et al., Inorganic mercury attenuates CD95-mediated apoptosis by interfering with formation of the death inducing signalling complex. *Toxicol Appl Pharmacol*, 2003. 190(2): p. 146-156.
- [87] McCabe, M.J., et al., Attenuation of CD95-induced apoptosis by inorganic mercury: caspase-3 is not a direct target of low levels of Hg2+. *Toxicol Lett*, 2005. 155(1): p. 161-170.
- [88] McCabe, M.J., et al., Low and nontoxic inorganic mercury burdens attenuate BCR-mediated signal transduction. *Toxicol Sci*, 2007. 99(2): p. 512-521.
- [89] McCabe, M.J., et al., Low and nontoxic levels of ionic mercury interfere with the regulation of cell growth in the WEHI-231 B-cell lymphoma. *Scand J Immunol*, 1999. 50(3): p. 233-241.
- [90] Valentino, M., et al., Effect of lead on the levels of some immunoregulatory cytokines in occupationally exposed workers. *Hum Exp Toxicol*, 2007. 26(7): p. 551-556.
- [91] Kirschvink, N., et al., Repeated cadmium nebulizations induce pulmonary MMP-2 and MMP-9 production and emphysema in rats. *Toxicology*, 2005. 211(1-2): p. 36-48.
- [92] Zhang, W., et al., Effects of formoterol and ipratropium bromide on repeated cadmium inhalation-induced pulmonary inflammation and emphysema in rats. *Eur J Pharmacol*, 2010. 647(1-3): p. 178-187.
- [93] Beatriz, M., et al., Cytokine Production by Human Peripheral Blood Mononuclear Cells after Exposure to Heavy Metals. *Journal of Health Science National Institute of Industrial Health*, 2000. 46: p. 6-21.
- [94] Flohé, S.B., et al., Enhanced proinflammatory response to endotoxin after priming of macrophages with lead ions. *J Leukoc Biol*, 2002. 71(3): p. 417-424.
- [95] Krocova, Z., et al., The immunomodulatory effect(s) of lead and cadmium on the cells of immune system in vitro. *Toxicol In Vitro*, 2000. 14(1): p. 33-40.
- [96] Hassoun, E.A., et al., Cadmium-induced production of superoxide anion and nitric oxide, DNA single strand breaks and lactate dehydrogenase leakage in J774A.1 cell cultures. *Toxicology*, 1996. 112(3): p. 219-226.
- [97] Ramirez, D.C., et al., Biphasic effect of cadmium in non-cytotoxic conditions on the secretion of nitric

- oxide from peritoneal macrophages. *Toxicology*, 1999. 139(1-2): p. 167-177.
- [98] Kataranovski, M., et al., Granulocyte and plasma cytokine activity in acute cadmium intoxication in rats. *Physiol Res*, 1998. 47(6): p. 453-461.
- [99] Reichard, J.F., et al., Long term low-dose arsenic exposure induces loss of DNA methylation. *Biochem Biophys Res Commun*, 2007. 352(1): p. 188-192.
- [100] Biagioli, M., et al., Endoplasmic reticulum stress and alteration in calcium homeostasis are involved in cadmium-induced apoptosis. *Cell Calcium*, 2008. 43(2): p. 184-195.
- [101] Viarengo, A., et al., Possible role of Ca^{2+} in heavy metal cytotoxicity. *Comp Biochem Physiol C*, 1991. 100(1-2): p. 81-84.
- [102] Kim, J., et al., Calcium-mediated activation of c-Jun NH2-terminal kinase (JNK) and apoptosis in response to cadmium in murine macrophages. *Toxicol Sci*, 2004. 81(2): p. 518-527.
- [103] Kawai, T., et al., Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity*, 2011. 34(5): p. 637-650.
- [104] Murphy, M., et al., Pellino-1 Positively Regulates Toll-like Receptor (TLR) 2 and TLR4 Signalling and Is Suppressed upon Induction of Endotoxin Tolerance. *J Biol Chem*, 2015. 290(31): p. 19218-19232.
- [105] Wada, J., et al., Innate immunity in diabetes and diabetic nephropathy. *Nat Rev Nephrol*, 2016. 12(1): p. 13-26.
- [106] Yoshino, H., et al., Involvement of reactive oxygen species in ionizing radiation-induced upregulation of cell surface Toll-like receptor 2 and 4 expression in human monocytic cells. *J Radiat Res*, 2017. 58(5): p. 626-635.
- [107] Parker, L.C., et al., Toll-like receptor (TLR)2 and TLR4 agonists regulate CCR expression in human monocytic cells. *J Immunol*, 2004. 172(8): p. 4977-4986.
- [108] Luna, A.L., et al., TLR4 is a target of environmentally relevant concentration of lead. *Toxicol Lett*, 2012. 214(3): p. 301-306.
- [109] Pollard, K.M., et al., Induction of Systemic Autoimmunity by a Xenobiotic Requires Endosomal TLR Trafficking and Signalling from the Late Endosome and Endolysosome but Not Type I IFN. *J Immunol*, 2017. 199(11): p. 3739-3747.
- [110] Rachmawati, D., et al., Transition metal sensing by Toll-like receptor-4: next to nickel, cobalt and palladium are potent human dendritic cell stimulators. *Contact Dermatitis*, 2013. 68(6): p. 331-338.
- [111] Rachmawati, D., et al., Dental metal-induced innate reactivity in keratinocytes. *Toxicol In Vitro*, 2015. 30(1 Pt B): p. 325-330.
- [112] Raghavan, B., et al., Metal allergens nickel and cobalt facilitate TLR4 homodimerization independently of MD2. *EMBO Rep*, 2012. 13(12): p. 1109-1115.
- [113] Saito, M., et al., Molecular Mechanisms of Nickel Allergy. *Int J Mol Sci*, 2016. 17(2).
- [114] Vijay, K., Toll-like receptors in immunity and inflammatory diseases: Past, present, and future. *Int Immunopharmacol*, 2018. 59: p. 391-412.
- [115] Schmidt, M., et al., Crucial role for human Toll-like receptor 4 in the development of contact allergy to nickel. *Nat Immunol*, 2010. 11(9): p. 814-819.
- [116] Hu, C.H., et al., Zinc oxide influences intestinal integrity, the expressions of genes associated with inflammation and TLR4-myeloid differentiation factor 88 signalling pathways in weanling pigs. *Innate Immun*, 2014. 20(5): p. 478-486.
- [117] Ahamed, M., et al., Low level lead exposure and oxidative stress: current opinions. *Clin Chim Acta*, 2007. 383(1-2): p. 57-64.
- [118] Ahamed, M., et al., Environmental lead toxicity and nutritional factors. *Clin Nutr*, 2007. 26(4): p. 400-408.
- [119] Kupsco, A., et al., Oxidative stress, unfolded protein response, and apoptosis in developmental toxicity. *Int Rev Cell Mol Biol*, 2015. 317: p. 1-66.
- [120] Fenga, C., et al., Immunological effects of occupational exposure to lead (Review). *Mol Med Rep*, 2017. 15(5): p. 3355-3360.
- [121] Farina, M., et al., Mechanisms of methylmercury-induced neurotoxicity: evidence from experimental studies. *Life Sci*, 2011. 89(15-16): p. 555-563.
- [122] Rosin, A., The long-term consequences of exposure to lead. *Isr Med Assoc J*, 2009. 11(11): p. 689-694.
- [123] Ercal, N., et al., In vivo indices of oxidative stress in lead-exposed C57BL/6 mice are reduced by treatment with meso-2,3-dimercaptosuccinic acid or N-acetylcysteine. *Free Radic Biol Med*, 1996. 21(2): p. 157-161.

2

ALTERATION OF THE NUMBER AND PERCENTAGE OF INNATE IMMUNE CELLS IN PRESCHOOL CHILDREN FROM AN E-WASTE RECYCLING AREA

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ABSTRACT

Heavy metals lead (Pb) and cadmium (Cd) are widespread environmental contaminants and exert detrimental effects on the immune system. We evaluated the association between Pb/Cd exposures and innate immune cells in children from an electronic waste (e-waste) recycling area. A total of 294 preschool children were recruited, including 153 children from Guiyu (e-waste exposed group), and 141 from Haojiang (reference group). Pb and Cd levels in peripheral blood were measured by a graphite furnace atomic absorption spectrophotometer, NK cell percentages were detected by a flow cytometer, and other innate immune cells including monocytes, eosinophils, neutrophils and basophils were immediately measured by an automated hematology analyzer. Results showed children in Guiyu had significantly higher Pb and Cd levels than in the reference group. Absolute counts of monocytes, eosinophils, neutrophils and basophils, as well as percentages of eosinophils and neutrophils, were significantly higher in the Guiyu group. In contrast, NK cell percentages were significantly lower in Guiyu group. Pb elicited a significant escalation in counts of monocytes, eosinophils and basophils, as well as percentages of monocytes, but a decline in percentages of neutrophils in different quintiles with respect to the first quintile of Pb concentrations. Cd induced a significant increase in counts and percentages of neutrophils in the highest quintile compared with the first quintile of Cd concentrations. We concluded that alterations in the number and percentage of innate immune cells are linked to higher levels of Pb and Cd, which indicates that Pb and Cd exposures might affect the innate and adaptive immune response in Guiyu children.

1. Introduction

Informal and uncontrolled electronic waste (e-waste) recycling often results in human exposure to harmful chemical contaminants [1]. Guiyu, a typical e-waste destination and recycling area in southern China, with nearly a 30-year history of unregulated e-waste disposal, has reported massive amounts of environmental toxicants, including heavy metals and organic pollutants, in environmental and human samples [2-7]. Our previous studies showed that higher Pb and Cd levels are present in the placenta, umbilical cord blood, peripheral blood and urine in the Guiyu populations [8-11].

Heavy metals Pb and Cd are widespread environmental contaminants, which cause extensive concern for their adverse effects on health [12, 13]. Pb is toxic to the central nervous, hematopoietic, renal, and immune systems [14-16]. Previous studies found that Pb exposure affects the humoral and innate immune responses, lymphocyte function and cytokine production [17, 18]. Cd is a highly hazardous metal which causes nephrotoxicity, teratogenicity, neurotoxicity, immunotoxicity, and endocrine and reproductive toxicities [19-21]. Cd exposure has also been associated with risk of cardiovascular disease and cancer [22, 23]. In recent years, Pb and Cd immunotoxicity on humoral and cell-mediated immunity has been well documented, as mentioned above, but few reports have characterized their impact on innate immunity.

Innate immune cells, including macrophages, dendritic cells, mast cells, neutrophils, eosinophils, natural killer (NK) cells and NKT cells, provide the first line of defense against pathogens and viral infections through recognition of pathogen-associated molecular patterns, via a limited number of germline-encoded pattern recognition receptors, and secretion of a series of cytokines and chemokines to eliminate pathogens and facilitate the adaptive response [24-26]. A handful of studies demonstrated that Pb exposure decreases host resistance to pathogens and viral infections [27]. Cd exposure suppresses specific immune responses while increasing neutrophil activity and macrophage phagocytosis [28, 29]. With regard to the mechanisms of Pb and Cd toxicity on innate immunity, current evidence suggests that Pb and Cd interact with DNA repair mechanisms, generate reactive oxygen species and induce apoptosis, as well as alter cytokine secretion [27, 30, 31].

The immune system of preschool children is in the process of maturing, making it particularly sensitive to environmental toxicants [2, 32-34]. To date, there have been no studies reporting

the alteration of the innate immune homeostasis affected by heavy metal exposure in Guiyu children. This study aimed to determine the blood Pb and Cd levels in children who reside in Guiyu area, and evaluate their toxic effects on innate immune cells, in order to get a deeper understanding of Pb and Cd immunotoxicity on susceptible populations, thereby making an early assessment of the risk for diseases.

2. Materials and Methods

2.1. Study population

A total of 294 preschool children, 3 to 7 years of age, were recruited from Guiyu (n = 153) and Haojiang (n = 141) in December, 2011. We selected Haojiang as the reference group, which has similarities with Guiyu in population, cultural background and socioeconomic status, but lacks electronic waste pollution. Questionnaires, including general characteristics of both parents and children, child behavior habits, diet and health physiological parameters, dwelling environments, parent education and jobs, were delivered to the participants and their parents who gave written informed consent prior to enrollment. The study protocol was approved by the Human Ethics Committee of Shantou University Medical College, China.

2.2. Sample collection

Whole blood samples were obtained from volunteers, collected in Pb-free tubes by trained nurses, and transported to the laboratory. Blood sample tubes containing EDTA were used for blood routine examination and heavy metal measurement.

2.3. Blood cell examination

Blood counts were immediately measured by an automated hematology analyzer (Sysmex XT-1800i, Japan) in a hospital not far from the laboratory. NK cells (CD3⁺CD56⁺CD16⁺) were detected by labeled antibodies: MultiTEST CD45/CD3/CD56/CD16 (BD Bioscience, America), and data were collected by an Aria II flow cytometer (BD Bioscience, America) and analyzed with DVIA software (version 6.1, BD Bioscience).

2.4. Pb and Cd measurement

Blood Pb and Cd levels were measured by graphite furnace atomic absorption spectrophotometer (Jena Zeenit 650, Germany), using detection methods according to our

previously described publication [35]. For Pb determination, the main parameters were: a wavelength of 283.3 nm, a lamp current of 4.0 mA, a slit width of 0.8 nm, drying at 90 °C, 105 °C, and 120 °C, ashing at 950 °C, and atomization at 1500 °C. The 0.5% nitric acid solution was used as blank, and the limit of detection (LOD) of this method was 0.51 µg/L (0.051 µg/dL). The accuracy of the method was controlled by recoveries between 95% and 107% from the spiked blood samples. For Cd determination, the main parameters were: a wavelength of 228.8 nm, a lamp current of 4.0 mA, a slit width of 1.2 nm, drying at 90 °C, 105 °C, and 120 °C, ashing at 300 °C, and atomization at 1300 °C. The 2.0 % nitric acid solution was used as blank, and the limit of detection (LOD) of this method was 0.05 µg/L. The accuracy of the detection method was controlled by recoveries between 100% and 103% from the spiked blood samples. Repeated analyses of standard solutions confirmed the method's precision.

2.5. Statistical analysis

Summary statistical analyses were performed using IBM SPSS 19.0 software. Mean $\pm$ SD were used to depict blood Pb and Cd concentrations, absolute counts and percentages of monocytes, eosinophils, neutrophils, basophils and NK cells. Chi-square and independent-sample t-tests were used to detect differences between the exposed and reference groups for categorical variables and continuous variables, respectively. We adopted a univariate linear regression analysis to analyze the impact of possible relevant factors on Pb and Cd exposure. Separate regression models were used to estimate the association between innate immune cell levels and Pb and Cd exposure, with each exposure categorized by quintiles. All linear models for group differences, in the changes of Pb and Cd concentrations and several innate immune cell levels, controlled for potential confounding variables, such as child gender, age and body mass index (BMI). A $p < 0.05$ in a two-tailed test was determined to be statistically significant.

3. Results

3.1. Characteristics of the study population

Descriptive statistics for the sample characteristics are presented in **Table 1**. The mean child age in the exposed group was 5.1 years, higher than the 4.4 years in the reference group ($p < 0.01$). In addition, the mean child body mass index (BMI) was significantly lower in the exposed group than that in the reference group (14.73 kg/m² versus 16.06 kg/m², $p < 0.01$). No

significant differences between two groups were found for gender. Differences between the two groups for categorical variables, such as the duration of the child outdoor play, habit of biting pencils and erasers, consumption of dairy and bean products, child e-waste contact, frequency of colds, the duration of residence of both child and parents in the local area, the distance of residence from the road, e-waste contamination within 50 meters of the residence, and using the residence as an e-waste recycling workplace, were all significant different (all $p < 0.05$).

3.2. Candidate factors associated with Pb and Cd levels

Pb and Cd both showed higher mean concentrations in the exposed group (10.34 ± 4.75 $\mu\text{g/dL}$ and 2.39 ± 1.16 $\mu\text{g/L}$, respectively) than the reference group (8.30 ± 3.01 $\mu\text{g/dL}$ and 1.79 ± 0.45 $\mu\text{g/L}$, respectively) (**Figure 1**). A subsequent univariate linear regression model was used to determine the relationship between possible factors and two heavy metal exposures (**Table 2**). We took into account the child's age, BMI and gender as potential covariates in the change of Pb and Cd concentrations. Therefore, these variables were being controlled. We found child blood Pb levels were positively associated with the residence duration of child in the local area ($\beta_{\text{unadjusted}} = 0.845$, 95% CI: 0.076, 1.615), the residence duration of father in the local area ($\beta_{\text{unadjusted}} = 1.087$, 95% CI: 0.383, 1.791), the residence duration of mother in the local area ($\beta_{\text{unadjusted}} = 1.070$, 95% CI: 0.544, 1.595), the residence as an e-waste recycling workplace ($\beta_{\text{unadjusted}} = 1.346$, 95% CI: 0.332, 2.361), and the child e-waste contact ($\beta_{\text{unadjusted}} = 2.636$, 95% CI: 1.492, 3.780). However, in the adjusted linear regression model, child blood Pb levels had no correlation with the residence duration of child in the local area ($\beta_{\text{adjusted}} = 0.622$, 95% CI: -0.160, 1.404). In addition, some factors negatively correlated with Pb levels, such as dairy product consumption frequency ($\beta_{\text{adjusted}} = -0.621$, 95% CI: -1.201, -0.041) and distance of residence from the road ($\beta_{\text{adjusted}} = -0.521$, 95% CI: -0.942, -0.100).

In terms of factors associated with blood Cd levels, we found positive correlations between blood Cd levels and certain variables, such as using the residence as an e-waste recycling workplace ($\beta_{\text{adjusted}} = 0.248$, 95% CI: 0.023, 0.474), and the residence duration of child in the local area ($\beta_{\text{adjusted}} = 0.183$, 95% CI: 0.016, 0.350) and the residence duration of mother in the local area ($\beta_{\text{adjusted}} = 0.136$, 95% CI: 0.019, 0.253). Other variables, such as the duration of the child outdoor play ($\beta_{\text{adjusted}} = -0.140$, 95% CI: -0.245, -0.036) and distance of residence from road ($\beta_{\text{adjusted}} = -0.141$, 95% CI: -0.231, -0.052) exhibited negative association with blood Cd levels.

Table 1. Descriptive statistics for the study population.

Characteristics	Exposed group (n = 153)	Reference group (n = 141)	<i>p</i>
Child age (years)	5.1 ± 1.2	4.4 ± 0.9	< 0.001 ^a
Gender [n (%)]	153	141	0.930 ^b
male	93 (60.8)	85 (60.3)	
female	60 (39.2)	56 (39.7)	
Child body mass index (BMI, kg/m ²)	14.73 ± 1.17	16.06 ± 1.41	< 0.001 ^a
Duration of the child outdoor play (h) [n (%)]			0.003 ^b
~0.5	26 (17.2)	6 (4.7)	
~1.0	51 (33.8)	40 (31.0)	
~2.0	53 (35.1)	48 (37.2)	
~3.0	16 (10.6)	27 (20.9)	
>3.0	5 (3.3)	8 (6.2)	
Habit of biting pencils and erasers [n (%)]			0.007 ^b
none	102 (67.5)	106 (79.7)	
occasionally	41 (27.2)	27 (20.3)	
frequently	8 (5.3)	0 (0)	
Dairy product consumption frequency [n (%)]			0.002 ^b
none	12 (7.9)	3 (2.2)	
1-3 times/month	36 (23.9)	33 (25.0)	
1-3 times/week	74 (49.0)	48 (36.4)	
daily	29 (19.2)	48 (36.4)	
Bean product consumption frequency [n (%)]			0.011 ^b
none	9 (5.9)	3 (2.4)	
1-3 times/month	93 (61.2)	64 (50.4)	
1-3 times/week	43 (28.3)	58 (45.7)	
daily	7 (4.6)	2 (1.5)	
Residence as e-waste recycling workplace [n (%)]			< 0.001 ^b
no	62 (41.1)	119 (89.5)	
yes	89 (58.9)	14 (10.5)	
Residence duration of child in local area (years) [n (%)]			< 0.001 ^b
~1	10 (6.5)	13 (9.2)	
~3	15 (9.8)	39 (27.6)	
~6	128 (83.7)	89 (63.2)	
Residence duration of father in local area (years) [n (%)]			< 0.001 ^b
~1	2 (1.4)	3 (2.3)	
~5	11 (7.4)	10 (7.6)	
~10	9 (6.1)	31 (23.7)	
>10	126 (85.1)	87 (66.4)	
Residence duration of mother in local area (years) [n (%)]			< 0.001 ^b
~1	4 (2.7)	10 (7.6)	
~5	11 (7.4)	38 (28.8)	
~10	26 (17.4)	40 (30.3)	
>10	108 (72.5)	44 (33.3)	
Distance of residence from road (m) [n (%)]			< 0.001 ^b
< 10	59 (39.0)	19 (14.6)	
~50	40 (26.5)	24 (18.5)	
~100	27 (17.9)	33 (25.4)	
>100	25 (16.6)	54 (41.5)	
E-waste contamination within 50 meters away from house [n (%)]			< 0.001 ^b
no	35 (23.2)	131 (96.3)	
yes	116 (76.8)	5 (3.7)	
Child e-waste contact [n (%)]			< 0.001 ^b
no	87 (59.6)	120 (93.8)	
yes	59 (40.4)	8 (6.2)	
Child vitamin and calcium intake in the past year [n (%)]			0.008 ^b
none	22 (14.6)	31 (24.1)	
rarely	69 (45.7)	68 (52.7)	
occasionally	53 (35.1)	23 (17.8)	
frequently	7 (4.6)	7 (5.4)	
Child cold frequency in the past year [n (%)]			0.013 ^b
0-3	74 (48.7)	54 (40.9)	
4-6	37 (24.3)	52 (39.4)	
7-9	23 (15.1)	20 (15.2)	
>10	18 (11.9)	6 (4.5)	

^aAnalysis by independent-sample t-test. ^bAnalysis by chi-square test.

3.3. Distribution of innate immune cells

A general description of several innate immune cell levels is presented in **Figure 2 (A - I)**. Monocytes, eosinophils, neutrophils, basophils and NK cells were estimated in our study, and expressed as a percentage and absolute value. There were higher mean absolute values of monocytes, eosinophils, neutrophils and basophils in the exposed group ($0.61 \times 10^9/L$, $0.29 \times 10^9/L$, $4.24 \times 10^9/L$ and $0.05 \times 10^9/L$, respectively) compared with the reference group ($0.51 \times 10^9/L$, $0.19 \times 10^9/L$, $3.30 \times 10^9/L$ and $0.04 \times 10^9/L$, respectively). Furthermore, there were higher eosinophil and neutrophil percentages in white blood cells and lower NK cell percentages among lymphocytes in the exposed group (3.26%, 47.50% and 14.87%, respectively) than those in the reference group (2.57%, 42.67% and 18.01%, respectively).

Figure 1. Pb and Cd levels in peripheral blood of children. Exposed group, n=153; Reference group, n=141. Results are presented as mean $\pm$ SD; analysis by independent-sample t-test. Value of *** $p < 0.001$ were considered statistically significant.

Table 2. Univariate linear regression analysis of factors related to blood Pb and Cd levels in children.

	Blood Pb levels		Blood Cd levels	
	Unadjusted β (95% CI)	Adjusted β (95% CI) ^a	Unadjusted β (95% CI)	Adjusted β (95% CI) ^a
Duration of the child outdoor play	-0.404 (-0.896, 0.088)	-0.143 (-0.901, 0.074)	-0.151 (-0.255, -0.047)**	-0.140 (-0.245, -0.036)**
Habit of biting pencils and erasers	0.158 (-0.802, 1.118)	0.043 (-0.902, 0.988)	0.063 (-0.142, 0.267)	0.035 (-0.167, 0.237)
Dairy product consumption frequency	-0.709 (-1.291, -0.127)*	-0.621 (-1.201, -0.041)*	-0.044 (-0.170, 0.081)	-0.014 (-0.139, 0.111)
Bean product consumption frequency	0.156 (-0.654, 0.965)	0.165 (-0.631, 0.961)	-0.144 (-0.316, 0.029)	-0.140 (-0.310, 0.029)
Residence as an e-waste recycling workplace	1.346 (0.332, 2.361)**	1.227 (0.176, 2.279)*	0.316 (0.100, 0.532)**	0.248 (0.023, 0.474)*
Residence duration of child in local area	0.845 (0.076, 1.615)*	0.622 (-0.160, 1.404)	0.244 (0.081, 0.407)**	0.183 (0.016, 0.350)*
Residence duration of father in local area	1.087 (0.383, 1.791)**	0.894 (0.185, 1.602)*	0.102 (-0.047, 0.252)	0.053 (-0.098, 0.240)
Residence duration of mother in local area	1.070 (0.544, 1.595)**	0.933 (0.393, 1.473)**	0.182 (0.068, 0.295)**	0.136 (0.019, 0.253)*
Distance of residence from the road	-0.642 (-1.061, -0.224)**	-0.521 (-0.942, -0.100)*	-0.166 (-0.255, -0.078)**	-0.141 (-0.231, -0.052)**
E-waste contamination within 50 meters away from house	0.470 (-0.615, 1.556)	0.122 (-0.979, 1.222)	0.177 (-0.062, 0.416)	0.089 (-0.155, 0.333)
Child e-waste contact	2.636 (1.492, 3.780)**	2.389 (1.237, 3.540)**	0.211 (-0.043, 0.464)	0.131 (-0.123, 0.385)
Child vitamin and calcium intake in the past year	-0.095 (-0.726, 0.536)	-0.202 (-0.826, 0.421)	0.001 (-0.135, 0.134)	-0.028 (-0.162, 0.106)
Child cold frequency in the past year	0.270 (-0.241, 0.782)	0.250 (-0.260, 0.760)	0.014 (-0.095, 0.124)	0.019 (-0.091, 0.128)

^a Model was adjusted by child age, gender and BMI. β : regression coefficient; CI: confidence interval. Values of * $p < 0.05$, ** $p < 0.01$ were considered statistically significant.

Figure 2. Innate immune cell counts and percentages in child peripheral blood. A, B, C and D represent innate immune cell counts; E, F, G, H and I show the percentage results between two groups. Exposed group, n=153; Reference group, n=141. Results are presented as mean $\pm$ SD; analysis by independent-sample t-test. Values of * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ were considered statistically significant.

3.4. Associations between the distribution of innate immune cells and heavy metal exposure

To estimate associations between distribution of different innate immune cells and exposures, we categorized Pb and Cd exposures into quintiles for statistical analysis, and adjusted separate regression models by the child's age (**Table 3**). For blood Pb, the lowest (2.64-6.31 $\mu\text{g/dL}$), second (6.31-7.69 $\mu\text{g/dL}$), third (7.69-9.60 $\mu\text{g/dL}$), fourth (9.60-12.04 $\mu\text{g/dL}$) and highest (12.04-37.09 $\mu\text{g/dL}$) quintile had average blood Pb concentrations of 5.10, 7.11, 8.65, 10.54 and 15.50 $\mu\text{g/L}$, respectively. We found monocyte ($\beta = 0.083$; 95% CI: 0.004, 0.162) and basophil ($\beta = 0.013$; 95% CI: 0.001, 0.026) counts were both on average higher in the third quintile of Pb concentration, and eosinophil counts ($\beta = 0.078$; 95% CI: 0.004, 0.152) in the fourth quintile compared with those in the lowest quintile (all $p < 0.05$). For percentages of monocytes, results showed increments in all quintiles (second quintile $\beta = 0.765$; 95% CI: 0.038, 1.491; third quintile $\beta = 0.976$; 95% CI: 0.248, 1.704; highest quintile $\beta = 0.766$; 95% CI: 0.036, 1.497) except the fourth quintile ($\beta = 0.358$; 95% CI: -0.371, 1.088). However, we observed

that neutrophil percentages were on average lower in Pb concentrations in the second quintile compared with the lowest quintile ($\beta = -4.153$; 95% CI: -7.792, -0.514) ($p < 0.05$). Neither neutrophil counts nor percentages of eosinophils, basophils and NK cells had significant associations with Pb exposure.

For blood Cd, the lowest (0.47-1.45 $\mu\text{g/L}$), second (1.45-1.69 $\mu\text{g/L}$), third (1.69-2.10 $\mu\text{g/L}$), fourth (2.10-2.59 $\mu\text{g/L}$) and highest (2.59-7.90 $\mu\text{g/L}$) quintile had an average Cd of 1.25, 1.56, 1.87, 2.36 and 3.51 $\mu\text{g/L}$, respectively. Concerning the relationship between innate immune cells and Cd exposure, we only found neutrophil counts ($\beta = 0.665$; 95% CI: 0.052, 1.278) increased $0.665 \times 10^9/\text{L}$, and neutrophil percentages ($\beta = 3.919$; 95% CI: 0.259, 7.579) increased 3.919% in association with the highest versus the lowest quintile of Cd levels ($p < 0.05$).

4. Discussion

4.1. Higher blood Pb and Cd concentrations in Guiyu children are associated with the candidate factors

Primitive and unregulated e-waste recycling and disposals, such as open incineration and acidic leaching, lead to the emission of heavy metals and persistent organic pollutants, and the accumulation of heavy metals in the dust, soil, water, vegetables and paddy rice [2, 36, 37]. Our results are consistent with prior studies demonstrating that elevated Pb and Cd concentrations in Guiyu children are mainly due to the severe pollution of the local environment, as well as the child's habits, diets, contaminant exposure sources and duration of exposure [8, 9, 38, 39]. Among candidate factors investigated, such as the child's contact with e-waste, parents' dwelling time in the local area and using the residence as an e-waste recycling workplace contribute to the elevated Pb and Cd levels in children. Our previous investigation confirmed that nearly 60–80% of families in Guiyu are engaged in e-waste recycling operations, and most of the recycling workplaces are often residences. These family-run e-waste dismantling activities increase the risk of exposure of children to Pb. Indeed, studies showed that the Pb concentration in the dust, collected from the workshops, is hundreds of times higher than indoor dust in other areas [8, 38, 40]. We also observed that Pb levels are positively associated with the residence duration of father in the local area, which suggests that fathers with a high risk of Pb exposure can expose children when in close contact. Another possibility is that, because

Table 3. Regression coefficients and 95% CIs from linear regression models for innate immune cell levels (10⁹/L, %) in children by quintiles of blood Pb (µg/dL) and Cd (µg/L) concentrations.

	n	Mean ± SD	Monocyte count β (95% CI)	Eosinophil count β (95% CI)	Neutrophil count β (95% CI)	Basophil count β (95% CI)	% Monocytes β (95% CI)	% Eosinophils β (95% CI)	% Neutrophils β (95% CI)	% Basophils β (95% CI)	% NK β (95% CI)
Blood Pb (µg/dL)											
2.64-6.31	59	5.10 ± 1.05									
6.31-7.69	59	7.11 ± 0.36	0.039 (-0.039, 0.118)	0.017 (-0.056, 0.091)	-0.450 (-1.062, 0.162)	0.008 (-0.005, 0.020)	0.765 (0.038, 1.491)*	0.085 (-0.681, 0.851)	-4.153 (-7.792, -0.514)*	0.076 (-0.085, 0.237)	0.301 (-2.211, 2.814)
7.69-9.60	59	8.65 ± 0.53	0.083 (0.004, 0.162)*	-0.015 (-0.089, 0.058)	-0.256 (-0.869, 0.358)	0.013 (0.001, 0.026)*	0.976 (0.248, 1.704)**	-0.247 (-1.015, 0.521)	-1.872 (-5.519, 1.775)	0.105 (-0.056, 0.267)	-0.107 (-2.625, 2.411)
9.60-12.04	59	10.54 ± 0.70	0.053 (-0.026, 0.131)	0.078 (0.004, 0.152)*	-0.052 (-0.667, 0.563)	0.007 (-0.006, 0.020)	0.358 (-0.371, 1.088)	0.657 (-0.113, 1.427)	-2.167 (-5.822, 1.489)	0.033 (-0.129, 0.195)	0.301 (-2.224, 2.825)
12.04-37.09	58	15.50 ± 4.61	0.074 (-0.005, 0.153)	0.009 (-0.065, 0.083)	-0.186 (-0.802, 0.430)	0.002 (-0.011, 0.015)	0.766 (0.036, 1.497)*	-0.024 (-0.795, 0.747)	-2.897 (-6.558, 0.763)	-0.014 (-0.176, 0.148)	0.830 (-1.697, 3.358)
Blood Cd (µg/L)											
0.47-1.45	59	1.25 ± 0.16									
1.45-1.69	59	1.56 ± 0.07	-0.002 (-0.080, 0.077)	0.004 (-0.070, 0.078)	0.138 (-0.471, 0.748)	-0.004 (-0.016, 0.009)	-0.184 (-0.913, 0.546)	-0.017 (-0.788, 0.753)	1.214 (-2.427, 4.856)	-0.028 (-0.189, 0.134)	1.864 (-0.620, 4.348)
1.69-2.10	59	1.87 ± 0.12	0.052 (-0.026, 0.131)	0.010 (-0.064, 0.084)	0.277 (-0.332, 0.887)	-0.003 (-0.015, 0.010)	0.346 (-0.383, 1.076)	0.070 (-0.700, 0.840)	1.169 (-2.470, 4.808)	-0.003 (-0.165, 0.158)	1.117 (-1.365, 3.600)
2.10-2.59	59	2.36 ± 0.16	-0.015 (-0.094, 0.0163)	0.004 (-0.070, 0.078)	0.266 (-0.344, 0.875)	0.001 (-0.012, 0.013)	-0.481 (-1.211, 0.249)	-0.075 (-0.846, 0.695)	2.518 (-1.124, 6.159)	0.006 (-0.155, 0.168)	0.114 (-2.340, 2.628)
2.59-7.90	58	3.51 ± 1.14	0.068 (-0.011, 0.146)	0.058 (-0.016, 0.133)	0.665 (0.052, 1.278)*	-0.002 (-0.015, 0.011)	0.190 (-0.544, 0.924)	0.494 (-0.281, 1.268)	3.919 (0.259, 7.579)*	-0.077 (-0.239, 0.086)	-1.396 (-3.893, 1.101)

Model was adjusted by age. Values of * $p < 0.05$, ** $p < 0.01$ were considered statistically significant.

smoking or passive smoking is a source of non-occupational Pb exposure for humans [41-43] and most fathers smoke, smoking could lead to passive smoking by children. Unfortunately, we did not take into account parental smoking as a candidate factor in the study. However, our previous studies have reported that parental smoking may promote an increase in child blood Pb and Cd levels [8].

We found factors that can hinder the accumulation of Pb levels, such as the intake of dairy products, which may be protective factors for Pb levels. Other dietary factors, such as bean products and supplements of calcium, iron, zinc and vitamin C, also can be a part of nutritional intervention to reduce Pb absorption [8]; however, we are very confused we do not draw such a conclusion. In terms of Cd exposure, our results show that outdoor play time negatively correlates with blood Cd levels for children, which is opposite to the early findings [3, 8]. We speculate that indoor exposure to heavy metals may be more serious than outdoor pollution in Guiyu because of the greater density of heavy metal accumulation in dust particles [44, 45].

4.2. Association between Pb and Cd exposures and innate immune cells in children

We also found blood Pb and Cd levels are associated with the altered distribution of innate immune cells in children. Prior studies conducted on immunotoxicity of heavy metals on human leukocytes *in vitro* suggest that Pb and Cd exposures can interfere with the viability and function of lymphocytes and monocytes [46-48]. Our results show that monocyte percentages positively correlate with Pb levels, which contrasts with a prior study of Pb exposure in male mice [49]. Pb and Cd promote inflammation as a consequence of ROS production, cell damage and apoptosis, and lead to increases in endogenous waste requiring elimination by monocytes and neutrophils [50-52]. Therefore, it is understandable that Pb exposure might induce increased levels of monocytes.

Strangely, the quintile with blood Pb levels from 6.31 µg/dL to 7.69 µg/dL exhibited a decrease of neutrophil percentages, in contrast to that in the group with Pb levels lower than 6.31 µg/dL. Di Lorenzo et al. have reported the mean absolute neutrophil count is significantly higher in Pb-exposed workers (geometric mean Pb levels: 20.5 µg/dL) with respect to a control group (geometric mean: 3.5 µg/dL) [53]. The different exposure pathways, duration and levels may be reasons for different results. We also observed an upward trend of eosinophil and basophile counts resulting from Pb exposure ($\beta = 0.078$ and $\beta = 0.013$, respectively) ($p < 0.05$). Few prior

studies among highly exposed populations have noted positive as well as negative correlations between Pb and eosinophils. In addition to different study populations, the variation in results may also be due to the analyses of divergent adjustment on covariates [54-56].

Our analyses indicate Cd exposure in the highest quintile (2.59-7.90 $\mu\text{g/L}$) will increase the percentage of neutrophils with respect to the first quintile (0.47-1.45 $\mu\text{g/L}$), which is similar to a study in rats by Mladenović and colleagues [57]. However, another research in workers has suggested that individuals with blood Cd levels higher than 1.2 $\mu\text{g/L}$ exhibits a decrease in neutrophil percentages with respect to individuals with Cd levels lower than 1.1 $\mu\text{g/L}$, which is inconsistent with our finding [58]. One possible reason for the difference in findings is that our subjects, preschool children, had higher blood Cd levels and lived in the typical e-waste dismantling area. As mentioned above, Cd may elevate monocyte and neutrophil levels via ROS generation, inducing cell apoptosis and increasing endogenous substances. Thus, it is plausible that higher Cd exposure can show a positive association with neutrophil counts and percentages ($\beta = 0.665$ and $\beta = 3.919$, respectively).

From our results, we found decreased percentages of NK cells in Guiyu children. NK cells play critical roles in innate and adaptive immune responses, including cytokine secretion, contact-dependent cell-cell signalling and direct killing of other immune cells, and are involved in combating tumors, viral infections, parasites and bacteria [59-62]. Therefore, lower percentages of NK cells might impair innate immune response, as well as an adaptive immune response in Guiyu children. Unfortunately, we cannot conclude the effects of Pb and Cd on NK cells, which is similar to previously reported population studies [63, 64]. However, Sata et al. found a negative correlation between CD16⁺ NK cells and blood Pb in Pb-exposed workers [65]. Studies *in vitro* also have shown that Pb and Cd can inhibit NK cell number and activity [66, 67].

4.3. Limitations

This study is the first investigation to explore the toxic effect of heavy metals on the innate immune system of preschool children in the e-waste dismantling area, Guiyu. However, there are several limitations of our study. First, we did not analyze some parameters like oxidative stress or innate immunity cytokines to sufficiently conclude that the immunotoxicity of Pb and Cd on innate immune cells. Second, we cannot eliminate the synergistic or antagonistic impact

of other environmental pollutants, such as mercury, arsenic, PCB, PBDE and BPA, which are widely spread in Guiyu [68-71] and can affect the innate immune response [72, 73]. It will be of importance to consider these interactions in our further research. Third, children in the reference group unexpectedly had a mean blood Pb level of 8.30 $\mu\text{g/dL}$, which exceeds the maximum safe level ($\leq 5 \mu\text{g/dL}$) established by the U.S. CDC [74]. Thus we analyzed the association between Pb and innate immune cells by quintiles of Pb concentrations in order to understand the effects of different exposure levels. Other information is required in our questionnaire, such as the child passive smoking and dietary sources, which can enhance the risk of exposure to Pb and Cd [38, 41-43].

5. Conclusions

Alteration of the numbers and percentages of several innate immune cells is linked to higher levels of Pb and Cd, indicating that Pb and Cd exposures might affect the innate immune response, as well as the adaptive immune response in Guiyu children.

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REFERENCES

- [1] Heacock, M., et al., E-waste and harm to vulnerable populations: a growing global problem. *Environ. Health Perspect.*, 2016. 124(5): p. 550-555.
- [2] Huo, X., et al., Elevated blood lead levels of children in Guiyu, an electronic waste recycling town in China. *Environ. Health Perspect.*, 2007. 115(7): p. 1113-1117.
- [3] Wu, K., et al., Polybrominated diphenyl ethers in umbilical cord blood and relevant factors in neonates from Guiyu, China. *Environ. Sci. Technol.*, 2010. 44(2): p. 813-819.
- [4] Xu, X., et al., Association of polycyclic aromatic hydrocarbons (PAHs) and lead co-exposure with child physical growth and development in an e-waste recycling town. *Chemosphere*, 2015. 139: p. 295-302.
- [5] Lu, S.-y., et al., Associations between polycyclic aromatic hydrocarbon (PAH) exposure and oxidative stress in people living near e-waste recycling facilities in China. *Environ. Int.*, 2016. 94: p. 161-169.
- [6] Lu, S.-y., et al., Effect of e-waste recycling on urinary metabolites of organophosphate flame retardants and plasticizers and their association with oxidative stress. *Environ. Sci. Technol.*, 2017. 51(4): p. 2427-2437.
- [7] Zhang, T., et al., Urinary concentrations of bisphenols and their association with biomarkers of oxidative stress in people living near e-waste recycling facilities in China. *Environ. Sci. Technol.*, 2016. 50(7): p. 4045-4053.
- [8] Zheng, L., et al., Blood lead and cadmium levels and relevant factors among children from an e-waste recycling town in China. *Environ. Res.*, 2008. 108(1): p. 15-20.
- [9] Guo, Y., et al., Monitoring of lead, cadmium, chromium and nickel in placenta from an e-waste recycling town in China. *Sci. Total Environ.*, 2010. 408(16): p. 3113-3117.
- [10] Zeng, X., et al., Children with health impairments by heavy metals in an e-waste recycling area. *Chemosphere*, 2016. 148: p. 408-415.
- [11] Zeng, X., et al., Decreased lung function with mediation of blood parameters linked to e-waste lead and cadmium exposure in preschool children. *Environ. Pollut.*, 2017. 230: p. 838-848.
- [12] Jorissen, A., et al., Impact of lead and mercuric ions on the interleukin-2-dependent proliferation and survival of T cells. *Arch. Toxicol.*, 2013. 87(2): p. 249-258.
- [13] Dudka, I., et al., Metabonomic analysis of serum of workers occupationally exposed to arsenic, cadmium and lead for biomarker research: a preliminary study. *Environ. Int.*, 2014. 68: p. 71-81.
- [14] García-Lestón, J., et al., Assessment of immunotoxicity parameters in individuals occupationally exposed to lead. *J. Toxicol. Environ. Health, A*, 2012. 75(13-15): p. 807-818.
- [15] Cabral, M., et al., Effects of environmental cadmium and lead exposure on adults neighboring a discharge: Evidences of adverse health effects. *Environ. Pollut.*, 2015. 206: p. 247-255.
- [16] Salamat, N., et al., Environmentally induced tissue responses of hematopoietic system in abu mullet (*Liza abu*) and tiger tooth croaker (*Otolithes ruber*) from the Persian Gulf. *Ecotoxicol. Environ. Saf.*, 2017. 136: p. 161-172.
- [17] Dyatlov, V.A., et al., Neonatal lead exposure potentiates sickness behavior induced by *Listeria monocytogenes* infection of mice. *Brain, Behav., Immun.*, 2002. 16(4): p. 477-492.
- [18] Lawrence, D.A., et al., Immunomodulation by metals. *Int. Immunopharmacol.*, 2002. 2(2-3): p. 293-302.
- [19] Wang, B., et al., Cadmium and its neurotoxic effects. *Oxid. Med. Cell. Longev.*, 2013. 2013: p. 1-10.
- [20] Rani, A., et al., Cellular mechanisms of cadmium-induced toxicity: a review. *Int. J. Environ. Health Res.*, 2014. 24(4): p. 378-399.
- [21] Priyadarshani, S., et al., Heavy metal mediated immunomodulation of the Indian green frog, *Euphlyctis hexadactylus* (Anura: Ranidae) in urban wetlands. *Ecotoxicol. Environ. Saf.*, 2015. 116: p. 40-49.
- [22] Maret, W., et al., The bioinorganic chemistry of cadmium in the context of its toxicity, in *Cadmium: From toxicity to essentiality*. 2013, Springer. p. 1-29.
- [23] Vilahur, N., et al., The epigenetic effects of prenatal cadmium exposure. *Current environmental health reports*, 2015. 2(2): p. 195-203.
- [24] Martin, S.F., Adaptation in the innate immune system and heterologous innate immunity. *Cell. Mol. Life Sci.*, 2014. 71(21): p. 4115-4130.
- [25] Ward, A.E., et al., Evolutionary responses of innate immunity to adaptive immunity. *Infect., Genet. Evol.*, 2014. 21: p. 492-496.
- [26] Iwasaki, A., et al., Control of adaptive immunity by the innate immune system. *Nat. Immunol.*, 2015. 16(4): p. 343-353.
- [27] Nain, S., et al., Subchronic lead exposure, immunotoxicology and increased disease resistance in Japanese quail (*Coturnix coturnix japonica*). *Ecotoxicol. Environ. Saf.*, 2011. 74(4): p. 787-792.
- [28] Sövényi, J., et al., Studies on the toxic and

- immunosuppressive effects of cadmium on the common carp. *Acta Vet. Hung.*, 1993. 41(3-4): p. 415-426.
- [29] Demenescu, J., et al., Strain differences of cadmium-induced toxicity in rats: Insight from spleen and lung immune responses. *Toxicol. Lett.*, 2016. 256: p. 33-43.
- [30] Breton, J., et al., Chronic ingestion of cadmium and lead alters the bioavailability of essential and heavy metals, gene expression pathways and genotoxicity in mouse intestine. *Arch. Toxicol.*, 2013. 87(10): p. 1787-1795.
- [31] Thévenod, F., et al., Cadmium and cellular signaling cascades: interactions between cell death and survival pathways. *Arch. Toxicol.*, 2013. 87(10): p. 1743-1786.
- [32] Xu, X., et al., Decreased blood hepatitis B surface antibody levels linked to e-waste lead exposure in preschool children. *J. Hazard. Mater.*, 2015. 298: p. 122-128.
- [33] Dai, Y., et al., Elevated lead levels and changes in blood morphology and erythrocyte CR1 in preschool children from an e-waste area. *Sci. Total Environ.*, 2017. 592: p. 51-59.
- [34] Lin, X., et al., Decreased vaccine antibody titers following exposure to multiple metals and metalloids in e-waste-exposed preschool children. *Environ. Pollut.*, 2017. 220: p. 354-363.
- [35] Yang, H., et al., Effects of lead and cadmium exposure from electronic waste on child physical growth. *Environmental Science and Pollution Research*, 2013. 20(7): p. 4441-4447.
- [36] Leung, A.O., et al., Heavy metals concentrations of surface dust from e-waste recycling and its human health implications in southeast China. *Environ. Sci. Technol.*, 2008. 42(7): p. 2674-2680.
- [37] Xu, P., et al., Levels, profiles and source identification of PCDD/Fs in farmland soils of Guiyu, China. *Chemosphere*, 2013. 91(6): p. 824-831.
- [38] Guo, P., et al., Blood lead levels and associated factors among children in Guiyu of China: a population-based study. *PLoS One*, 2014. 9(8): p. e105470.
- [39] Xu, X., et al., Blood concentrations of lead, cadmium, mercury and their association with biomarkers of DNA oxidative damage in preschool children living in an e-waste recycling area. *Environ. Geochem. Health*, 2018. 40(4): p. 1481-1494.
- [40] Srivastava, A.K., et al., Evaluating risks of acquired clinical vulnerability among subjects exposed to e-waste, in *Rev. Environ. Contam. Toxicol.* 2012, Springer. p. 1-14.
- [41] Liu, J., et al., Blood lead levels and associated sociodemographic factors among preschool children in the South Eastern region of China. *Paediatr. Perinat. Epidemiol.*, 2012. 26(1): p. 61-69.
- [42] Lutz, P.M., et al., Environmental cigarette smoke exposure modulates IgE levels of Pb-exposed children. *Toxicology*, 2012. 291(1-3): p. 43-50.
- [43] Behera, S.N., et al., Human health risk associated with exposure to toxic elements in mainstream and sidestream cigarette smoke. *Sci. Total Environ.*, 2014. 472: p. 947-956.
- [44] Argyraki, A., Garden soil and house dust as exposure media for lead uptake in the mining village of Stratoni, Greece. *Environ. Geochem. Health*, 2014. 36(4): p. 677-692.
- [45] Lau, W.K.Y., et al., Human health risk assessment based on trace metals in suspended air particulates, surface dust, and floor dust from e-waste recycling workshops in Hong Kong, China. *Environmental Science and Pollution Research*, 2014. 21(5): p. 3813-3825.
- [46] Steffensen, I., et al., Cytotoxicity and accumulation of Hg, Ag, Cd, Cu, Pb and Zn in human peripheral T and B lymphocytes and monocytes in vitro. *General Pharmacology: The Vascular System*, 1994. 25(8): p. 1621-1633.
- [47] Valentino, M., et al., Effect of lead on the levels of some immunoregulatory cytokines in occupationally exposed workers. *Hum. Exp. Toxicol.*, 2007. 26(7): p. 551-556.
- [48] Fortier, M., et al., Effects of physiological concentrations of heavy metals both individually and in mixtures on the viability and function of peripheral blood human leukocytes in vitro. *J. Toxicol. Environ. Health, A*, 2008. 71(19): p. 1327-1337.
- [49] Sharma, V., et al., The effect of oral administration of *Allium sativum* extracts on lead nitrate induced toxicity in male mice. *Food Chem. Toxicol.*, 2010. 48(3): p. 928-936.
- [50] Hebeda, C.B., et al., Intracellular mechanisms of hydroquinone toxicity on endotoxin-activated neutrophils. *Arch. Toxicol.*, 2012. 86(11): p. 1773-1781.
- [51] Pineda-Zavaleta, A.P., et al., Nitric oxide and superoxide anion production in monocytes from children exposed to arsenic and lead in region Lagunera, Mexico. *Toxicol. Appl. Pharmacol.*, 2004. 198(3): p. 283-290.
- [52] Cheng, Y.-J., et al., Lead increases lipopolysaccharide-induced liver injury through tumor necrosis factor- α overexpression by monocytes/macrophages: role of protein kinase C and p42/44 mitogen-activated protein kinase. *Environ. Health Perspect.*, 2006. 114(4): p. 507-513.
- [53] Di Lorenzo, L., et al., Evaluation of peripheral blood neutrophil leucocytes in lead-exposed workers. *Int.*

- Arch. Occup. Environ. Health, 2006. 79(6): p. 491-498.
- [54] Karmaus, W., et al., Immune function biomarkers in children exposed to lead and organochlorine compounds: a cross-sectional study. *Environ. Health*, 2005. 4(1): p. 5.
- [55] Ukaejiofo, E., et al., Haematological assessment of occupational exposure to lead handlers in Enugu urban, Enugu State, Nigeria. *Niger. J. Clin. Pract.*, 2009. 12(1).
- [56] Wells, E.M., et al., The relationship of blood lead with immunoglobulin E, eosinophils, and asthma among children: NHANES 2005–2006. *Int. J. Hyg. Environ. Health*, 2014. 217(2-3): p. 196-204.
- [57] Mladenović, J., et al., Protective effects of oestradiol against cadmium-induced changes in blood parameters and oxidative damage in rats. *Archives of Industrial Hygiene and Toxicology*, 2014. 65(1): p. 37-46.
- [58] Ciarrocca, M., et al., Correlation between cadmium and blood counts in workers exposed to urban stressor. *Arch. Environ. Occup. Health*, 2015. 70(2): p. 70-76.
- [59] Van Loo, P., et al., T-cell/histiocyte-rich large B-cell lymphoma shows transcriptional features suggestive of a tolerogenic host immune response. *Haematologica*, 2010. 95(3): p. 440-448.
- [60] Holgate, S.T., Innate and adaptive immune responses in asthma. *Nat. Med.*, 2012. 18(5): p. 673-683.
- [61] Deckers, J., et al., Innate immune cells in asthma. *Trends Immunol.*, 2013. 34(11): p. 540-547.
- [62] Stringaris, K., et al., Leukemia-induced phenotypic and functional defects in natural killer cells predict failure to achieve remission in acute myeloid leukemia. *Haematologica*, 2014. 99(5): p. 836-847.
- [63] Yücesoy, B., et al., Simultaneous effects of lead and cadmium on NK cell activity and some phenotypic parameters. *Immunopharmacol. Immunotoxicol.*, 1997. 19(3): p. 339-348.
- [64] Sarasua, S.M., et al., Serum immunoglobulins and lymphocyte subset distributions in children and adults living in communities assessed for lead and cadmium exposure. *J Toxicol Environ Health Part A*, 2000. 60(1): p. 1-15.
- [65] Sata, F., et al., Changes in natural killer cell subpopulations in lead workers. *Int. Arch. Occup. Environ. Health*, 1997. 69(5): p. 306-310.
- [66] Cifone, M., et al., Effects of cadmium on cytotoxic functions of human natural killer cells. *Toxicol. In Vitro*, 1991. 5(5-6): p. 525-528.
- [67] Skoczyńska, A., et al., The impact of lead and cadmium on the immune system. *Med. Pr.*, 2002. 53(3): p. 259-264.
- [68] Guo, Y., et al., Heavy metal contamination from electronic waste recycling at Guiyu, Southeastern China. *J. Environ. Qual.*, 2009. 38(4): p. 1617-1626.
- [69] Xing, G.H., et al., Environmental impact and human exposure to PCBs in Guiyu, an electronic waste recycling site in China. *Environ. Int.*, 2009. 35(1): p. 76-82.
- [70] Wu, K., et al., In utero exposure to polychlorinated biphenyls and reduced neonatal physiological development from Guiyu, China. *Ecotoxicol. Environ. Saf.*, 2011. 74(8): p. 2141-2147.
- [71] Ni, W., et al., Hair mercury concentrations and associated factors in an electronic waste recycling area, Guiyu, China. *Environ. Res.*, 2014. 128: p. 84-91.
- [72] Miller, R.L., et al., Environmental effects on immune responses in patients with atopy and asthma. *J. Allergy Clin. Immunol.*, 2014. 134(5): p. 1001-1008.
- [73] Thompson, P.A., et al., Environmental immune disruptors, inflammation and cancer risk. *Carcinogenesis*, 2015. 36(Suppl_1): p. S232-S253.
- [74] Betts, K.S., CDC updates guidelines for children's lead exposure. 2012, National Institute of Environmental Health Sciences.

**ELEVATED LEAD LEVELS AND ADVERSE EFFECTS
ON NATURAL KILLER CELLS IN CHILDREN
FROM AN ELECTRONIC WASTE RECYCLING AREA**

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ABSTRACT

Lead (Pb) has been proved to exert immunotoxicity to influence immune homeostasis in humans. To monitor the internal Pb level and evaluate its effect on natural killer (NK) cells and cytokine/chemokine concentrations, we recruited 285 preschool children from Guiyu, one of the largest electronic waste (e-waste) destinations and recycling areas in the world, and known to have high concentrations of Pb in the air, soil, water, sediment and plants. A total of 126 preschool children were selected from Haojiang as a reference group. Results showed that children in Guiyu, the exposed area, had higher blood Pb levels and lower percentages of NK cells than children from the reference area. A significantly negative association was found between the percentage of NK cells and increasing Pb levels. Moreover, children in Guiyu area had higher platelet counts and IL-1 β concentrations, and lower levels of IL-2, IL-27, MIP-1 α and MIP-1 β were observed in the exposed children. These changes might not be conducive to the development and differentiation of NK cells. Taken together, the elevated Pb level results in the lower percentages of NK cells, but also alters the levels of platelets, IL-1 β and IL-27, which might be unconducive to the activity and function of NK cells.

1. Introduction

Electronic waste (e-waste) has been regarded as leading source of environmental contamination and human health problems, which arouses wide concern in recent years [1, 2]. Our previous studies showed that a large amount of environmental toxicants, including heavy metals and organic pollutants, were found in Guiyu, a typical e-waste site in southern China with nearly a 30-year history of unregulated e-waste disposal [3, 4]. Lead (Pb), as one of the conventional environmental chemicals, has been constantly discovered in the air, soil, water, sediment and plants in Guiyu, and its level is usually much higher than other non-polluted areas [5-7]. Besides, high blood Pb levels were observed among local preschool children in our cross-sectional studies [8, 9]. *In vivo* and *in vitro* experiments demonstrated that Pb can stir up developmental toxic outcomes, such as low cognitive competence, poor memory and study, reduced birth weight [10-12]. Children are particularly vulnerable to this xenobiotic and are prone to have a long-term accumulation, resulting in many potential health risks [13-15].

Pb is known to produce neurotoxicity and dysfunction of the hematopoietic system [16]. It has also been proved to exert immunotoxicity to immune homeostasis in humans, increasing opportunities to suffer from infections or diseases [17-20]. A prior study conducted for *in vitro* immunotoxic effects of heavy metals on human blood shows that Pb and other metal co-exposures can interrupt the viability and immune competence of human peripheral lymphocytes and mononuclear cells [21]. Natural killer (NK) cells, as a unique subset of lymphocytes, can spontaneously lyse certain virus-infected cells and tumor cells without the requirement for prior sensitization, which plays a key role in innate immunity and exhibits immunoregulatory functions in adaptive immune responses [22]. Pb exposure has been verified as a significant contributor to the NK cell cytotoxicity. This is especially true for high doses of acute and chronic Pb exposure to rat spleen cells *in vitro* that NK cell cytolytic activity is largely suppressed, which may to some extent explain the tumor-promoting effect of this chemical through compromising immunosurveillance [23, 24]. There are some population-based studies revealing that environmental pollutants have an impact on children's immune system development, following decreases in the numbers of NK cells and NK cell receptor expressions, and increases of other inflammatory mediators [25, 26]. However, data are scarcely available focusing on epidemiological investigations of Pb exposure effect on NK cell cytotoxicity,

especially for those susceptible populations. Thus, efforts are needed to find out the chemical-specific effects on abnormal immunoregulatory responses during which perturbations in quantity and function of NK cells may be changed.

In addition to the likely imbalance of lymphocytes including NK cells induced by Pb exposure, current studies have identified that certain critical immune molecules like cytokines are tightly correlated with NK cell functions *in vivo* [27-31]. Cytokines and chemokines, such as interleukin (IL)-2, IL-12, IL-15, IL-18, IL-21, IFN- α , TNF- α , IL-1, IL-6, IL-7, IL-27, MIP-1 α and MIP-1 β , are important signals for regulating NK cell development, survival and functioning actions [32-35], as well as promoting NK cell cytotoxicity and/or production of IFN- γ [36-38]. Several experimental reports have indicated that increased interleukin levels including IL-1 β , IL-6, IL-8 and TNF- α are associated with varying doses of Pb exposure, causing neurodevelopmental impairments, angiogenesis and tumor metastasis [39-42]. However, an inverse result is found that secretions of IFN- γ and IL-4 are significantly decreased after the guinea pigs are exposed to Pb inhaling, resulting in asthma-like changes [34, 43]. Taken collectively, the existed observations highlight the pivotal roles of cytokines in mediating inflammation or anti-inflammatory immunoregulation induced by Pb exposure.

To date, human health risk assessment, of particular in children, performed to link exposure to trace levels of environmental chemicals with probable abnormal immune regulation and status is unproven and remains a matter of speculation, even though some evidence has been gained by rodent experiments. Our previous study showed a decreased immune response to the hepatitis B vaccine with chronic Pb exposure in the e-waste polluted area of Guiyu [44]. In this respect, we consider the immune components or immunoregulatory chain of local children are partly injured. Again combined with the current context of Pb-induced NK cell cytotoxicity, we hypothesize that Pb might affect the development and activity of NK cells by inducing changes in the levels of accessory cells and cytokines/chemokines involved in NK cell survival and function. This study aims to determine child blood Pb level and evaluate its effect on NK cells and cytokine/chemokine concentrations, to offer a deeper understanding of the cellular and molecular networks of cytokine–cytokine receptor interactions by activating the anti-tumor potential of NK cells.

2. Materials and Methods

2.1. Study population

A total of 411 preschool children with a 3- to 7-years of age, were recruited from Guiyu (n = 285) and Haojiang (n = 126) in December, 2014. Haojiang was selected as the reference area because of its similarities to Guiyu in population, cultural background and socioeconomic status, and lacks electronic waste pollution. All participants were given the written informed consent prior to enrollment. A questionnaire was delivered to the participants, including both parents' and children's general characteristics, dwelling environments, parent education and jobs, child behavior habits, diet and health physiological parameters. The study protocol was approved by the Human Ethics Committee of Shantou University Medical College, China.

2.2. Sample collection

Peripheral blood samples were obtained from volunteers and collected in Pb-free tubes by trained nurses. The blood sample tube containing EDTA, as an anticoagulant, was used for routine blood examination and Pb testing. The other tube of blood was anticoagulant-free and was centrifuged at room temperature for 10 min at 855 g; serum supernatant was stored at -70°C until assay for cytokines and chemokines.

2.3. Determination of blood Pb

For Pb detection, 100 μL whole blood and 900 μL of 0.5% nitric acid (analytical reagent) were combined, followed by vortexing, and subsequent digestion for 10 min at room temperature. The blood Pb level was analyzed by graphite furnace atomic absorption spectrophotometry (Jena Zeenit 650, Germany), using detection methods according to our previous publication [10].

2.4. Flow cytometry analysis

To determine the $\text{CD3}^+\text{CD56}^{\text{bright}}\text{CD16}^{\text{low/-}}$ and $\text{CD3}^+\text{CD56}^{\text{dim}}\text{CD16}^+$ phenotype of NK cells, 100 μL whole blood was added to a tube with 2 mL wash buffer, and cells were pelleted by centrifugation at room temperature for 5 min at 500 g. The supernatant was decanted and cells were rewashed. After washing, cells were resuspended in 100 μL wash buffer by gentle vortexing. The appropriate volume of the following monoclonal antibodies were added: CD3-APC-CY7, CD56-PE, CD16-PerCP-Cy5.5 (BD Bioscience, USA). The cell suspension was

gently vortexed, after adding all antibodies, and incubated for 15 minutes in the dark at room temperature, then 2 mL of 1× FACS lysing solution (BD Bioscience, USA) was added, and the cell suspension was vortexed gently and incubated for 10 min in the dark at room temperature. Cells were pelleted by centrifugation at 500 g for 5 minutes, and the supernatant was removed. Cells were washed twice as above, followed by thorough resuspension in 200 µL wash buffer. Cells were analyzed by FACS using an Aria I flow cytometer (BD Bioscience). Data were analyzed with FlowJo and DIVA software (version 6.1, BD bioscience).

2.5. Cytokine and chemokine assays

Cytokines and chemokines were detected with the use of a ProcartaPlex Human Cytokine & Chemokine Panel 1A (eBioscience, USA). Beads coated with anti-human IL-12, IL-23, IL-27, MCP-1, RANTES, GRO α , SDF-1 α , IP-10, eotaxin, GM-CSF, IFN- α , IFN- γ , IL-1 α , IL-1 β , IL-1 receptor antagonist, IL-10, IL-13, IL-15, IL-17A, IL-18, IL-2, IL-21, IL-22, IL-31, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, MIP-1 α , MIP-1 β , TNF- α and TNF- β were incubated with the serum samples and analysis was based on the manufacturer's instructions. Data acquisition was performed using a Luminex 200 analyzer (Luminex, USA).

2.6. Statistical analysis

All statistical analyses were performed with SPSS 19.0 and GraphPad Prism 5.0 software. The comparison between two groups was made by an independent-sample *t* test, Mann-Whitney U test and chi-square test. Median or mean $\pm$ SD were used to depict blood Pb levels, percentages of NK cell subsets, cell counts and cytokine/chemokine concentrations. Spearman rank correlation analysis was used to examine relationships between blood Pb levels, the number of blood cells, and cytokine and chemokine concentrations. Multiple linear regression analysis was used to assess the relevant factors contributing to Pb exposure, and association of blood Pb levels with percentage of NK cell subsets. A *p* < 0.05 in a two-tailed test was considered as statistically significant.

3. Results

3.1. Characteristics of the study population

A total of 411 children were enrolled in the study (**Table 1**), the mean age of children in the exposed group ($n = 285$) was 4.5 ± 0.9 years, and 4.3 ± 1.0 years in the reference group ($n = 126$) ($p < 0.01$). Gender, height, weight and body mass index (BMI) of children did not show significant differences (all $p > 0.05$) between the exposed and reference group. The mean blood Pb level in the exposed group was higher than that in the reference group (6.00 ± 2.75 $\mu\text{g/dL}$ vs. 3.92 ± 1.96 $\mu\text{g/dL}$) ($p < 0.01$). More than 57% of children from exposed group exceeded the limit value of Pb level (>5 $\mu\text{g/dL}$) according to the U.S. CDC [45]. Besides, significant differences between two groups were found for smoking by a family member, housing ventilation, using the residence as a workplace, distance of residence from the road, mother's work associated with e-waste, and e-waste contamination within 50 meters from residence ($p < 0.01$).

3.2. Blood Pb levels and related factors

Multiple linear regression analysis was used to estimate whether specific factors were related to blood Pb levels (**Table 2**). We found ventilation of housing, using the residence as a workplace, distance of residence from the road, mother's work associated with e-waste and e-waste contamination within 50 meters away from the residence were all positively associated with blood Pb levels ($\beta = 0.142$, $\beta = 0.143$, $\beta = 0.115$, $\beta = 0.126$ and $\beta = 0.147$, respectively), among which e-waste contamination within 50 meters away from living house predominated, contributing more to the elevated Pb levels in children.

Table 1. Descriptive statistics for the study population.

Characteristics	Exposed group (n = 285)	Reference group (n = 126)	<i>p</i>
Child's age (years)	4.5 ± 0.9	4.3 ± 1.0	0.006 ^a
Gender [n (%)]			0.780 ^b
male	153 (53.9)	66 (52.4)	
female	131 (46.1)	60 (47.6)	
Height (cm)	105.12 ± 7.22	105.01 ± 8.21	0.894 ^a
Weight (kg)	16.80 ± 2.73	16.91 ± 3.44	0.751 ^a
Body mass index (BMI, kg/m ²)	15.12 ± 1.24	15.27 ± 2.03	0.457 ^a
Blood Pb levels (µg/dL)	6.00 ± 2.75	3.92 ± 1.96	< 0.001 ^a
> 5 µg/dL [n (%)]	164 (57.5)	21 (16.7)	< 0.001 ^b
≤ 5 µg/dL [n (%)]	121 (42.5)	105 (83.3)	
Family member daily smoking [n (%)]			0.001 ^b
non-smoking	66 (24.8)	54 (43.2)	
~ 2 cigarettes	49 (18.4)	19 (15.2)	
~ 10 cigarettes	59 (22.2)	30 (24.0)	
~ 20 cigarettes	61 (22.9)	13 (10.4)	
> 20 cigarettes	31 (11.7)	9 (7.2)	
Ventilation of house [n (%)]			< 0.001 ^b
occasionally	42 (15.3)	1 (0.8)	
frequently	233 (84.7)	125 (99.2)	
Residence as workplace [n (%)]			< 0.001 ^b
no	134 (51.9)	120 (96.0)	
yes	124 (48.1)	5 (4.0)	
Distance of residence from road (m) [n (%)]			< 0.001 ^b
< 10	103 (37.9)	12 (9.5)	
~ 50	59 (21.7)	30 (23.8)	
~ 100	57 (21.0)	17 (13.5)	
> 100	53 (19.5)	67 (53.2)	
Mother's work associated with e-waste dismantling [n (%)]			0.001 ^b
no	242 (91.0)	125 (100.0)	
yes	24 (9.0)	0 (0)	
E-waste contamination within 50 meters away from living house [n (%)]			< 0.001 ^b
no	169 (65.5)	121 (96.0)	
yes	89 (34.5)	5 (4.0)	

^aAnalysis by independent-sample *t* test. ^bAnalysis by chi-square test. Values of *p* < 0.05 were considered statistically significant.

Table 2. Multiple linear regression analysis of factors related to blood Pb levels in children.

	Blood Pb level		
	B ^a	β^b	95% CI for B
Ventilation of house	1.344	0.142	(0.398, 2.290)**
Residence as workplace	0.825	0.143	(0.217, 1.432)**
Distance of residence from road	0.255	0.115	(0.023, 0.487)*
Mother's work associated with e-waste dismantling	1.548	0.126	(0.233, 2.862)*
E-waste contamination within 50 meters away from living house	0.930	0.147	(0.231, 1.628)**

Data was adjusted by age, family members daily smoking. ^aB: unstandardized coefficients; ^b β : standardized coefficients. CI, confidence interval; Values of * $p < 0.05$, ** $p < 0.01$ were considered statistically significant.

3.3. Percentage of NK cell subsets in peripheral blood

We used a gating strategy to investigate different NK cell subsets in child peripheral blood via CD3⁻ populations with the exception of CD3⁺ cells. Further, the identified subsets were analyzed based on CD16 and CD56 expression (**Table 3**). Differentiated human peripheral blood NK cells were characterized by surface expression of CD56, with approximately 2 - 10% of these NK cells designated as CD56^{bright}, and more than 90% of cells classified as CD56^{dim}. The CD56^{dim} cells expressing CD16 were responsible for the cytotoxic effect. Results showed that the percentage of CD3⁻CD56⁺ NK cells in the CD3⁻ population was significantly lower in the exposed group (mean 8.51%) compared with that in the reference group (mean 11.69%) ($p < 0.01$). The percentages of CD3⁻CD56^{bright}CD16^{low/-} and CD3⁻CD56^{dim}CD16⁺ subpopulations in CD3⁻CD56⁺ NK cells were both lower in the exposed group (mean 3.64% and 84.10%, respectively) than in the reference group (mean 4.62% and 87.21%, respectively) (both $p < 0.05$).

Table 3. NK cell subset distributions (%) in peripheral blood of children.

	Exposed group		Reference group		<i>p</i>
	N	Mean $\pm$ SD	N	Mean $\pm$ SD	
CD3 ⁻ CD56 ⁺	56	8.51 $\pm$ 3.49	65	11.69 $\pm$ 5.24	< 0.001
CD3 ⁻ CD56 ^{bright} CD16 ^{low/-}	56	3.64 $\pm$ 2.24	65	4.62 $\pm$ 2.72	0.032
CD3 ⁻ CD56 ^{dim} CD16 ⁺	56	84.10 $\pm$ 7.25	65	87.21 $\pm$ 6.34	0.014

Analysis by independent-sample *t* test. Values of $p < 0.05$ were considered statistically significant.

3.4. The association between blood Pb levels and NK cells

The relationship between blood Pb levels and NK cells were shown in **Table 4**. We found that the percentages of CD3⁺CD56⁺ and CD3⁺CD56^{bright}CD16^{low/-} NK cells were both negatively correlated with blood Pb levels ($R_s = -0.199, p = 0.031$ and $R_s = -0.229, p = 0.013$, respectively). Univariate linear regression analysis between blood Pb levels and NK cells further showed that increased Pb levels mainly affected the CD3⁺CD56^{bright}CD16^{low/-} NK cells, which led to a decline in the percentage and differentiation of these subgroups ($B = -0.182, 95\% \text{ CI: } -0.357, -0.007, p < 0.05$) (**Table 5**).

Table 4. Spearman correlations of blood Pb levels and NK cells in children.

	CD3 ⁺ CD56 ⁺		CD3 ⁺ CD56 ^{bright} CD16 ^{low/-}		CD3 ⁺ CD56 ^{dim} CD16 ⁺	
	R_s	p	R_s	p	R_s	p
Blood Pb levels	-0.199	0.031	-0.229	0.013	-0.097	0.298

R_s : Spearman correlation coefficient. Values of $p < 0.05$ were considered statistically significant.

Table 5. Univariate linear regression analysis of the association between blood Pb levels and NK cells in children.

	CD3 ⁺ CD56 ⁺		CD3 ⁺ CD56 ^{bright} CD16 ^{low/-}		CD3 ⁺ CD56 ^{dim} CD16 ⁺	
	B	95% CI for B	B	95% CI for B	B	95% CI for B
Blood Pb levels	-0.183	(-0.514, 0.148)	-0.182	(-0.357, -0.007)*	-0.252	(-0.733, 0.229)

Data was adjusted by age. B: unstandardized coefficients. CI: confidence interval; Values of $*p < 0.05$ were considered statistically significant.

3.5. Determinations in indices of blood cells and cytokines/chemokines

In order to understand the NK cell microenvironment conditions that were associated with the proliferation and activation of NK cells, we measured blood corpuscles and cytokines/chemokines in child peripheral blood. Blood cells including lymphocytes, platelets, erythrocytes, monocytes and neutrophils were analyzed. We found that lymphocytes were 10.6% in the Guiyu group (median $3.71 \times 10^9/\text{L}$), lower than that in the reference group (median

$4.15 \times 10^9/\text{L}$) ($p < 0.01$). In contrast, platelets were 10.5% in the Guiyu group (median $315.00 \times 10^9/\text{L}$), higher than that in the reference group (median $285.00 \times 10^9/\text{L}$) ($p < 0.01$). In addition, erythrocyte and monocyte counts were both lower in the exposed group (median $4.78 \times 10^{12}/\text{L}$ and $0.50 \times 10^9/\text{L}$, respectively) compared with the reference group (median $4.73 \times 10^{12}/\text{L}$ and $0.51 \times 10^9/\text{L}$, respectively), while there was no significance for the number of neutrophils (median $3.81 \times 10^9/\text{L}$ vs. $3.85 \times 10^9/\text{L}$) between two groups ($p > 0.05$) (**Figure 1**).

Cytokines and chemokines, including IL-2, IL-12, IL-15, IL-18, IL-21, IFN- α , TNF- α , IL-1 β , IL-6, IL-7, IL-27, MIP-1 α and MIP-1 β , were determined in this study (**Figure 2**). We found that IL-2, IL-27, MIP-1 α and MIP-1 β concentrations were significantly lower in the exposed group (median 7.97 pg/mL, 50.40 pg/mL, 9.17 pg/mL and 758.06 pg/mL, respectively) compared to the reference group (median 8.41 pg/mL, 106.28 pg/mL, 10.16 pg/mL and 791.44 pg/mL, respectively) (all $p < 0.05$). IL-1 β concentration was increased in the exposed group (median 0.81 pg/mL) compared with that in the reference group (median 0.80 pg/mL) ($p < 0.05$). IL-12 secretions were not detectable (data not shown). The concentrations of IL-15, IL-18, IL-21, IFN- α , TNF- α , IL-6 and IL-7 did not show statistically significant differences between two groups.

Figure 1. Blood cell counts in peripheral blood. Exposed group, $n=80$; Reference group, $n=80$. Results are presented as medians; analysis by the Mann-Whitney U test. Values of $*p < 0.05$, $**p < 0.01$ were considered statistically significant.

Figure 2. Levels of cytokines and chemokines in peripheral blood. Exposed group, $n=80$; Reference group, $n=80$. Data were presented by medians. P values are the result of a Mann-Whitney U test. Values of $*P < 0.05$, $**P < 0.01$ were considered statistically significant.

3.6. The association between Pb exposure and blood cells, cytokine/chemokine concentrations

The blood cells, cytokines and chemokines were assessed to link with Pb levels. We found that platelet, neutrophil and monocyte counts correlated with blood Pb levels ($R_s = 0.112$, $R_s = 0.139$ and $R_s = 0.120$, respectively; all $p < 0.05$). In terms of cytokines, serum IL-1 β and IL-6 concentrations were positively associated with blood Pb levels ($R_s = 0.162$ and $R_s = 0.222$, respectively; $p < 0.05$ and $p < 0.01$, respectively). By contrast, IL-27 concentrations were negatively associated with blood Pb levels ($R_s = -0.306$, $p < 0.01$) (Table 6).

Table 6. Spearman correlations between blood Pb level and immune parameters.

	Blood Pb level	
	R_s	p
Lymphocyte	-0.015	0.763
Erythrocyte	0.059	0.235
Platelet	0.112	0.024
Neutrophil	0.139	0.037
Monocyte	0.120	0.015
IL-2	-0.033	0.685
IL-15	0.087	0.280
IL-18	-0.068	0.399
IL-21	0.050	0.562
IFN- α	0.008	0.924
TNF- α	0.052	0.537
IL-1 β	0.162	0.046
IL-6	0.222	0.005
IL-27	-0.306	0.000
MIP-1 α	-0.048	0.557
MIP-1 β	-0.006	0.940

R_s : Spearman correlation coefficient. Values of $p < 0.05$ were considered statistically significant.

4. Discussion

In this study, we explored the changes of NK cells and NK cell activation-associated cytokines and chemokines, and estimated the associations of these level changes with Pb exposure in preschool children from Guiyu, a typical e-waste site. Our results show that children in Guiyu still suffer a high Pb level compared to the reference population [6, 8, 10-12, 15]. We analyzed

certain factors including ventilation of housing, using the residence as a workplace, distance of residence from the road, mother's work associated with e-waste and e-waste contamination within 50 meters away from residence, were all positively associated with blood Pb levels, suggesting that Pb accumulation from the mainly preliminary e-waste polluting sources continues affecting local people, particularly for children. In the present data, percentages of NK cell subsets encompassing $CD3^+CD56^+$, $CD3^+CD56^{bright}CD16^{low/-}$ and $CD3^+CD56^{dim}CD16^+$ NK cells in the peripheral blood of Guiyu children are reduced compared with the reference area. $CD3^+CD56^+$ and $CD3^+CD56^{bright}CD16^{low/-}$ NK cells both exhibit a negatively significant correlation with blood Pb levels. These observations indicate that decreased percentages of NK cells to some extent may be related to elevated blood Pb levels. Heavy metal Pb exposure can partly interrupt child immune state and development through alterations of different NK cell subsets. A former cross-sectional study conducted by Stata et al. demonstrated that there is a negatively significant correlation between the amount changes of NK cells and blood Pb levels in Pb-exposed workers [23, 46]. Their results showed that the number and percentage of $CD16^+$ NK cells in the high-Pb group are significantly lower than those in the control and in the low-Pb group, which is consistent with our finding for children in the e-waste site. The difference is that they found a negative correlation between the number of $CD16^+$ NK cell subpopulations and Pb exposure, instead of $CD3^+CD56^{bright}CD16^{low/-}$ NK cells in our results. From the internal fate of Pb exposure in children, approximately 73% of Pb eventually accumulates in bone [47]. Although NK cells are derived from bone marrow cells, previous results *in vitro* showed that Pb exposure reduces somatic growth, longitudinal bone growth, and bone strength during the pubertal period [48]. The status of skeletal growth and development will affect bone marrow cell survival and differentiation. In addition, one of the most important trace elements, zinc, influences the proliferation and differentiation of $CD34^+$ progenitors and plays a crucial role in the development and differentiation of NK cells [49, 50]. Pb, as an antagonist that competes with zinc at zinc binding sites, can inhibit the biological function of zinc [51, 52]. From this point of view, it can be explained that Pb may be uncondusive to the development and proliferation of NK cells.

Certainly, NK cells may also be influenced by multiple factors, such as T lymphocytes, B lymphocytes, red blood cells and several cytokines, all of which can alter the number and function of NK cells [30, 53]. We determined blood cell quantities and cytokine concentrations

to explore the possible factors related to the change in percentage of NK cells. In this study, we found lower lymphocyte quantities in children from Guiyu. As a kind of lymphocytes, CD3⁺CD56^{dim}CD16⁺ is the most abundant subpopulation of NK cells in human peripheral blood. Unexpectedly, we did not find any reduction of lymphocyte counts associated with elevated Pb levels. Another blood cell type, platelets, may attenuate cytokine secretion and immunosuppressive responses of Th cells, and enhance Tc cell proliferation and cytotoxicity, as well as impeding the anti-tumor activity of NK cells [54]. Our results show that increased platelet counts are associated with elevated Pb levels, affecting NK cell activity. Neutrophils are potent inflammatory effector cells essential to host defense, but they may also result in tissue damage [55]. Our findings show that children living in Guiyu have a higher percentage of neutrophils, and exhibit a negative correlation between the percentage of neutrophils and CD3⁺CD56^{dim}CD16⁺ NK cells (data not shown). It has shown that even low exposure to Pb can cause an increase in the number of neutrophils in Pb-exposed workers[56]. Thus, our finding of the increased percentage of neutrophils may indicate a higher risk of inflammation in Guiyu children. Thorén et al. provided evidence that NK cells may have the ability to induce neutrophil apoptosis via NKp46- and Fas-dependent mechanisms [57]. Interestingly, we found monocyte numbers are negatively correlated with blood Pb levels, but there is no significant difference between the exposed group and the reference group. Possible reason could be that the effects of Pb on monocytes are too low to be detected by our methods, or the effects of Pb on monocytes are neutralized by other factors.

Research on the anti-tumor effect and cytotoxicity of NK cells has been a hot topic in recent decades. The killing activity of NK cells is associated with the levels of cytokines and chemokines, the numbers of activated NK cells in the body, and the expression of MHC class I molecules on the surface of tumor cells [37, 38]. NK cells express a variety of cytokine and chemokine receptors. Cytokines and chemokines can regulate the development and function of NK cells, and promote NK cell-mediated anti-tumor immune response [58]. In our study, we found that IL-1 β concentrations are higher in exposed group ($p < 0.05$). This is similar to Li et al. who found that high expression of IL-1 β and TNF- α were associated with maternal Pb exposure in mouse pups. IL-1 β is a pleiotropic cytokine that promotes angiogenesis, tumor invasiveness and carcinogenesis, and Elkabets et al. reported IL-1 β can confer myeloid-derived suppressor cells with enhanced capacity to inhibit NK cell activity [39, 59]. Meanwhile, we

also found that levels of IL-2, IL-27, MIP-1 α and MIP-1 β are lower in the exposed group. CD56^{bright} NK cells can constitutively express the high-affinity heterotrimeric IL-2 receptor (IL-2R $\alpha\beta\gamma$) and expand *in vitro* and *in vivo* in response to IL-2 [25]. This suggests that IL-2 levels are related to the development and proliferation of CD56^{bright} NK cells. As a member of the IL-12 family of cytokines, IL-27 induces the production of IFN- γ by human NK cells and increases cytotoxicity in mouse NK cells. It also potentiates antibody-dependent cell-mediated cytotoxicity against target cells and stimulates NK-cell effector functions [2, 60]. MIP-1 α and MIP-1 β are involved in the recruitment of NK cells to various tissues and sites of chronic inflammation, and may facilitate or potentiate NK cell cytotoxic activity [58, 61]. In addition, correlation analysis shows that the concentrations of IL-1 β and IL-27 are both related to blood Pb levels ($R_s = 0.162$ and $R_s = -0.306$, respectively). An explanation could be that Pb induced excessive ROS, thus stimulating MPAKs and NF- κ B signalling pathways to alter cytokine production, which has been demonstrated by Liu and Cabral et al. [62, 63]. Taken collectively, Pb may affect the expressions of IL-1 β and IL-27, which are not conducive to the number and function of NK cells (**Figure 3**).

There are still some limitations in this study. Firstly, Guiyu children are exposed to multiple contaminants, including other heavy metals and organic pollutants, but we only investigate the effect of Pb on NK cells because of the insufficient blood samples. However, Pb is the most widespread environmental contaminant and hinders immune response, lymphocyte function and cytokine production reported in extensive research [46, 64, 65]. Secondly, there exist minor differences between the two groups and weak correlations between Pb and relevant immune parameters. Taking into account the duration and levels of exposure, the influence of Pb on these immune parameters may be more notable in the future. Finally, we did not directly measure the number of NK cell populations in peripheral blood. To better interpret the effects of Pb on the activity and function of NK cells, we need to detect more direct indicators, such as NK cell numbers, receptor expressions and NK cell-secreted granzyme/perforin. In addition, the toxicological effects of multiple contaminants on NK cells should be noticed in our further investigation.

Figure 3. Main effects of Pb exposure on immune parameters in this study. Black arrows: higher levels in Guiyu children; Green arrows: lower levels in Guiyu children; Red arrows: the positive correlations between Pb and parameters; Blue arrows: the negative correlations between Pb and parameters.

5. Conclusions

To summarize, we demonstrate higher blood Pb levels and lower percentages of NK cells in Guiyu children. Our findings suggest that the elevated Pb levels result in the lower percentages of NK cells, but also alter the levels of platelets, IL-1 β and IL-27, which might be uncondusive to the activity and function of NK cells. More indicators related to the activity and function of NK cells should be measured to elucidate Pb toxicological effect in the future studies.

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REFERENCES

- [1] Wang, J.P., et al., Impact of electronic wastes recycling on environmental quality. *Biomedical and environmental sciences: BES*, 2006. 19(2): p. 137.
- [2] Ziblat, A., et al., IL-27 stimulates human NK-cell effector functions and primes NK cells for IL-18 responsiveness. *Eur. J. Immunol.*, 2015. 45(1): p. 192-202.
- [3] Leung, A.O., et al., Heavy metals concentrations of surface dust from e-waste recycling and its human health implications in southeast China. *Environ. Sci. Technol.*, 2008. 42(7): p. 2674-2680.
- [4] Xu, L., et al., Polybrominated diphenyl ethers in human placenta associated with neonatal physiological development at a typical e-waste recycling area in China. *Environ. Pollut.*, 2015. 196: p. 414-422.
- [5] Bi, X., et al., Lead in Chinese villager house dust: Geographical variation and influencing factors. *Environ. Pollut.*, 2015. 207: p. 183-189.
- [6] Huo, X., et al., Elevated blood lead levels of children in Guiyu, an electronic waste recycling town in China. *Environ. Health Perspect.*, 2007. 115(7): p. 1113-1117.
- [7] Wu, K., et al., Polybrominated diphenyl ethers in umbilical cord blood and relevant factors in neonates from Guiyu, China. *Environ. Sci. Technol.*, 2010. 44(2): p. 813-819.
- [8] Zheng, L., et al., Blood lead and cadmium levels and relevant factors among children from an e-waste recycling town in China. *Environ. Res.*, 2008. 108(1): p. 15-20.
- [9] Li, Y., et al., Monitoring of lead load and its effect on neonatal behavioral neurological assessment scores in Guiyu, an electronic waste recycling town in China. *J. Environ. Monit.*, 2008. 10(10): p. 1233-1238.
- [10] Guo, Y., et al., Monitoring of lead, cadmium, chromium and nickel in placenta from an e-waste recycling town in China. *Sci. Total Environ.*, 2010. 408(16): p. 3113-3117.
- [11] Xu, X., et al., Birth outcomes related to informal e-waste recycling in Guiyu, China. *Reprod. Toxicol.*, 2012. 33(1): p. 94-98.
- [12] Yang, H., et al., Effects of lead and cadmium exposure from electronic waste on child physical growth. *Environmental Science and Pollution Research*, 2013. 20(7): p. 4441-4447.
- [13] Cao, S., et al., Isotopic ratio based source apportionment of children's blood lead around coking plant area. *Environ. Int.*, 2014. 73: p. 158-166.
- [14] Cao, S., et al., Health risk assessment of various metal (loid) s via multiple exposure pathways on children living near a typical lead-acid battery plant, China. *Environ. Pollut.*, 2015. 200: p. 16-23.
- [15] Zheng, G., et al., Association between lung function in school children and exposure to three transition metals from an e-waste recycling area. *J. Expo. Sci. Environ. Epidemiol.*, 2013. 23(1): p. 67-72.
- [16] Turgeon O'Brien, H., et al., Exposure to toxic metals and persistent organic pollutants in Inuit children attending childcare centers in Nunavik, Canada. *Environ. Sci. Technol.*, 2012. 46(8): p. 4614-4623.
- [17] García-Lestón, J., et al., Assessment of immunotoxicity parameters in individuals occupationally exposed to lead. *J. Toxicol. Environ. Health, A*, 2012. 75(13-15): p. 807-818.
- [18] Kakuschke, A., et al., Immunological impact of metals in harbor seals (*Phoca vitulina*) of the North Sea. *Environ. Sci. Technol.*, 2005. 39(19): p. 7568-7575.
- [19] Kasten-Jolly, J., et al., Impact of developmental lead exposure on splenic factors. *Toxicol. Appl. Pharmacol.*, 2010. 247(2): p. 105-115.
- [20] Laffon, B., et al., Endocrine and immunological parameters in individuals involved in Prestige spill cleanup tasks seven years after the exposure. *Environ. Int.*, 2013. 59: p. 103-111.
- [21] Fortier, M., et al., Effects of physiological concentrations of heavy metals both individually and in mixtures on the viability and function of peripheral blood human leukocytes in vitro. *J. Toxicol. Environ. Health, A*, 2008. 71(19): p. 1327-1337.
- [22] Hayakawa, Y., et al. NKG2D and cytotoxic effector function in tumor immune surveillance. in *Semin. Immunol.* 2006. Elsevier.
- [23] Sata, F., et al., Changes in natural killer cell subpopulations in lead workers. *Int. Arch. Occup. Environ. Health*, 1997. 69(5): p. 306-310.
- [24] Talcott, P.A., et al., The effect of lead and polychlorinated biphenyl exposure on rat natural killer cell cytotoxicity. *Int. J. Immunopharmacol.*, 1985. 7(2): p. 255-261.
- [25] Calderón-Garcidueñas, L., et al., Immunotoxicity and environment: immunodysregulation and systemic inflammation in children. *Toxicol. Pathol.*, 2009. 37(2): p. 161-169.
- [26] De Celis, R., et al., Expression of NK cells activation receptors after occupational exposure to toxics: a preliminary study. *Immunol. Lett.*, 2008. 118(2): p. 125-131.
- [27] Brady, J., et al., The interactions of multiple cytokines control NK cell maturation. *J. Immunol.*, 2010. 185(11): p. 6679-6688.
- [28] Exon, J., et al., Effect of lead, polychlorinated biphenyls, and cyclophosphamide on rat natural killer cells, interleukin 2, and antibody synthesis. *Toxicol. Sci.*, 1985. 5(1): p. 158-164.

- [29] Farag, S.S., et al., Human natural killer cell development and biology. *Blood Rev.*, 2006. 20(3): p. 123-137.
- [30] Fu, B., et al., Subsets of human natural killer cells and their regulatory effects. *Immunology*, 2014. 141(4): p. 483-489.
- [31] McKenna, H.J., et al., Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. *Blood, The Journal of the American Society of Hematology*, 2000. 95(11): p. 3489-3497.
- [32] Fauriat, C., et al., Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood*, 2010. 115(11): p. 2167-2176.
- [33] Loza, M.J., et al., Expression of type 1 (interferon gamma) and type 2 (interleukin-13, interleukin-5) cytokines at distinct stages of natural killer cell differentiation from progenitor cells. *Blood, The Journal of the American Society of Hematology*, 2002. 99(4): p. 1273-1281.
- [34] Marcenaro, E., et al., IL-12 or IL-4 prime human NK cells to mediate functionally divergent interactions with dendritic cells or tumors. *J. Immunol.*, 2005. 174(7): p. 3992-3998.
- [35] Neta, G., et al., Fetal exposure to chlordane and permethrin mixtures in relation to inflammatory cytokines and birth outcomes. *Environ. Sci. Technol.*, 2011. 45(4): p. 1680-1687.
- [36] Fodil, N., et al., Specific dysregulation of IFN γ production by natural killer cells confers susceptibility to viral infection. *PLoS Pathog.*, 2014. 10(12): p. e1004511.
- [37] Lee, S.K., et al., The role of natural killer cells in cancer therapy. *Front Biosci (Elite Ed)*, 2010. 2: p. 380-391.
- [38] Suck, G. Novel approaches using natural killer cells in cancer therapy. in *Semin. Cancer Biol.* 2006. Elsevier.
- [39] Li, N., et al., The effects of early life Pb exposure on the expression of IL1- β , TNF- α and A β in cerebral cortex of mouse pups. *J. Trace Elem. Med Biol.*, 2014. 28(1): p. 100-104.
- [40] Lin, Y.C., et al., Pb²⁺ induced IL-8 gene expression by extracellular signal-regulated kinases and the transcription factor, activator protein 1, in human gastric carcinoma cells. *Environ. Toxicol.*, 2015. 30(3): p. 315-322.
- [41] Liu, M.-C., et al., Involvement of microglia activation in the lead induced long-term potentiation impairment. *PLoS One*, 2012. 7(8): p. e43924.
- [42] Luna, A.L., et al., TLR4 is a target of environmentally relevant concentration of lead. *Toxicol. Lett.*, 2012. 214(3): p. 301-306.
- [43] Farkhondeh, T., et al., The effect of lead exposure on tracheal responsiveness to methacholine and ovalbumin, total and differential white blood cells count, and serum levels of immunoglobulin E, histamine, and cytokines in guinea pigs. *Hum. Exp. Toxicol.*, 2014. 33(3): p. 325-333.
- [44] Xu, X., et al., Decreased blood hepatitis B surface antibody levels linked to e-waste lead exposure in preschool children. *J. Hazard. Mater.*, 2015. 298: p. 122-128.
- [45] Betts, K.S., CDC updates guidelines for children's lead exposure. 2012. National Institute of Environmental Health Sciences.
- [46] Mishra, K.P., et al., Effect of lead exposure on the immune response of some occupationally exposed individuals. *Toxicology*, 2003. 188(2-3): p. 251-259.
- [47] DeSilva, P., Determination of lead in plasma and studies on its relationship to lead in erythrocytes. *Occup. Environ. Med.*, 1981. 38(3): p. 209-217.
- [48] Ronis, M.J., et al., Skeletal effects of developmental lead exposure in rats. *Toxicol. Sci.*, 2001. 62(2): p. 321-329.
- [49] Abbas, S., et al., Developmental exposure to As, Cd, and Pb mixture diminishes skeletal growth and causes osteopenia at maturity via osteoblast and chondrocyte malfunctioning in female rats. *Toxicol. Sci.*, 2013. 134(1): p. 207-220.
- [50] Muzzioli, M., et al., Zinc improves the development of human CD34⁺ cell progenitors towards natural killer cells and induces the expression of GATA-3 transcription factor. *The international journal of biochemistry & cell biology*, 2007. 39(5): p. 955-965.
- [51] Goering, P., Lead-protein interactions as a basis for lead toxicity. *Neurotoxicology*, 1993. 14(2-3): p. 45.
- [52] Hsu, P.-C., et al., Antioxidant nutrients and lead toxicity. *Toxicology*, 2002. 180(1): p. 33-44.
- [53] Jeannot, G., et al., T and B lymphocytes exert distinct effects on the homeostasis of NK cells. *Eur. J. Immunol.*, 2006. 36(10): p. 2725-2734.
- [54] Li, N., Platelet-lymphocyte cross-talk. *J. Leukocyte Biol.*, 2008. 83(5): p. 1069-1078.
- [55] Li, R., et al., Reactive oxygen species alteration of immune cells in local residents at an electronic waste recycling site in northern China. *Environ. Sci. Technol.*, 2013. 47(7): p. 3344-3352.
- [56] Di Lorenzo, L., et al., Evaluation of peripheral blood neutrophil leucocytes in lead-exposed workers. *Int. Arch. Occup. Environ. Health*, 2006. 79(6): p. 491-498.
- [57] Thorén, F.B., et al., Human NK Cells induce neutrophil apoptosis via an NKp46-and Fas-dependent mechanism. *J. Immunol.*, 2012. 188(4): p. 1668-1674.
- [58] Taub, D.D., et al., Alpha and beta chemokines induce NK cell migration and enhance NK-mediated cytotoxicity. *J. Immunol.*, 1995. 155(8): p. 3877-3888.
- [59] Elkabets, M., et al., IL-1 β regulates a novel myeloid-derived suppressor cell subset that impairs NK cell development and function. *Eur. J. Immunol.*, 2010. 40(12): p. 3347-3357.

- [60] Laroni, A., et al., IL-27 imparts immunoregulatory function to human NK cell subsets. *PLoS One*, 2011. 6(10): p. e26173.
- [61] Baschuk, N., et al., NK cell intrinsic regulation of MIP-1 α by granzyme M. *Cell Death Dis.*, 2014. 5(3): p. e1115-e1115.
- [62] Cabral, M., et al., Effects of environmental cadmium and lead exposure on adults neighboring a discharge: Evidences of adverse health effects. *Environ. Pollut.*, 2015. 206: p. 247-255.
- [63] Liu, C.-M., et al., Protective role of quercetin against lead-induced inflammatory response in rat kidney through the ROS-mediated MAPKs and NF- κ B pathway. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 2012. 1820(10): p. 1693-1703.
- [64] Dudka, I., et al., Metabonomic analysis of serum of workers occupationally exposed to arsenic, cadmium and lead for biomarker research: a preliminary study. *Environ. Int.*, 2014. 68: p. 71-81.
- [65] Dyatlov, V.A., et al., Neonatal lead exposure potentiates sickness behavior induced by *Listeria monocytogenes* infection of mice. *Brain, Behav., Immun.*, 2002. 16(4): p. 477-492.

4

EXPOSURE TO LEAD AND CADMIUM AND THEIR EFFECTS ON CD19⁺ B CELL APOPTOSIS IN CHILDREN FROM AN E-WASTE RECYCLING AREA

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ABSTRACT

Dysregulated apoptosis can disturb the survival and function of B cells, affecting the humoral immune response. However, the effects of exposure to heavy metal on B cell apoptosis in humans are not fully investigated. This study explored whether lead (Pb) and cadmium (Cd) exposure are associated with B cell apoptosis in preschool children. A total of 147 preschool children, 3 - 7 years of age, were recruited from Guiyu (an electronic waste recycling area) and a reference group. Individual blood Pb and Cd levels were measured by a graphite furnace atomic absorption spectrometer. Proportions of cells in apoptosis and caspase-9 activation of CD19⁺ B cells were assessed by a flow cytometer. The detection of NF- κ B transcription factor *c-Rel* and *p65* mRNA level in CD19⁺ B cells was performed by real-time polymerase chain reaction (RT-qPCR). Plasma cytokines (IL-4, IFN- γ and IL-6) involved in B cell development were tested by a Luminex 200 analyzer. Guiyu children had increased blood Pb and Cd levels but lower apoptosis in CD19⁺ B cells. The levels of caspase-9 activation and IL-4 were decreased, while *c-Rel* mRNA level and IFN- γ /IL-4 ratio were elevated in Guiyu group compared with the reference group. Notably, we present novel findings on the inverse association between heavy metal exposure and B cell apoptosis in children. Decreased caspase-9 activation and raised *c-Rel* mRNA level link Pb and Cd exposure to inhibited B cell apoptosis. Aberrant IL-4 production and Th1 bias may suggest a disturbance in the development and functioning of B cells.

1. Introduction

Environmental contamination by heavy metal lead (Pb) and cadmium (Cd) resulted from uncontrolled electronic waste (e-waste) recycling activities have aroused increasing attention in recent years [1, 2]. Concentrations of Pb and Cd were found to be considerably greater in the surrounding air, dust, soils, sediments, and plants of the e-waste recycling areas than in other regions [2-4]. Our prior studies reported elevated Pb and Cd levels among pregnant women and preschool children of Guiyu, a typical e-waste area with nearly a 30-year history of unregulated e-waste disposal in southern China [5-7]. Evidence of the association of exposure to Pb and Cd with human health was established. Epidemiological studies have shown that Pb and Cd toxicities are associated with numerous adverse outcomes of humans, such as cancers, neurological disorders, hypertension, cognitive impairments, immunodeficiency and specific organ dysfunctions [8-10]. Mechanism research indicates that Pb and Cd are able to induce oxidative stress and mitochondrial dysfunction, subsequently promoting the process of carcinogenesis or apoptosis [11-13].

B cells, also known as B lymphocytes, originate from multipotent hematopoietic stem cells in the bone marrow throughout life and act as the effector of humoral immunity providing defense against pathogens [14]. Apart from contributing to the immune system by producing antigen-specific antibodies, B cells also function in the processes of antigen presentation, cytokine generation, lymphoid organogenesis, and T cell differentiation [15]. It has been acknowledged that the development and maturation of B cells are dependent upon the participation of multiple cytokines, such as interleukin (IL)-4, IL-6, and interferon (IFN)- γ [16, 17]. During this process of maturation, apoptosis plays an essential role in the development of B cells, which contributes to check their affinity for self-antigens to delete those B cells expressing potentially self-reactive BCRs or failing any maturation steps [18, 19].

Investigations regarding the immunotoxicity of Pb and Cd on B cells have been established. For instance, *in vivo* and *in vitro* studies indicated that Pb may promote the activation and proliferation of B cells, and increase the major histocompatibility complex activity and antibody production [20, 21]. In contrast to Pb, *in vitro* exposure to Cd reduces the activity and percentage of B cells from humans and mice [22, 23]. However, the effects of Pb and Cd exposure on B cells are not fully elucidated in population studies. Our previous study reporting

decreased vaccine antibody titers (diphtheria, pertussis, tetanus, hepatitis B, Japanese encephalitis, polio, measles) in children exposed to Pb, Cd and other heavy metals and metalloids [24], indicates that the antibody-producing capacity of B cells (plasma cells) may have been affected by heavy metal exposure. This study aimed to evaluate the survival and development of B cells in preschool children following Pb and Cd exposure, and explore the potential role of intracellular activated caspase-9, nuclear factor- κ B (NF- κ B) transcription factors (c-Rel and p65) and cytokines (IL-4, IFN- γ and IL-6) in B cell apoptosis induced by Pb and Cd.

2. Materials and Methods

2.1. Study participants

A total of 147 preschool children, 3 - 7 years of age, were recruited from Guiyu (23°32'N, 116°34'E, the e-waste exposed group, n = 80) and Haojiang (23°17'N, 116°43'E, the reference group, n = 67) between December 2013 and January 2014. Guiyu is located in southeast China and is a typical e-waste destination with a more than 30-year e-waste recycling history [25]. Haojiang, around 31.6 km away from Guiyu, has a similar latitude, number of inhabitants, socioeconomic status and cultural background to Guiyu but without any e-waste disposal actions. Questionnaires were delivered to all parents or guardians of participants and their written informed consent was received before the enrollment. A baseline survey of the standardized questionnaire and physiological health parameters was carried out by trained workers, which include general information on demographics, child's age, gender, body mass index (BMI), outdoor time, frequency of washing hands before meals, milk consumption frequency, calcium/iron/zinc supplementation frequency, parents' education level, family member daily cigarette consumption, monthly household income, distance of the road from the residence. Participants enrolled were healthy children without having family medical history, or chronic diseases such as asthma, or acute cough or cold recently. The study protocol was approved and identified by the institutional review boards of the Human Ethics Committee of Shantou University Medical College, China.

2.2. Measurement of blood Pb and Cd exposure

Peripheral venous blood samples from participants were collected in vacuum tubes with EDTA anticoagulant by trained nurses from the First Affiliated Hospital of Shantou University Medical College. Blood samples were immediately transferred to the lab within 2 hours after acquisition.

The measurement of blood Pb and Cd levels were performed by using a graphite furnace atomic absorption spectrometry (GFASS, Jena Zeenit 650, Germany) and has been described in our previous reports [7, 25]. Briefly, for blood Pb detection, the GFASS were set at the condition of a wavelength of 283.3 nm, a lamp current of 4.0 mA, a slit width of 0.8 nm, drying at 90 °C, 105 °C, and 120 °C, ashing at 950 °C, and atomization at 1500 °C. The accuracy of the method was controlled by recoveries between 92.5% and 104.6% from the spiked blood samples. For Cd, the similar procedure was followed with a wavelength of 228.8 nm, a lamp current of 4.0 mA, a slit width of 1.2 nm, drying at 90 °C, 105 °C, and 120 °C, ashing at 300 °C, and atomization at 1300 °C. The accuracy of the detection method was controlled by recoveries between 91.7% and 106.3% from the spiked samples. A relative standard deviation (RSD%) before each run was acquired less than 15% as a warranty for the instrumental accuracy, and the linearity of a five-point calibration curve for Pb and Cd ($r^2 > 0.99$) indicated the GFASS running under good performance.

2.3. Isolation and detachment of CD19⁺ B lymphocytes

B cells in the peripheral blood of healthy participants were positively selected by using a Dynabeads CD19 Pan B kit (ThermoFisher Scientific, USA) according to the manufacturer's instructions or as previously described [26]. Briefly, each fresh blood sample was mixed with Ca²⁺ and Mg²⁺ free PBS supplemented with 0.1% BSA and 2 mM EDTA (Isolation Buffer) at a ratio of 1:2. After centrifugation at 600 × g for 10 min at 4°C, Dynabeads were added and incubated with blood cells by a ratio of 1:40 for 20 min at 4°C, then rosetted cells were captured using a magnet (ThermoFisher Scientific, USA) and washed 4 times with Isolation Buffer. Obtained cell pellet was resuspended in RPMI 1640 medium with 1% FCS. To release B cells from Dynabeads, DETACHaBEAD CD19 (ThermoFisher Scientific, USA) was added to the cell suspension at a ratio of 1:10. After incubation for 45 min at room temperature with gentle mixing, detached cells were thoroughly washed by resuspending the cells in a total volume of

10 mL RPMI 1640 medium with 1% FCS and centrifuged for 6 min at $400 \times g$ to remove beads. The isolated CD19⁺ cells will be applied in the downstream flow cytometry for the tests of CD19⁺ B cell apoptosis and caspase-9 activation proportion.

2.4. Apoptosis and caspase-9 activation in CD19⁺ B cells

Cell apoptosis was measured by using an FITC-conjugated Annexin V/PI apoptosis detection kit (BioVision, USA). In brief, after spinning at $300 \times g$ for 10 min, cell pellet in each tube was resuspended in 500 μ L $1 \times$ Annexin V binding buffer (BioVision, USA) with 5 μ L Annexin V and 5 μ L propidium iodide (PI) Staining Solution. Cell suspension was incubated for 15 min at room temperature in the dark. A total of 10,000 events was quantified to score each run. Flow cytometry data were plotted by flow cytometer (BD Accuri C6, BD biosciences, USA) and analyzed by BD CellQuest Software (BD biosciences, USA).

Apoptosis is known to be executed by a series of sequentially activated caspases, in which caspase-9 is a key initiator in the intrinsic or mitochondrial pathway that responds to harmful cellular stimuli [27, 28]. The CaspGLOW Fluorescein Active Caspase-9 Staining Kit (BioVision, USA) was used to assess the activation of caspase-9 according to the manufacturer's instructions. CD19⁺ B cells in 300 μ L culture medium were incubated with 1 μ L cell membrane-permeable fluorogenic caspase-9 specific substrate (FITC-LEHD-FMK, BioVision, USA), while negative control was treated with the caspase inhibitor Z-VAD-fmk (BioVision, USA). Afterward, cells were incubated with the fluorogenic substrates for 1 h at 37°C incubator with 5 % CO₂, then centrifuged at $855 \times g$ for 5 minutes. Obtained cell pellet was resuspended in 500 μ L washing buffer (BioVision, USA). A total of 10,000 events were collected for each sample. Data were acquired by flow cytometer (BD Accuri C6, BD biosciences, USA) and analyzed by BD CellQuest Software (BD biosciences, USA).

2.5. Detection of NF- κ B transcription factors *c-Rel* and *p65* mRNA level

NF- κ B transcription factors were first identified as a regulator of κ B light chain expression in mature B and plasma cells by Sen and Baltimore [29]. It consists of a family of transcription factors RelA/p65, c-Rel, RelB, p50 and p52 that play critical roles in regulating cell apoptosis, proliferation, differentiation and development (Oeckinghaus and Ghosh 2009). To evaluate whether NF- κ B transcription factors were altered in CD19⁺ B cells of children following Pb

and Cd exposure, *c-Rel* and *p65* transcriptional levels were measured by real-time polymerase chain reaction (qRT-PCR) in this study.

Total RNA was isolated from obtained CD19⁺ B cells using a Dynabeads Oligo(dT)₂₅ kit (Thermofisher Scientific, USA) according to the manufacturer's instructions and previous literature [30, 31]. In brief, cells were lysed for 10 min on ice in a Lysis/Binding Buffer containing 100 mM Tris-HCl, pH 7.5, 500 mM LiCl, 10 mM EDTA, 1% LiDS, 5 mM dithiothreitol (Thermofisher Scientific, USA). A 10 µL prewashed Dynabeads Oligo (dT)₂₅ was added and the mixture was incubated for 15 min at room temperature with continuous shaking. Afterward, the mixture was placed on a magnet to collect beads, washed twice with 10 mM Tris-HCl (pH 7.5), 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS, and twice with a similar washing buffer containing 10 mM Tris-HCl (pH 7.5), 0.15 M LiCl, 1 mM EDTA 10 mM Tris-HCl (pH 7.5) (both from Thermofisher Scientific). The captured mRNA mixture was eluted from the beads in a 10 µL of 10 mM Tris-HCl in 2 min at 75 °C, then was placed on the magnet and the supernatant containing the mRNA was quickly transferred to a new RNase-free tube.

The one-step real-time PCR was conducted using a Quant One Step qRT-PCR Kit (TIANGEN Biotech, China) according to the manufacturer's protocol. Briefly, each 10 ng mRNA sample was used to perform a PCR reaction in a final volume of 20 µL containing buffer 10 µL of 2 × Quant One-Step SYBR qRT-PCR Master Mix (probe), 1 µL of 2.5 U/µL HotMaster Taq Polymerase, 0.16 µL Quant RTase (for one-step), 0.25 µM of each oligonucleotide primer and few RNase-Free ddH₂O. The qRT-PCR reaction was undertaken by an Applied Biosystems™ ABI 7500 real-time thermal cycler (Thermofisher Scientific, USA). The cycling procedure involved reverse transcription at 50 °C for 30 min, denaturation at 95 °C for 2 min, followed by 40 cycles of 94 °C for 20 s, 58 °C for 20 s and 68 °C for 30 s. Relative transcript levels of *c-Rel* and *p65* were normalized to the endogenous reference *β-Actin*. The primers used in this study were presented as below:

<i>c-Rel</i>	forward: 5'-ACGCTCAGGCAATACAAACC-3', reverse: 5'-CGCTTCCATTCCGACTATGT-3';
<i>p65</i>	forward: 5'-GGAGCACAGATACCACCAAGA-3', reverse: 5'-CGGCAGTCCTTTCTCACAAG -3';
<i>β-Actin</i>	forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-GGGCCGGACTCGTCATAC-3';

2.6. Cytokine production

Cytokines, such as IL-4, IFN- γ and IL-6, play a vital role in the process of B cell development, survival and immunoglobulin isotype switching [16, 17]. In this study, we measured plasma levels of these cytokines to determine whether there exists a relationship between B cell apoptosis and cytokine production. Beads coated with anti-human IL-4, IL-6 and IFN- γ were incubated with the plasma samples according to the manufacturer's recommendations. The assay was carried out by a ProcartaPlex Th1/Th2 Cytokine Panel (11 plex) (ThermoFisher Scientific, USA) and a Luminex 200 multiplex immunoassay instrument (Luminex, USA).

2.7. Statistic analysis

All statistical analyses were performed by using R v3.6.3, SPSS 25.0 and GraphPad Prism 5.0 software. Before data analysis, the one-sample Kolmogorov-Smirnov test was used to identify the specific distribution of data. Categorical and continuous variables were compared using the chi-square test and t-test, respectively. Normal distributions were presented as mean $\pm$ standard deviation (SD), and data between the two groups were compared using the independent-sample t-test. Abnormal distributions were presented as the median, and comparisons between two groups were made by the Mann-Whitney U test. For hypothesis testing, a $p < 0.05$ in a two-tailed test was considered statistically significant.

A multiple correspondence analysis (MCA) was performed to explore the simultaneous relationships between variables, including child gender, BMI and age group, heavy metal Pb and Cd, percentages of apoptosis and caspase-9 activation, and relative mRNA levels of *c-Rel* and *p65* in CD19⁺ B cells, cytokine IL-4, IL-6 and IFN- γ as well as the IFN- γ /IL-4 ratio from participants. Except for child age group, data of variables were processed into binary variables according to their medians. Based on the points located approximately in the same direction of the origin and the same quadrant of space by variables, MCA analysis can interpret the simultaneous relationships between them [32].

Multiple linear regression analysis was used to evaluate the association of Pb and Cd exposure with B cell apoptosis. For this analysis, data of CD19⁺ B cell apoptosis percentage were ln-transformed to normalize the distribution, blood Pb and Cd levels were split into binary variables by their medians, and potential confounders were adjusted in 3 different models. The

model 1 represents a crude model without any adjustment; the model 2 was adjusted for child age, gender, head circumference, chest circumference and BMI; the model 3 was adjusted for child age, head circumference, chest circumference, gender, BMI, outdoor time, frequency of washing hands before meals, milk consumption frequency, calcium/iron/zinc supplementation frequency, parents' education level, family member daily cigarette consumption, monthly household income, distance of the road from the residence.

3. Results

3.1. Characteristics from the study population

Participant characteristics are described in **Table 1**. No differences in child age, gender, body mass index (BMI), outdoor time, frequency of calcium/ iron/zinc supplementation, and monthly household income were found in the reference and exposed group. Children from the exposed group were more likely to have a lower frequency of hand-washing before meals, and milk consumption compared with children in the reference group. Moreover, the exposed group had significantly higher family member daily cigarette consumption and distance between residence and road, but significantly lower parents' educational level than those in the reference group ($p < 0.05$).

3.2. Blood Pb and Cd levels in children

Pb and Cd levels in child peripheral blood were examined. As shown in **Figure 1**, the median [interquartile range (IQR)] blood Pb and Cd values in the exposed group was 5.40 (3.81 - 6.97) $\mu\text{g/dL}$ and 0.54 (0.36 - 0.66) $\mu\text{g/dL}$ greater than those in the reference group 4.10 (3.24 - 5.26) $\mu\text{g/dL}$ and 0.43 (0.31 - 0.68) $\mu\text{g/dL}$, respectively (both $p < 0.05$).

Table 1. Descriptive statistics for the study population.

Characteristics	Reference group (n = 67)	Exposed group (n = 80)	<i>p</i>
Child age [n (%), years]	4.2 ± 0.9	4.3 ± 0.9	0.473 ^a
2 ~	9 (13.6)	6 (7.5)	0.479 ^b
3 ~	22 (33.3)	24 (30.0)	
4 ~	17 (25.8)	31 (38.8)	
5 ~	16 (24.2)	17 (21.3)	
6 ~	3 (3.0)	2 (2.5)	
Gender [n (%)]			0.316 ^b
male	32 (48.5)	45 (57.7)	
female	34 (51.5)	33 (42.3)	
Child body mass index (BMI, kg/m ²)	15.4 ± 1.5	15.5 ± 3.0	0.626 ^a
Outdoor time [n (%), hour]			0.139 ^b
≤ 0.5	4 (6.0)	10 (12.5)	
~ 1	15 (22.4)	26 (32.5)	
~ 2	29 (43.3)	23 (28.8)	
~ 3	14 (20.9)	13 (16.2)	
> 3	5 (7.4)	8 (10.0)	
Frequency of hand-washing before meals [n (%)]			0.001 ^b
never	1 (1.5)	5 (6.2)	
sometimes	17 (25.4)	42 (52.5)	
often	37 (55.2)	27 (33.8)	
always	12 (17.9)	6 (7.5)	
Milk consumption frequency [n (%)]			0.021 ^b
never	1 (1.5)	1 (1.2)	
1-3 times per month	12 (17.9)	18 (22.5)	
1-3 times per week	22 (32.8)	42 (52.5)	
everyday	32 (47.8)	19 (23.8)	
Calcium/iron/zinc supplementation frequency [n (%)]			0.262 ^b
never	32 (47.8)	32 (40.0)	
1-3 times per month	21 (31.3)	36 (45.0)	
1-3 times per week	14 (20.9)	12 (15.0)	
Family member daily cigarette consumption [n (%)]			0.026 ^b
non-smoking	29 (43.3)	16 (20.0)	
~ 2 cigarettes	7 (10.4)	17 (21.2)	
~ 10 cigarettes	16 (23.9)	19 (23.8)	
~ 20 cigarettes	12 (17.9)	22 (27.5)	
> 20 cigarettes	3 (4.5)	6 (7.5)	
Paternal educational level [n (%)]			< 0.001 ^b
primary school or lower	2 (3.0)	6 (7.5)	
junior high school	14 (20.9)	49 (62.0)	
technical secondary	11 (16.4)	12 (15.2)	
senior high school	11 (16.4)	5 (6.3)	
college/university	29 (43.3)	8 (10.1)	
Maternal educational level [n (%)]			0.001 ^b
primary school or lower	2 (3.0)	13 (16.2)	
junior high school	17 (25.4)	37 (46.8)	
technical secondary	15 (22.4)	16 (20.3)	
senior high school	12 (17.9)	5 (6.3)	
college/university	21 (31.3)	9 (11.4)	
Monthly household income [n (%), CNY]			0.391 ^b
< 1500	2 (3.0)	5 (6.2)	
~ 3000	12 (17.9)	17 (21.2)	
~ 4500	14 (20.9)	23 (28.8)	
~ 6000	16 (23.9)	13 (16.3)	
> 6000	23 (34.3)	22 (27.5)	
Distance between residence and road [n (%), m]			0.012 ^b
< 10	11 (16.4)	29 (36.7)	
~ 50	11 (16.4)	17 (21.5)	
~ 100	16 (23.9)	17 (21.5)	
> 100	29 (43.3)	16 (20.3)	

^aAnalysis by the independent-sample t-test; ^bAnalysis by the chi-square test; Value of *p* < 0.05 was considered statistically significant.

Figure 1. Blood Pb and Cd levels in study populations. Data are expressed as the median, minimum, maximum, 25th percentile and 75th percentile. Analysis by the Mann-Whitney U test. Reference group: n = 67, exposed group: n = 80; Values of *** $p < 0.001$ and * $p < 0.05$ were considered statistically significant.

3.3. Alterations of apoptosis and caspase-9 activation in CD19⁺ B cells

Proportions of CD19⁺ B cell apoptosis and caspase-9 activated cells were determined as shown in the density plot (**Figure 2**). According to our observation, the exposed group had a lower median apoptosis rate compared with the reference group (3.6% vs. 4.9%, $p = 0.030$). Caspase-9 is the intrinsic initiator that induces apoptosis by harmful cellular stimuli; therefore, its activation probably indicates intrinsic apoptosis happens [27, 28]. We found the median caspase-9 activated level is significantly decreased in the exposed group (5.8%) compared with that in the reference group (9.0%) ($p = 0.011$).

3.4. Relative mRNA levels of *c-Rel* and *p65* in CD19⁺ B cells

The subunits *c-Rel* and *p65* are two essential transcription factors of NF- κ B, which was reported to play critical roles in regulating cell apoptosis and development [33]. In the present study, the relative mRNA levels of *c-Rel* and *p65* in CD19⁺ B cells were quantified compared to β -actin mRNA level and results are shown in **Figure 3**. We found that the exposed group had an elevated median *c-Rel* mRNA level compared with the reference group (fold-change: 2.30 vs. 1.01, $p = 0.001$). No significant difference was found in *p65* mRNA level between the two groups (median fold-change: exposed group 0.55 vs. reference group 0.47, $p = 0.572$).

Figure 2. Proportions of CD19⁺ B cell apoptosis and caspase-9 positive cells in children. (A) Gating strategy to select apoptotic and caspase-9 activated percentage in CD19⁺ B cells. Apoptotic cells were stratified by Annexin V positive and propidium iodide (PI) negative; caspase-9 activated population was gated by caspase-9 positive. (B) Percentages of apoptotic and caspase-9 activated cells in the reference and exposed group. All data are expressed as the median, minimum, maximum, 25th percentile and 75th percentile. Analysis by the Mann-Whitney U test. Reference group: n = 67, exposed group: n = 80; Value of * $p < 0.05$, was considered statistically significant.

3.5. Alterations in B cell development-related cytokines

We further determined the production of several cytokines involved in B cell proliferation, survival and immunoglobulin isotype switching, such as IL-4, IL-6 and IFN- γ [17]. Details are provided in **Figure 4**. Lower median IL-4 level was found in the exposed group (2.49 pg/mL) compared with the reference group (3.16 pg/mL) ($p < 0.05$). The individual IFN- γ /IL-4 ratio was calculated to estimate the balance of T helper 1/T helper 2 (Th1/Th2)-type of responses [34]. According to our results, the median IFN- γ /IL-4 ratio in the exposed group (0.41) is

significantly greater than in the reference group (0.32) ($p = 0.037$). No significant differences in IFN- γ and IL-6 were observed within two groups.

Figure 3. Relative mRNA levels of NF- κ B transcription factor *c-Rel* and *p65* in CD19⁺ B cells. Relative transcript levels of *c-Rel* and *p65* were normalized to the endogenous reference β -*Actin*. All data in fold-change are expressed as the median, minimum, maximum, 25th percentile and 75th percentile. Analysis by the Mann-Whitney U test. Reference group: $n = 67$, exposed group: $n = 80$; Value of ** $p < 0.01$ was considered statistically significant.

Figure 4. Plasma cytokine production in study populations. Results of IL-4, IL-6, IFN- γ and IFN- γ /IL-4 ratio are presented as the median, minimum, maximum, 25th percentile and 75th percentile; analysis by the Mann-Whitney U test. Reference group: $n = 67$, exposed group: $n = 80$; Value of * $p < 0.05$ was considered statistically significant.

3.6. Relationships between heavy metal, apoptosis, caspase-9 activation, NF- κ B transcription factors and cytokines

To explore the complex interrelationships between heavy metal exposure and alterations in apoptosis, caspase-9 activation, NF- κ B transcription factor *c-Rel* and *p65* in CD19⁺ B cells, and

cytokine IL-4, IL-6 and IFN- γ as well as the IFN- γ /IL-4 ratio from participants, a multiple correspondence analysis was performed. As shown in **Figure 5**, the MCA map shows that dimension 1 and dimension 2 explain 52.1% and 33.2% of data variability, respectively. Points located in the same quadrant reflect the close correlation between these variables. For example, low BMI (12.6 - 15.4 kg/m²), high Pb level (4.8 - 16.3 μ g/dL), high Cd level (0.49 - 1.77 μ g/dL), low apoptosis proportion (0.8 - 4.4%), low caspase-9 activation (1.1 - 7.9%), high *c-Rel* mRNA level (1.46 - 9.43 fold-change), low *p65* mRNA level (0.21 - 0.49 fold-change), and low IFN- γ concentration (0.2 - 1.1 pg/mL) were scattered in the upper left quadrant. It prompts that high blood Pb and Cd levels are prone to be found in children with low BMI values, while low levels seem to exist in children with high BMI correspondently (in the lower right quadrant). Pb and Cd exposure is negatively correlated with CD19⁺ B cell apoptosis as well as caspase-9 activation according to the results in the upper left and lower right quadrants. Moreover, the MCA figure manifests high caspase-9 activation is related to high apoptosis of B cells, which also has a close relationship with low levels of *c-Rel* transcription and IFN- γ (in the upper left quadrant). It's worth noting that child age and gender might not disturb or be implicated in these interrelationships.

Furthermore, to evaluate the association between heavy metal exposure and B cell apoptosis, we performed a multiple linear regression analysis. Data of blood Pb and Cd levels were split into binary variables as abovementioned. Based on our results (**Table 2**), all 3 models show a descent for CD19⁺ B cell apoptosis percentage in high Pb group (4.8 - 16.3 μ g/dL) as compared with low Pb group (1.5 - 4.8 μ g/dL) when stratifying by median [β (95% confidence intervals or CIs in model 1, 2 and 3 are -0.376 (-0.701, -0.051), -0.326 (-0.643, -0.010) and -0.380 (-0.716, -0.045), respectively, all $p < 0.05$]. In model 1, CD19⁺ cell apoptosis had a decrease [β (95% CI): -0.272 (-0.597, -0.053), $p < 0.05$] in high Cd group (0.49 - 1.77 μ g/dL) in comparison with that in low Cd group (0.18 - 0.49 μ g/dL). Nevertheless, after adjustment, the decreased trends remained but no statistical significance in the model 2 and 3 for the association between Cd and cell apoptosis.

Figure 5. Multiple correspondence analysis (MCA) of relevant parameters in children. Parameters include child gender (female and male), body mass index (BMI, low: 12.6 - 15.4 kg/m², high: 15.4 - 19.8 kg/m²) and age group (y: years; 2 y ~, 3 ~ 4 y, 4 ~ 5 y, 5 y ~), heavy metal Pb (low: 1.5 - 4.8 µg/dL, high: 4.8 - 16.3 µg/dL) and Cd (low: 0.18 - 0.49 µg/dL, high: 0.49 - 1.77 µg/dL), apoptosis proportion (low: 0.8 - 4.4%, high: 4.4 - 27.0%) and caspase-9 activation (low: 1.1 - 7.9%, high: 7.9 - 42.0%), relative mRNA levels of *c-Rel* (presented as cRel in this MCA figure, low: 0.15 - 1.46 fold-change, high: 1.46 - 9.43 fold-change) and *p65* (low: 0.21 - 0.49 fold-change, high: 0.49 - 16.5 fold-change), and cytokine IL-4 (low: 1.51 - 2.79 pg/mL, high: 2.79 - 8.64 pg/mL), IFN-γ (low: 0.2 - 1.1 pg/mL, high: 1.1 - 4.4 pg/mL) and IL-6 (low: 0.4 - 6.9 pg/mL, high: 6.9 - 22.6 pg/mL), IFN-γ/IL-4 ratio (low: 0.06 - 0.36 pg/mL, high: 0.36 - 1.67 pg/mL), were processed into binary variables according to their medians.

Table 2. Estimates and 95% CIs for the association between blood Pb and Cd level and CD19⁺ B cell apoptosis percentage.

	CD19 ⁺ B cell apoptosis percentage		
	<i>Model 1</i>	<i>Model 2</i>	<i>Model 3</i>
Blood Pb level			
1.5 - 4.8 µg/dL	Reference	Reference	Reference
4.8 - 16.3 µg/dL	-0.376 (-0.701, -0.051)*	-0.326 (-0.643, -0.010)*	-0.380 (-0.716, -0.045)*
Blood Cd level			
0.18 - 0.49 µg/dL	Reference	Reference	Reference
0.49 - 1.77 µg/dL	-0.272 (-0.597, -0.053)*	-0.223 (-0.539, 0.092)	-0.127 (-0.458, 0.204)

Note: Values in this table, including heavy metal Pb and Cd, and CD19⁺ B cell apoptosis percentage, were ln-transformed to normalize the distribution. Data are presented as estimates (95% confidence intervals, CI). Data of CD19⁺ B cell apoptosis percentage were ln-transformed to normalize the distribution, blood Pb (low: 1.5 - 4.8 µg/dL, high: 4.8 - 16.3 µg/dL) and Cd (low: 0.18 - 0.49 µg/dL, high: 0.49 - 1.77 µg/dL) levels were categorized into binary variables according to their medians. The estimates (β value) were calculated by multiple linear regression models. Model 1 represents a crude model without any adjustment; model 2 was adjusted for child age, gender, head circumference, chest circumference and BMI; model 3 was adjusted for child age, head circumference, chest circumference, gender, BMI, outdoor time, frequency of washing hands before meals, milk consumption frequency, calcium/iron/zinc supplementation frequency, parents' education level, family member daily cigarette consumption, monthly household income, distance of the road from the residence. Value of * $p < 0.05$ was considered statistically significant.

4. Discussion

This study was carried out focusing on the spot of Pb and Cd exposure and humoral immunity in children. Children from the e-waste recycling area had higher exposure to heavy metal Pb and Cd, with a lower proportion of apoptosis and caspase-9 activation but up-regulated *c-Rel* mRNA level in CD19⁺ B cells compared with the reference area. Using an MCA analysis, the complex interrelationships between relevant parameters, such as child gender, BMI and age group, Pb and Cd exposure, alterations in apoptosis, caspase-9 activation, and NF- κ B transcription factor *c-Rel* and *p65* in CD19⁺ B cells, plasma cytokine IL-4, IL-6 and IFN- γ as well as the IFN- γ /IL-4 ratio, were revealed. We also discovered inverse associations of blood Pb and Cd level with CD19⁺ B cell apoptosis, which suggests that exposure to Pb and Cd might be detrimental to B cell survival and development.

Our prior work has consistently shown elevated blood Pb and Cd levels in children from the e-waste recycling area [5, 35, 36]. E-waste recycling activities, such as incineration and acidic

leaching, are a major source of the emission of Pb and Cd exposure for vulnerable populations, especially children and pregnant women [5, 36]. Based on MCA analysis, we observed an inverse association between BMI values and blood Pb and Cd levels in children in our study. Earlier literature has previously reported similar findings [37, 38], which suggests Pb and Cd exposure may limit or delay child growth and development. Although many other heavy metals and organic pollutants resulting from e-waste recycling activities have been previously reported [24, 39], blood samples were not available for assessing multiple pollutants simultaneously due to the large measurement of markers in the aspect of B cells in the present study. Because of their high degree of toxicity and since they are ranked among the priority metals with a public health concern [40, 41], Pb and Cd could be a kind of representative pollutant to study environmental pollution and human health risk assessment.

B cells are at the center of the humoral immunity component of the adaptive immune system [14]. To the best of our knowledge, this study is the first to observe a decreased apoptosis in CD19⁺ B cells of Guiyu children. B cell functioning is dependent on its appropriate development and proliferation, in which apoptosis is a counterbalance to cell proliferation and is a crucial process to select cell populations undergoing functional demise and to eliminate cells that are no longer required or potentially autoreactive [18, 42]. It has been demonstrated that decreased apoptosis in B cells may induce the disruption of immune homeostasis, promoting the incidence of autoimmunity or lymphoma [18]. Even though we hardly drew a conclusion on what adverse health consequences will emerge in the future, the fact of aberrant B cell apoptosis in Guiyu children should be cause for concern.

The decreased caspase-9 activation in CD19⁺ B cells found in this study suggests that the caspase-9 activity of B cells in Guiyu children might be suppressed. Evidence from genetic knockout studies showed that abnormal suppression of caspase-9 activity is not only associated with developmental aberrance and proliferative diseases but also considered as a tumor escape mechanism of apoptosis [27, 43, 44]. As a key initiator, caspase-9 is required for intrinsic apoptosis to obliterate irreparable cells and maintain cell homeostasis [27, 28]. Our finding shows an association between the proportion of apoptotic CD19⁺ B cells and caspase-9 activation, which provides a piece of evidence to support the mechanism of apoptosis attributed to caspase-9 activation. Besides the intrinsic pathway, apoptosis is also known to be initiated by an extrinsic pathway mediated by the death receptors, including Fas receptors, tumor

necrosis factor (TNF) receptors, and TNF-related apoptosis-inducing ligand (TRAIL) receptors [45]. It is a limitation of this study that the evidence in terms of extrinsic apoptosis remains unexplained since the insufficient blood amounts of participants we obtained have fallen short of the assessment of experiments. Further studies are warranted to explain the alterations in the aspects of extrinsic apoptosis.

The results of the present study also suggest the potential role of c-Rel in B cell apoptosis. In mammals, c-Rel and p65 serve as essential transcription factors in the NF- κ B family, which function in regulating B-cell development and apoptosis [46, 47]. Prior studies in genetic knockout mice demonstrated that mitogen-activated *c-Rel*^{-/-} B cells failed to proliferate, by contrast, they underwent enhanced apoptosis compared with normal B cells [48]. Another former model stimulated with mitogen revealed that *p65*^{-/-} B cell proliferation was impaired, whereas apoptosis did not change significantly [49]. Such evidence offers a plausibility that the existence of c-Rel is more critical than p65 for B cells to inhibit apoptosis induced by particular mitogenic signals [50]. Our observation shows an increased *c-Rel* mRNA level coinciding with a decrease of B cell apoptosis in Guiyu group, which is consistent with the explanation above. Meanwhile, the MCA map signifies affirmatively that low *c-Rel* mRNA level is associated with high B cell apoptosis (in the lower right quadrant of **Figure 5**), in other words, high *c-Rel* mRNA level is associated with low B cell apoptosis (in the lower right quadrant of **Figure 5**). Additionally, the association of *c-Rel* mRNA level with blood Pb and Cd value was found, possibly providing a link of B cell apoptosis with heavy metal exposure.

The development, proliferation, isotype switching and immunoregulation ability of B cells are heavily dependent on cytokine production in the microenvironment [17, 51]. The plasma IL-4 level presently observed is lower, and concomitantly, the IFN- γ /IL-4 ratio was increased in Guiyu group, indicating a bias toward Th1 immune responses. IL-4, mainly produced by activated Th2 cells, was initially described as a B cell growth factor attributing stimulating B cell proliferation, survival and the preferential secretion of certain immunoglobulin isotypes, such as IgG1 and IgE [17, 52]. The Th1 response was reported to be responsible for IFN- γ primary source and has generally 5- to 10-fold less efficient than Th2 in inducing B cell proliferation [17, 53]. IFN- γ /IL-4 ratio reflects the balance of Th1/Th2, which is also known to be related to B cell proliferation and isotype switching [54]. Based on the observation in the MCA map, we found a negative interaction between IFN- γ and B cell apoptosis, which suggests

that IFN- γ or Th1 bias is likely to decrease B cell proliferation relative to Th2 or IL-4. Accordingly, alterations in IL-4 and IFN- γ /IL-4 ratio indicate that there is an unfavorable microenvironment to support the development and immunoglobulin isotype switching of B cells in Guiyu children.

It is worth noting that our findings are varied from previous studies. In the present study, our results show that exposure to Pb and Cd is inversely associated with B cell apoptosis level, suggesting that exposure to Pb and Cd might contribute to the inhibition of apoptosis in CD19⁺ B cells. However, in vitro and animal studies have shown that Pb and/or Cd stimulation can promote cell apoptosis [20, 55-57]. The inconsistency may be related to the difference in exposure dosages and duration; on the other hand, our study populations were exposed to the superimposition of two or more pollutants, thus our findings may differ from in vitro and animal studies. We also explored the impact of individuals' characteristics and social-behavioral factors on the association between heavy metal exposure and B cell apoptosis by multiple linear regression models (**Table 2**). For example, a negative association between high Cd exposure and B cell apoptosis was found in model 1 without adjustment, while neither model 2 nor model 3 shows a significant estimate, which suggests that not only individuals' characteristics (child age, gender, head circumference, chest circumference and BMI) but also social-behavioral factors (outdoor time, frequency of washing hands before meals and milk consumption, parents' education level, family member daily cigarette consumption, etc.) may pose an implication in their relationship. Our previous studies have reported that social-behavioral factors, such as parents' education level, milk consumption and frequency of washing hands before meals, were negatively associated with Pb and Cd in child blood due to their favorable role in the prevention of and protection from heavy metal exposure [58, 59]. Meanwhile, as aforementioned, child BMI was found to be inversely associated with blood Pb and Cd levels [37, 38]. Therefore, adjusting for these possible confounders is likely to remove bias and show a relatively credible relationship between heavy metal exposure and B cell apoptosis.

In conclusion, children living in the e-waste recycling area suffered from relatively high exposure to Pb and Cd. The association between CD19⁺ B cell apoptosis and Pb and Cd exposure provides significant evidence suggesting the role of Pb and Cd exposure in suppressing CD19⁺ B cell apoptosis in a population study. Our preliminary investigations into the mechanisms showed that caspase-9 activation and NF- κ B transcription factor *c-Rel* mRNA

level link the suppression of B cell apoptosis to Pb and Cd exposure. Decreased IL-4 production and Th1 bias probably point to the existence of adverse disturbance in the development and functioning of either B cells or T cells, which however needs further health surveillance and assessment. Based on our results, we are concerned about the consequences of the inhibition of B cell apoptosis in Guiyu children in the future.

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REFERENCES

- [1] Xu, X., et al., E-waste environmental contamination and harm to public health in China. *Front. Med.*, 2015. 9(2): p. 220-228.
- [2] Leung, A.O., et al., Heavy metals concentrations of surface dust from e-waste recycling and its human health implications in southeast China. *Environ. Sci. Technol.*, 2008. 42(7): p. 2674-2680.
- [3] Huo, X., et al., Elevated blood lead levels of children in Guiyu, an electronic waste recycling town in China. *Environ. Health Perspect.*, 2007. 115(7): p. 1113-1117.
- [4] Song, Q., et al., Environmental effects of heavy metals derived from the e-waste recycling activities in China: a systematic review. *Waste Manage.*, 2014. 34(12): p. 2587-2594.
- [5] Guo, Y., et al., Monitoring of lead, cadmium, chromium and nickel in placenta from an e-waste recycling town in China. *Sci. Total Environ.*, 2010. 408(16): p. 3113-3117.
- [6] Kim, S.S., et al., Birth outcomes associated with maternal exposure to metals from informal electronic waste recycling in Guiyu, China. *Environ. Int.*, 2020. 137: p. 105580.
- [7] Xu, L., et al., Hearing loss risk and DNA methylation signatures in preschool children following lead and cadmium exposure from an electronic waste recycling area. *Chemosphere*, 2020. 246: p. 125829.
- [8] Patrick, L., Lead toxicity part II: the role of free radical damage and the use of antioxidants in the pathology and treatment of lead toxicity. *Altern. Med. Rev.*, 2006. 11(2).
- [9] Pimentel, D., et al., Ecology of increasing diseases: population growth and environmental degradation. *Hum. Ecol.*, 2007. 35(6): p. 653-668.
- [10] Wu, X., et al., A review of toxicity and mechanisms of individual and mixtures of heavy metals in the environment. *Environmental Science and Pollution Research*, 2016. 23(9): p. 8244-8259.
- [11] Tchounwou, P.B., et al., Cytotoxicity and transcriptional activation of stress genes in human liver carcinoma cells (HepG2) exposed to cadmium chloride. *Mol. Cell. Biochem.*, 2001. 222(1-2): p. 21-28.
- [12] Valko, M., et al., Redox- and non-redox-metal-induced formation of free radicals and their role in human disease. *Arch Toxicol*, 2016. 90(1): p. 1-37.
- [13] Yedjou, C.G., et al., N-acetyl-L-cysteine affords protection against lead-induced cytotoxicity and oxidative stress in human liver carcinoma (HepG2) cells. *Int. J. Env. Res. Public Health*, 2007. 4(2): p. 132-137.
- [14] Tsai, D.-Y., et al., Regulatory mechanisms of B cell responses and the implication in B cell-related diseases. *J. Biomed. Sci.*, 2019. 26(1): p. 64.
- [15] Yoshizaki, A., et al., Abnormal B lymphocyte activation and function in systemic sclerosis. *Annals of dermatology*, 2015. 27(1): p. 1-9.
- [16] Takatsu, K., Cytokines involved in B-cell differentiation and their sites of action. *Proc. Soc. Exp. Biol. Med.*, 1997. 215(2): p. 121-133.
- [17] Vazquez, M.I., et al., B cells responses and cytokine production are regulated by their immune microenvironment. *Cytokine*, 2015. 74(2): p. 318-326.
- [18] Rathmell, J.C., et al., Pathways of apoptosis in lymphocyte development, homeostasis, and disease. *Cell*, 2002. 109(2): p. S97-S107.
- [19] Tobón, G.J., et al., B lymphocytes: development, tolerance, and their role in autoimmunity—focus on systemic lupus erythematosus. *Autoimmune Dis.*, 2013. 2013.
- [20] Kasten-Jolly, J., et al., Impact of developmental lead exposure on splenic factors. *Toxicol. Appl. Pharmacol.*, 2010. 247(2): p. 105-115.
- [21] Razani-Boroujerdi, S., et al., Lead stimulates lymphocyte proliferation through enhanced T cell-B cell interaction. *J. Pharmacol. Exp. Ther.*, 1999. 288(2): p. 714-719.
- [22] Jelovcan, S., et al., Effects of low concentrations of cadmium on immunoglobulin E production by human B lymphocytes in vitro. *Toxicology*, 2003. 188(1): p. 35-48.
- [23] Lafuente, A., et al., Differential effects of cadmium on blood lymphocyte subsets. *BioMetals*, 2004. 17(4): p. 451-456.
- [24] Lin, X., et al., Decreased vaccine antibody titers following exposure to multiple metals and metalloids in e-waste-exposed preschool children. *Environ. Pollut.*, 2017. 220: p. 354-363.
- [25] Zeng, X., et al., Decreased lung function with mediation of blood parameters linked to e-waste lead and cadmium exposure in preschool children. *Environ. Pollut.*, 2017. 230: p. 838-848.
- [26] Rasmussen, A.-M., et al., A new method for detachment of Dynabeads from positively selected B lymphocytes. *J. Immunol. Methods*, 1992. 146(2): p. 195-202.
- [27] Li, P., et al., Caspase-9: structure, mechanisms and clinical application. *Oncotarget*, 2017. 8(14): p. 23996.
- [28] Li, Y., et al., Mechanistic insights into caspase-9 activation by the structure of the apoptosome holoenzyme. *Proceedings of the National Academy of Sciences*, 2017. 114(7): p. 1542-1547.
- [29] Sen, R., et al., Inducibility of κ immunoglobulin enhancer-binding protein NF- κ B by a posttranslational mechanism. *Cell*, 1986. 47(6): p. 921-928.
- [30] Ferone, D., et al., In vitro characterization of somatostatin receptors in the human thymus and effects of somatostatin and octreotide on cultured thymic epithelial cells. *Endocrinology*, 1999. 140(1): p. 1-9.

- p. 373-380.
- [31] Mann, M.R., et al., Disruption of imprinted gene methylation and expression in cloned preimplantation stage mouse embryos. *Biol. Reprod.*, 2003. 69(3): p. 902-914.
 - [32] LEANDRO-MERHI, V.A., et al., Relationship between nutritional status and the clinical outcomes of patients with and without neoplasms according to multiple correspondence analysis. *Arq. Gastroenterol.*, 2017. 54(2): p. 148-155.
 - [33] Oeckinghaus, A., et al., The NF- κ B family of transcription factors and its regulation. *Cold Spring Harb. Perspect. Biol.*, 2009. 1(4): p. a000034.
 - [34] Gabrielson, S., et al., Influence of atopic heredity on IL-4, IL-12 and IFN- γ -producing cells in in vitro activated cord blood mononuclear cells. *Clin. Exp. Immunol.*, 2001. 126(3): p. 390-396.
 - [35] Yang, H., et al., Effects of lead and cadmium exposure from electronic waste on child physical growth. *Environmental Science and Pollution Research*, 2013. 20(7): p. 4441-4447.
 - [36] Zhang, Y., et al., Alteration of the number and percentage of innate immune cells in preschool children from an e-waste recycling area. *Ecotoxicol. Environ. Saf.*, 2017. 145: p. 615-622.
 - [37] Wang, Q., et al., Cadmium affects blood pressure and negatively interacts with obesity: findings from NHANES 1999–2014. *Sci. Total Environ.*, 2018. 643: p. 270-276.
 - [38] Zeng, X., et al., Heavy metal exposure has adverse effects on the growth and development of preschool children. *Environ. Geochem. Health*, 2019. 41(1): p. 309-321.
 - [39] Xu, L., et al., Polybrominated diphenyl ethers in human placenta associated with neonatal physiological development at a typical e-waste recycling area in China. *Environ. Pollut.*, 2015. 196: p. 414-422.
 - [40] Anyanwu, B.O., et al., Heavy metal mixture exposure and effects in developing nations: an update. *Toxics*, 2018. 6(4): p. 65.
 - [41] Jan, A.T., et al., Heavy metals and human health: mechanistic insight into toxicity and counter defense system of antioxidants. *Int. J. Mol. Sci.*, 2015. 16(12): p. 29592-29630.
 - [42] Lopez-Hoyos, M., et al., Regulation of B cell apoptosis by Bcl-2 and Bcl-XL and its role in the development of autoimmune diseases. *Int. J. Mol. Med.*, 1998. 1(2): p. 475-558.
 - [43] Hakem, R., et al., Differential requirement for caspase 9 in apoptotic pathways in vivo. *Cell*, 1998. 94(3): p. 339-352.
 - [44] Kuida, K., et al., Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. *Cell*, 1998. 94(3): p. 325-337.
 - [45] Elmore, S., Apoptosis: a review of programmed cell death. *Toxicol. Pathol.*, 2007. 35(4): p. 495-516.
 - [46] Grumont, R.J., et al., The subunit composition of NF- κ B complexes changes during B-cell development. *Cell growth & differentiation: the molecular biology journal of the American Association for Cancer Research*, 1994. 5(12): p. 1321.
 - [47] Hayden, M.S., et al., NF- κ B and the immune response. *Oncogene*, 2006. 25(51): p. 6758-6780.
 - [48] Grumont, R.J., et al., B lymphocytes differentially use the Rel and nuclear factor κ B1 (NF- κ B1) transcription factors to regulate cell cycle progression and apoptosis in quiescent and mitogen-activated cells. *J. Exp. Med.*, 1998. 187(5): p. 663-674.
 - [49] Doi, T.S., et al., NF- κ B RelA-deficient lymphocytes: normal development of T cells and B cells, impaired production of IgA and IgG1 and reduced proliferative responses. *The Journal of experimental medicine*, 1997. 185(5): p. 953-962.
 - [50] Gasparini, C., et al., NF- κ B pathways in hematological malignancies. *Cell. Mol. Life Sci.*, 2014. 71(11): p. 2083-2102.
 - [51] Rowland, S.L., et al., Antigen and cytokine receptor signals guide the development of the naive mature B cell repertoire. *Immunol. Res.*, 2013. 55(1-3): p. 231-240.
 - [52] Rush, J.S., et al., B cells activated via CD40 and IL-4 undergo a division burst but require continued stimulation to maintain division, survival and differentiation. *Eur. J. Immunol.*, 2001. 31(4): p. 1150-1159.
 - [53] Rothermel, A.L., et al., Differential abilities of Th1 and Th2 to induce polyclonal B cell proliferation. *Cell. Immunol.*, 1991. 135(1): p. 1-15.
 - [54] Smith, K.M., et al., Th1 and Th2 CD4+ T cells provide help for B cell clonal expansion and antibody synthesis in a similar manner in vivo. *J. Immunol.*, 2000. 165(6): p. 3136-3144.
 - [55] He, L., et al., Bcl-xL overexpression blocks bax-mediated mitochondrial contact site formation and apoptosis in rod photoreceptors of lead-exposed mice. *Proceedings of the National Academy of Sciences*, 2003. 100(3): p. 1022-1027.
 - [56] Skipper, A., et al., Cadmium chloride induces DNA damage and apoptosis of human liver carcinoma cells via oxidative stress. *Int. J. Env. Res. Public Health*, 2016. 13(1): p. 88.
 - [57] Zhou, F., et al., Toxicity assessment due to prenatal and lactational exposure to lead, cadmium and mercury mixtures. *Environ. Int.*, 2019. 133: p. 105192.
 - [58] Zhang, Y., et al., Exposure to multiple heavy metals associate with aberrant immune homeostasis and inflammatory activation in preschool children. *Chemosphere*, 2020: p. 127257.
 - [59] Zheng, L., et al., Blood lead and cadmium levels and relevant factors among children from an e-waste recycling town in China. *Environ. Res.*, 2008. 108(1): p. 15-20.

**EXPOSURE TO MULTIPLE HEAVY METALS
ASSOCIATES WITH
ABERRANT IMMUNE HOMEOSTASIS
AND INFLAMMATORY ACTIVATION
IN PRESCHOOL CHILDREN**

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Abstract

Heavy metals generate adverse health effects by interfering with immune homeostasis and promoting inflammation in individuals. Our objective was to explore the induction of immune and inflammatory responses by multiple heavy metals in children living in the e-waste contaminated area. A total of 147 preschool children were recruited, including 73 children from Guiyu, a typical e-waste recycling area, and 74 from a reference group. Blood levels of heavy metals, including lead (Pb), cadmium (Cd), mercury (Hg) and arsenic (As), were detected using inductively coupled plasma mass spectrometry (ICP-MS). Immune cell counts (neutrophils, monocytes, lymphocytes) were determined by an automatic blood cell analyzer, pro-inflammatory cytokines (IL-1 β , IL-6, IL-8, TNF- α) and anti-inflammatory cytokines (IL-1RA, IL-4, IL-10, IL-13) were analyzed by a Luminex 200 multiplex immunoassay instrument. Multiple correspondences and linear regression analyses were applied to investigate the relationships between heavy metal exposure and relevant parameters. Results showed that Guiyu children had higher levels of Pb, Cd, Hg, As, IL-1 β and IL-6, but decreased lymphocyte, IL-1RA and IL-13. Neutrophil count was positively correlated with Pb, Cd and Hg exposure. Anti-inflammatory IL-1RA concentration was negatively related with Pb, Cd, Hg and As, while pro-inflammatory IL-1 β and IL-6 were positively correlated with Pb. Guiyu children may have dysregulated immune response and high inflammation risk. Exposure to Pb, Cd, Hg and As could be harmful to immune response and inflammatory regulation. Our finding of decreased IL-RA production in children exposed to Pb, Cd, Hg, and As is novel and could be an opportunity for future research.

1. Introduction

Electronic waste (e-waste) is defined as those discarded electrical and electronic equipment and components, which has become a rapidly increasing global problem [1, 2]. E-waste recycling, especially informal and unprotected recycling processes such as acid dissolution, incineration, pyrolysis and gasification, allows the emission of dangerous chemicals, including heavy metals and persistent organic pollutants [1]. Generally, residents living in close vicinity of an e-waste recycling area are also at risk of environmental exposure due to the contamination in the environment, food and water [1]. As one of the largest e-waste destinations and recycling areas in China and worldwide, Guiyu has a 30-year history of e-waste disposal and recycling [3, 4]. Elevated Pb and Cd levels were found in the placenta, umbilical cord blood, peripheral blood and urine of the Guiyu population [2, 5, 6]. Recently, our studies demonstrated that exposure to Pb may contribute to cardiovascular endothelial inflammation and restricted physical growth and development, and cause adverse changes in blood morphology, hemoglobin synthesis and erythrocyte complement receptor 1 expression in Guiyu children [6-9].

Growing evidence indicates that heavy metals exert toxicity to individuals by interfering with immune homeostasis and promoting inflammation [10-12]. Heavy metals, such as lead (Pb), cadmium (Cd), mercury (Hg) and arsenic (As), have been sufficiently underscored by prior mechanistic studies. They can cause oxidative stress by inducing the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [13], and promote DNA damage and cell necrosis [14-16]. Due to the loss of membrane integrity, necrotic cells release intracellular inflammatory molecules to the extracellular space and consequentially provoke inflammation activation [13, 17]. Epidemiological studies and animal models have indicated that chronic inflammatory diseases, such as type 2 diabetes, gout, recurrent pericarditis, heart failure, rheumatoid arthritis and smoldering myeloma, are strictly related to high exposure to environmental contaminants [18-22].

Inflammation is a part of the complex immune response in the body initiated by injurious stimuli, such as damaged cells, pathogens and stimulants. Evidence suggests that inflammation is strictly linked to the genesis of diseases in humans [23]. As a protective response, the function of inflammation is to eliminate injury-inducing agents, clear out necrotic cells, tissues damaged from the original insult, and initiate repair processes and restore the physiological function of

the tissue or organ affected [24, 25]. However, prolonged inflammation may contribute to a wide range of chronic diseases, including cancer, diabetes, and cardiovascular, neurodegenerative and autoimmune diseases [26-31]. Inflammation involving the innate and adaptive immune systems requires proper coordination of blood vessels, immune cells and molecular mediators in the development and maintenance of inflammation. In the acute inflammatory phase, neutrophils respond to various harmful stimuli and tissue damage. Studies have confirmed that monocytes/macrophages and lymphocytes (T, B and NK cells) constitute the predominant immune cell infiltrates in chronic inflammation [32-35]. As the largest corpuscle of leukocytes, monocytes in the bloodstream migrate to tissue and differentiate into macrophages and dendritic cells, both of which are capable of phagocytosis, antigen presentation and cytokine production involved in the ongoing inflammatory process and immune response [36-38]. In addition, numerous studies have demonstrated that critical cytokines, including pro-inflammatory or anti-inflammatory interleukin (IL)-1 β , IL-6, IL-8, tumor necrosis factor (TNF)- α and anti-inflammatory cytokines IL-1 receptor antagonist (IL-1 RA), IL-4, IL-10 and IL-13, play pivotal roles in mediating inflammatory responses as well as in regulating immune homeostasis [39, 40]. Consequently, inflammation-related immune cells and cytokines circulating in the bloodstream can be used to monitor inflammation in the body.

Concern about the toxicity of heavy metal exposure from e-waste is increasing. In our previous studies, parameters involved in immune and inflammatory regulation, such as aberrant alteration of cytokines, increased memory T cell, low percentages of natural killer (NK) cells and decreased concentrations of hepatitis B vaccine titers, have been reported to be associated with exposure to trace levels of environmental chemicals [41-43]. Even so, unequivocal evidence of immune and inflammation activation in children suffering from early-childhood exposure to heavy metals remains little. Hence, we carried out this study to explore and reveal the alterations in immune and inflammatory response resulting from heavy metal exposure.

2. Materials and Methods

2.1. Recruitment and questionnaire survey of the study population

This analysis used partial data from our earlier study [42]. A total of 147 preschool children, 3- to 7-years of age, were recruited from Guiyu (23°32'N, 116°34'E, the e-waste-exposed area,

n = 73) and Haojiang (23°17'N, 116°43'E, the reference area, n = 74) in December, 2014. Haojiang is about 31.6 km to the east of Guiyu, without any e-waste pollution. The two regions both belong to Shantou, Guangdong province, and has similar characteristics in latitude, demographic characteristics, life-styles, eating habits, traffic conditions, cultural background and socioeconomic status, as described in our previous study [44]. All enrolled participants from both Guiyu and Haojiang in this study were somatically healthy and free from medications, inflammation or disease history within 1 month before sampling. All parents or guardians of participants provided written informed consent prior to enrollment. The questionnaire collected detailed information on general characteristics of parents and children: child age, gender and BMI, distance of the road from the residence, milk consumption frequency, parents' education level, daily smoking by family members, parents' residence time, frequency of washing hands before meals, child born locally (Guiyu or Haojiang), child e-waste contact, ventilation of house, e-waste pollution within 50 meters (m) of residence, maternal occupation associated with e-waste dismantling, residence used as a workshop. The study protocol was approved by the Human Ethics Committee of Shantou University Medical College, China.

2.2. Collection and processing of blood samples

Venous blood samples were obtained from participants and collected in vacuum tubes by trained nurses from the First Affiliated Hospital of Shantou University Medical College. Blood samples in EDTA-containing vacuum tubes were used for the detection of heavy metals and inflammation-related cells. Blood without anticoagulants was centrifuged for 10 min at $855 \times g$ at room temperature, and the isolated serum supernatant was stored at -80°C until assayed for inflammatory cytokines.

2.3. Determination of heavy metals

The detection methods of heavy metals (Cd, Hg, Pb and As) were according to Lu et al. and Wu et al. [45, 46]. Metal concentrations were detected using inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7700x; Agilent, USA) in the Shenzhen Center for Disease Control and Prevention. A standard solution of heavy metals was obtained from the National Institute of Meteorology of China (NIM, Beijing, China).

2.4. Analysis of immune cells

Venous blood samples collected in EDTA-containing vacuum tubes were immediately sent to the First Affiliated Hospital of Shantou University Medical College after drawing from

participants. Counts of neutrophils, monocytes and lymphocytes were determined by an automatic blood cell analyzer (XE-2100, Sysmex, Japan).

2.5. Measurement of immune and inflammation regulation-related cytokines

In our previous study, we described multiple immunoassays of 34 cytokines [43]. Immune and inflammation regulatory cytokines in serum, including four pro-inflammatory [interleukin (IL)-1 β , IL-6, IL-8, tumor necrosis factor (TNF)- α] and four anti-inflammatory cytokines [IL-1 receptor antagonist (IL-1 RA), IL-4, IL-10 and IL-13] were quantified by a ProcartaPlex Human Cytokine & Chemokine Panel 1A (Thermo Fisher Scientific, USA). Concentrations of these cytokines were analyzed by Luminex 200 multiplex immunoassay instrument (Luminex, USA).

2.6. Statistical analysis

All statistical analyses were carried out using R v3.6.3, SPSS 25.0 and GraphPad Prism 5.0 software. Categorical and continuous variables were compared using the chi-square test and *t*-test, respectively. Abnormal distributions, including heavy metal level, cell count and cytokine concentration, were presented as the median, and comparisons between two groups were made by the Mann-Whitney U test.

Multiple correspondence analysis was conducted to investigate the pattern of relationships between heavy metal exposure and socioeconomic factors from questionnaires. It can provide a structural organization for the variables and categories in a dimensional space that is useful for identifying patterns in the data and associations between the investigated parameters [47]. Multiple categories, such as child gender, distance of the road from the residence, milk consumption frequency, parents' education level, daily smoking by family members, parents' residence time, frequency of washing hands before meals, child born locally (Guiyu or Haojiang), child e-waste contact, ventilation of house, e-waste pollution within 50 m of residence, maternal occupation associated with e-waste dismantling, residence used as a workshop (see **Table 1**), as well as quintile data of heavy metal levels were all located as a point in a two-dimensional space. These factors reflect the situation in the aspects of children's behavior habits, diet, nutritional status, physical growth and development, dwelling environment as well as parents' care awareness. On the other hand, most of them have been reported to have an inverse association with blood Pb levels. For example, milk consumption may be a part of nutritional intervention to reduce Pb absorption and is considered as a protective factor for Pb exposure [6].

Multiple linear regression analysis was used to evaluate the association of heavy metal exposure with immune and inflammation regulatory cells/cytokines. For this analysis, data of heavy metals, cell counts and cytokines were ln-transformed to normalize the distribution. Potential confounders including child age, gender and BMI were adjusted in the models. Model 1 represents a crude model without any adjustment; model 2 was adjusted for child gender, age and BMI; model 3 was adjusted for child age, gender and BMI, distance of the road from the residence, milk consumption frequency, parents' education level, daily smoking by family members, parents' residence time, frequency of washing hands before meals, child born locally (Guiyu or Haojiang), child e-waste contact, ventilation of house, e-waste pollution within 50 m of residence, maternal occupation associated with e-waste dismantling, residence used as a workshop. Meanwhile, the interaction effects of Pb, Cd, Hg and As on immune cells and cytokines were assessed by a centering methodology in the multiple linear regression models. For hypothesis testing, a $p < 0.05$ in a two-tailed test was considered statistically significant.

3. Results

3.1. Characteristics of the study population

Study participant characteristics for the exposed and reference groups are provided in **Table 1**. The mean child's age in the exposed group was 5.0 years and 4.6 years in the reference group ($p = 0.007$). The mean child BMI was not significantly different between the two groups. All categories were significantly different between exposed and reference groups with the exception of child gender and smoking by family members.

3.2. Elevated heavy metal levels in Guiyu children

Blood concentrations of heavy metals are shown in **Figure 1**. The median values of Pb, Cd, Hg and As in the exposed group (37.23 µg/L, 0.33 µg/L, 1.46 µg/L and 5.91 µg/L, respectively) were significantly higher than those in the reference group (22.74 µg/L, 0.23 µg/L, 1.10 µg/L and 4.18 µg/L, respectively) (all $p < 0.001$). It indicates that the risk of heavy metal exposure in the e-waste-exposed area is more serious than in the reference group.

Table 1. Descriptive statistics for the study population (n = 147).

Characteristics	Exposed group (n = 73)	Reference group (n = 74)	<i>p</i>
Child age (years)	5.0 ± 0.8	4.6 ± 0.9	0.007 ^a
Gender [n (%)]	73 (100)	74 (100)	0.509 ^b
male	38 (52.1)	43 (53.1)	
female	35 (47.9)	31 (41.9)	
Child body mass index (BMI, kg/m ²)	14.97 ± 1.06	15.09 ± 1.99	0.654 ^a
Washing hands before meals [n (%)]	63 (100)	72 (100)	< 0.001 ^b
sometimes	37 (58.7)	15 (20.8)	
often	21 (33.3)	37 (51.4)	
always	5 (8.0)	20 (27.8)	
Child milk consumption frequency [n (%)]	68 (100)	72 (100)	0.002 ^b
never	3 (4.4)	1 (1.4)	
1-3 times per month	25 (36.8)	10 (13.9)	
1-3 times per week	30 (44.1)	35 (48.6)	
everyday	10 (14.7)	26 (36.1)	
Child born locally [n (%)]	68 (100)	72 (100)	< 0.001 ^b
no	7 (10.3)	33 (45.8)	
yes	61 (89.7)	39 (54.2)	
Child e-waste contact [n (%)]	63 (100)	71 (100)	< 0.001 ^b
never	47 (74.6)	69 (97.2)	
occasionally	9 (14.3)	2 (2.8)	
e-waste in residence	7 (11.1)	0 (0)	
Family member daily smoking [n (%)]	65 (100)	72 (100)	0.090 ^b
non-smoking	15 (23.1)	32 (44.4)	
~ 2 cigarettes	14 (21.5)	10 (13.9)	
~ 10 cigarettes	16 (24.6)	17 (23.6)	
~ 20 cigarettes	13 (20.0)	8 (11.1)	
> 20 cigarettes	7 (10.8)	5 (7.0)	
Residence used as workshop [n (%)]	63 (100)	71 (100)	< 0.001 ^b
no	34 (54.0)	69 (97.2)	
yes	29 (46.0)	2 (2.8)	
E-waste pollution within 50 m of residence [n (%)]	60 (100)	72 (100)	< 0.001 ^b
no	33 (55.0)	69 (95.8)	
yes	27 (45.0)	3 (4.2)	

Characteristics	Exposed group (n = 73)	Reference group (n = 74)	<i>p</i>
Paternal education level [n (%)]	68 (100)	72 (100)	< 0.001 ^b
primary	7 (10.3)	1 (1.4)	
junior	38 (55.9)	10 (13.9)	
technical secondary	7 (10.3)	15 (20.8)	
senior	6 (8.8)	9 (12.5)	
college	10 (14.7)	37 (51.4)	
Maternal education level [n (%)]	67 (100)	72 (100)	< 0.001 ^b
primary	9 (13.4)	3 (4.2)	
junior	39 (58.2)	16 (22.2)	
technical secondary	12 (17.9)	11 (15.3)	
senior	3 (4.5)	15 (20.8)	
college	4 (6.0)	27 (37.5)	
Paternal residence time [n (%)]	65 (100)	72 (100)	0.001 ^b
< 1 year	0 (0)	0 (0)	
~ 5 years	5 (7.7)	3 (4.2)	
~ 10 years	3 (4.6)	21 (29.2)	
> 10 years	57 (87.7)	48 (66.7)	
Maternal residence time [n (%)]	65 (100)	72 (100)	< 0.001 ^b
< 1 year	1 (1.5)	1 (1.4)	
~ 5 years	5 (7.7)	19 (26.4)	
~ 10 years	16 (24.6)	31 (43.1)	
> 10 years	43 (66.2)	21 (29.1)	
Distance to road from residence (m) [n (%)]	66 (100)	72 (100)	< 0.001 ^b
< 10	24 (36.4)	5 (6.9)	
~ 50	20 (30.3)	19 (26.4)	
~ 100	15 (22.7)	9 (12.5)	
> 100	7 (10.6)	39 (54.2)	
Ventilation of house [n (%)]	68 (100)	72 (100)	0.015 ^b
always	60 (88.2)	71 (98.6)	
occasionally	8 (11.8)	1 (1.4)	
Maternal occupation associated with e-waste dismantling [n (%)]	66 (100)	72 (100)	< 0.001 ^b
no	44 (66.7)	72 (100)	
yes	22 (33.3)	0 (0)	

^aAnalysis by the independent-sample *t*-test. ^bAnalysis by the chi-square test. Values of *p* < 0.05 were considered statistically significant.

Figure 1. Level of heavy metals ($\mu\text{g/L}$) in child peripheral blood. A. Blood Pb levels; B. Blood Cd levels; C. Blood Hg levels; D. Blood As levels. Exposed group: $n = 73$; Reference group: $n = 74$. Data are presented as medians, 25th percentile and 75th percentile. Analysis was by the Mann–Whitney U test. Values of $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ were considered statistically significant.

3.3. Impact of contributing factors on heavy metal exposure

Multiple correspondence analysis was performed to determine which socioeconomic factors were linked to changes in heavy metal levels. Data of heavy metals from all participants were processed into different percentiles, which are presented in **Table S1** (see Supplementary Material). Some categorical variables did not show significant differences in each sub-group of heavy metals determined by the chi-square test (data not shown) and were excluded in the following analysis. A joint plot of category points for the first and second dimensions showed an association of socioeconomic factors to heavy metal distributions (**Figure 2-A, B, C, and D**). For instance, in **Figure 2-A**, dimension 1 contributed with 53.2% of the inertia, while dimension 2 contributed 33.7%. The first quartile of Pb was likely to be associated with milk consumption frequency and not being born locally, and the fourth quartile of Pb was probably associated with residence used as a workshop, e-waste pollution existing within 50 m of residence, and a lack of milk consumption. For Cd, the first tertile was associated with a residence as not being a workshop, and the highest tertile was associated with occasional ventilation of the house. In addition, the highest tertile of Hg was associated with a long residence time of the mother (>10 years), and the highest tertile of As was inclined to be associated with variables such as maternal education level (primary) and occasional child hand washing before meals.

Figure 2. A joint plot of category points for the first two dimensions showing the association of relevant variables to blood Pb (A), Cd (B), Hg (C), and As (D) levels. Various identified points represent different variables. The relative distance of points in the same quadrant indicates the correlation level between variables.

The median lymphocyte count was lower in the exposed group than in the reference group (3.71×10^9 cells/L versus 4.17×10^9 cells/L) ($p = 0.007$); whereas there were no significant differences in neutrophil and monocyte counts between the two groups (**Figure 3**). The median concentration of IL-1 β and IL-6 were higher in the exposed group (0.88 pg/mL and 6.12 pg/mL, respectively) compared with those in the reference group (0.67 pg/mL and 4.51 pg/mL,

respectively) (both $p < 0.05$) (**Figure 4**). However, anti-inflammatory cytokines IL-1RA and IL-13 were both lower in the exposed group (83.72 pg/mL and 3.67 pg/mL, respectively) compared with those in the reference group (162.21 pg/mL and 4.41 pg/mL, respectively) (both $p < 0.001$). The concentrations of IL-8, TNF- α , IL-4 and IL-10 did not differ significantly between the two groups.

Figure 3. Counts of immune cells in child peripheral blood. A. Neutrophil counts; B. Monocyte counts; C. Lymphocyte counts. Exposed group: $n = 73$; Reference group: $n = 74$. Data are presented as medians, 25th percentile and 75th percentile. Analysis was by the Mann–Whitney U test. Values of $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ were considered statistically significant.

Figure 4. Concentration of inflammatory cytokines in child serum. Production of pro-inflammatory cytokines including IL-1 β , IL-6, IL-8 and TNF- α , are shown in A, B, C and D, respectively. Production of anti-inflammatory cytokines including IL-1RA, IL-4, IL-10 and IL-13, are shown in E, F, G and H, respectively. Exposed group: $n = 73$; Reference group: $n = 74$. Data are presented as medians, 25th percentile and 75th percentile. Analysis was by the Mann–Whitney U test. Values of $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ were considered statistically significant. IL: interleukin; TNF: tumor necrosis factor; RA: receptor antagonist.

3.5. Association between heavy metals and immune cells

Ln-transformed data supported the use of multiple linear regression models for the association of heavy metals and immune cells (**Table 2**). In the unadjusted model 1, neutrophil count was positively related with Pb, Cd and Hg exposure [β (95% confidence intervals or CI): 0.154 (0.004, 0.304), 0.109 (0.019, 0.237) and 0.157 (0.020, 0.293), respectively]. In model 2 neutrophil count was associated with Pb and Hg [β (95% CI): 0.153 (0.006, 0.309) and 0.177 (0.040, 0.313), respectively]. While in model 3, their relationships still existed [Pb: 0.196 (0.003, 0.394), Hg: 0.157 (0.002, 0.313)]. In addition, Cd also exhibited a positive association with neutrophil count [β (95%): 0.117 (0.028, 0.263)] ($p < 0.05$). Meanwhile, the positive association of monocyte level with Cd exposure [β (95%): 0.109 (0.009, 0.226)] was found in model 3 ($p < 0.05$). The interaction effects of 4 metals on immune cells was also analyzed, however, we did not discover a significant interaction between them (**Table S2** in Supplementary Materials).

3.6. Association between heavy metals and immune and inflammation regulatory cytokines

In the linear regression analysis (**Table 3**), the association between heavy metals and cytokines involved in immune and inflammation regulatory processes was explored. Both Pb and Cd positively correlated with IL-1 β production in the crude model 1 [β (95% CI): 0.225 (0.002, 0.447) and 0.172 (0.013, 0.357), respectively]; however, only Cd remained a positive relationship with IL-1 β level after adjusting for child age, gender and BMI [β (95% CI): 0.165 (0.023, 0.352)] ($p < 0.05$). Results showed that the association between Pb and IL-6 maintained positive in all 3 models [model 1: 0.287 (0.056, 0.518), model 2: 0.264 (0.038, 0.491) and model 3: 0.327 (0.036, 0.617), respectively] (all $p < 0.05$). The association between heavy metals and anti-inflammatory cytokines (**Table 4**) indicates that all four heavy metals were negatively related to IL-1RA in Model 1 and 2 (all $p < 0.05$). Interaction associations between metals and pro/anti-inflammatory cytokines were not found (**Table S3** in Supplementary Materials).

Table 2. Associations between heavy metals and immune cells (n = 147).

	Neutrophil			Monocyte			Lymphocyte		
	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3
Pb	0.154 (0.004, 0.304)*	0.153 (0.006, 0.309)*	0.196 (0.003, 0.394)*	0.002 (-0.127, 0.130)	0.001 (-0.132, 0.133)	0.019 (-0.144, 0.183)	-0.113 (-0.269, 0.044)	-0.149 (-0.307, 0.008)	-0.045 (-0.244, 0.155)
Cd	0.109 (0.019, 0.237)*	0.089 (-0.043, 0.221)	0.117 (0.028, 0.263)*	0.061 (-0.045, 0.167)	0.066 (-0.045, 0.176)	0.109 (0.009, 0.226)*	0.026 (-0.104, 0.157)	0.002 (-0.132, 0.135)	0.026 (-0.119, 0.171)
Hg	0.157 (0.020, 0.293)*	0.177 (0.040, 0.313)*	0.157 (0.002, 0.313)*	0.033 (-0.081, 0.148)	0.035 (-0.082, 0.152)	0.013 (-0.117, 0.142)	0.018 (-0.123, 0.159)	0.023 (-0.118, 0.164)	0.112 (-0.044, 0.268)
As	0.014 (-0.134, 0.163)	0.006 (-0.145, 0.158)	-0.049 (-0.217, 0.120)	0.016 (-0.106, 0.138)	0.010 (-0.116, 0.137)	-0.055 (-0.191, 0.082)	0.116 (-0.033, 0.264)	0.119 (-0.032, 0.269)	0.182 (-0.019, 0.346)

Immune cells: lymphocyte, neutrophil and monocyte; heavy metals: Pb, Cd, Hg and As. Data of heavy metals and immune cell counts were ln-transformed to normalize the distribution. Data in this table are presented as estimates (95% confidence intervals). The estimates (β value) were calculated by linear regression models. Model 1 represents a crude model without any adjustment; model 2 was adjusted for child age, gender and BMI; model 3 was adjusted for child age, gender and BMI; model 3 was adjusted for child age, gender and BMI, distance of the road from the residence, milk consumption frequency, parents' education level, daily smoking by family members, parents' residence time, frequency of washing hands before meals, child born locally (Guiyu or Haojiang), child e-waste contact, ventilation of house, e-waste pollution within 50 meters (m) of residence, maternal occupation associated with e-waste dismantling, residence used as a workshop. Values of $*p < 0.05$ was considered statistically significant.

Table 3. Associations between heavy metals and pro-inflammatory cytokines (n = 147).

	IL-1 β			IL-6			IL-8			TNF- α		
	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3
Pb	0.225 (0.002, 0.447)*	0.187 (- 0.038, 0.411)	0.186 (- 0.083, 0.454)	0.287 (0.056, 0.518)*	0.264 (0.038, 0.491)*	0.327 (0.036, 0.617)*	-0.110 (- 0.377, 0.158)	-0.134 (- 0.398, 0.130)	0.048 (- 0.277, 0.373)	-0.041 (- 0.247, 0.166)	-0.082 (- 0.289, 0.125)	-0.179 (- 0.440, 0.082)
Cd	0.172 (0.013, 0.357)*	0.165 (0.023, 0.352)*	0.151 (- 0.044, 0.346)	0.071 (- 0.124, 0.267)	0.059 (- 0.134, 0.253)	0.055 (- 0.161, 0.271)	-0.045 (- 0.268, 0.177)	-0.131 (- 0.352, 0.089)	-0.053 (- 0.289, 0.183)	-0.112 (- 0.282, 0.058)	-0.126 (- 0.299, 0.046)	-0.149 (- 0.339, 0.040)
Hg	-0.099 (- 0.300, 0.102)	-0.094 (- 0.294, 0.105)	-0.132 (- 0.344, 0.080)	-0.198 (- 0.406, 0.010)	-0.179 (- 0.381, 0.023)	-0.245 (- 0.639, 0.082)	-0.158 (- 0.396, 0.080)	-0.110 (- 0.343, 0.124)	-0.094 (- 0.350, 0.162)	-0.062 (- 0.246, 0.122)	-0.062 (- 0.245, 0.121)	-0.143 (- 0.349, 0.063)
As	0.008 (- 0.207, 0.223)	-0.037 (- 0.253, 0.179)	-0.056 (- 0.282, 0.170)	0.026 (- 0.199, 0.251)	-0.056 (- 0.276, 0.164)	-0.114 (- 0.362, 0.133)	-0.059 (- 0.314, 0.196)	-0.094 (- 0.346, 0.157)	-0.111 (- 0.382, 0.160)	0.008 (- 0.188, 0.204)	-0.030 (- 0.228, 0.167)	-0.139 (- 0.358, 0.079)

Pro-inflammatory cytokines: IL-1 β , IL-6, IL-8 and TNF- α ; IL: interleukin; TNF: tumor necrosis factor. Heavy metals: Pb, Cd, Hg and As. Data of heavy metals and pro-inflammatory cytokines were ln-transformed to normalize the distribution. Data in this table are presented as estimates (95% confidence intervals). The estimates (β value) were calculated by linear regression models. Model 1 represents a crude model without any adjustment; model 2 was adjusted for child age, gender and BMI; model 3 was adjusted for child age, gender and BMI, distance of the road from the residence, milk consumption frequency, parents' education level, daily smoking by family members, parents' residence time, frequency of washing hands before meals, child born locally (Guiyu or Haojiang), child e-waste contact, ventilation of house, e-waste pollution within 50 meters (m) of residence, maternal occupation associated with e-waste dismantling, residence used as a workshop. Values of * $p < 0.05$ was considered statistically significant.

Table 4. Associations between heavy metals and anti-inflammatory cytokines (n = 147).

	IL-1RA			IL-4			IL-10			IL-13		
	Model 1		Model 2	Model 1		Model 2	Model 1		Model 2	Model 1		Model 2
	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3
Pb	-0.336 (-0.583, -0.089)**	-0.392 (-0.639, -0.144)**	-0.188 (-0.499, 0.124)	-0.056 (-0.178, 0.067)	-0.096 (-0.216, 0.025)	-0.118 (-0.270, 0.033)	-0.005 (-0.290, 0.279)	-0.005 (-0.296, 0.286)	0.075 (-0.286, 0.437)	-0.090 (-0.253, 0.073)	-0.123 (-0.288, 0.042)	-0.086 (-0.289, 0.117)
Cd	-0.181 (-0.389, 0.027)*	-0.207 (-0.419, 0.006)*	-0.150 (-0.377, 0.076)	-0.026 (-0.127, 0.076)	-0.043 (-0.144, 0.058)	-0.072 (-0.182, 0.039)	-0.167 (-0.401, 0.067)	-0.139 (-0.381, 0.104)	-0.182 (-0.443, 0.079)	-0.007 (-0.143, 0.128)	-0.020 (-0.160, 0.119)	-0.033 (-0.181, 0.115)
Hg	-0.286 (-0.507, 0.065)*	-0.288 (-0.505, 0.070)*	-0.163 (-0.426, 0.100)	-0.082 (-0.191, 0.027)	-0.082 (-0.189, 0.024)	-0.106 (-0.255, 0.014)	-0.102 (-0.355, 0.151)	-0.125 (-0.381, 0.131)	-0.029 (-0.314, 0.256)	-0.114 (-0.258, 0.031)	-0.116 (-0.262, 0.030)	-0.101 (-0.261, 0.058)
As	-0.173 (-0.412, 0.067)*	-0.223 (-0.464, 0.019)*	-0.138 (-0.399, 0.123)	-0.023 (-0.140, 0.094)	-0.043 (-0.159, 0.072)	-0.070 (-0.198, 0.058)	-0.177 (-0.446, 0.091)	-0.190 (-0.465, 0.085)	-0.162 (-0.463, 0.139)	0.023 (-0.133, 0.178)	0.012 (-0.146, 0.171)	-0.008 (-0.179, 0.162)

Anti-inflammatory cytokines: IL-1RA, IL-4, IL-10 and IL-13; RA: receptor antagonist. Heavy metals: Pb, Cd, Hg and As. Data of heavy metals and pro-inflammatory cytokines were ln-transformed to normalize the distribution. Data in this table are presented as estimates (95% confidence intervals). The estimates (β value) were calculated by linear regression models. Model 1 represents a crude model without any adjustment; model 2 was adjusted for child age, gender and BMI; model 3 was adjusted for child age, gender and BMI, distance of the road from the residence, milk consumption frequency, parents' education level, daily smoking by family members, parents' residence time, frequency of washing hands before meals, child born locally (Guiyu or Haojiang), child e-waste contact, ventilation of house, e-waste pollution within 50 meters (m) of residence, maternal occupation associated with e-waste dismantling, residence used as a workshop. Values of $*p < 0.05$, $**p < 0.01$ were considered statistically significant.

4. Discussion

In this study, children from the e-waste recycling area had higher heavy metal levels (Pb, Cd, Hg and As) but lower lymphocyte counts in peripheral blood. Altered production of cytokines involved in the immune response and inflammatory regulation were observed. Pro-inflammatory IL-1 β and IL-6 were elevated, but anti-inflammatory IL-1RA and IL-13 were decreased. These changes might suggest aberrant immune homeostasis and inflammatory activation in children living in the exposed area. Moreover, our results revealed that exposure to Pb, Cd, Hg and As is associated with the alterations in neutrophils, IL-1 β , IL-6 and IL-1RA.

Our data demonstrated the associations between heavy metal exposure and relevant factors in questionnaires (**Figure 2**). Although previous studies have performed similar analyses with regard to the association between confounding factors and heavy metal exposure by linear regression analysis or spearman correlation analysis [43, 48], this study applied MCA analysis because it is a multivariate exploratory analysis method for visualizing large datasets of categorical variables [47]. The contributing factors, such as residence used as an e-waste workshop, maternal occupation associated with e-waste dismantling, and e-waste pollution within 50 m of the residence, were related to the highest quartile of Pb levels. In multiple correspondence analysis, education levels of parents were found to be negatively linked to heavy metal As concentration. We speculated that parents with higher educational levels have healthier awareness to prevent their children from e-waste hazards. Some behaviors involved in the prevention of and protection from e-waste exposure, such as high frequency of milk consumption, hand washing before meals, and house ventilation, were respectively associated with the lowest blood levels of Pb, As and Cd. Milk consumption frequency may reflect child nutritional status and intervene with Pb accumulation, it thus is considered as a protective factor for Pb exposure to some extent [6]. Heavy metals can accumulate on dust particles and other materials in the environment [49]. Ventilation and handwashing may decrease the contact opportunity of exposure to heavy metals. Furthermore, the maternal residence time in the e-waste area also determines heavy metal exposure to children, especially those who were born in Guiyu. Previous epidemiological studies have confirmed that those mothers with a long-term residence in an e-waste recycling area had relatively high levels of heavy metals in their blood and milk; these toxins can enter the body of the offspring through the placental barrier or breastfeeding process, increasing child exposure to heavy metals, accordingly [5, 50-53].

Immune cells and cytokines play an indispensable role in immune function and inflammatory regulation. We observed that different heavy metals exhibit distinct impacts on neutrophil counts. In the early stage of inflammation, neutrophils in the blood are initially recruited by sensing chemoattractant gradient, then traverse the vascular endothelium, and ultimately reach the sites of infection or inflammatory stimuli within minutes [54, 55]. Thus, sufficient amounts of neutrophils are required for surveilling and eliminating heterologous microbes and irritants. Although no remarkable difference in neutrophil counts between the two groups exists, our results showed positive relationships between neutrophil counts and exposure to Pb and Hg in all 3 models (**Table 2**). It suggests that the impact of individuals' characteristics and social-behavioral factors on the association between neutrophil counts and exposure to Pb and Hg is not obvious. Interestingly, a positive association between Cd exposure and neutrophil counts was found in both model 1 and model 3, while it disappeared in model 2, which may be due to the variation of child age, gender and BMI. A previous population study by Di Lorenzo et al. found an increased number of blood neutrophils in lead-exposed workers [56], which is in accordance with our result. The animal studies concerning Cd toxicity suggested Cd-treated mice had increased monocytes and neutrophils in the bone marrow and spleens, while a significantly higher neutrophil count was observed in the peripheral blood of Cd-exposed rats [43, 57]. Our finding is also in line with a prior animal study showing enhanced neutrophil counts concerning increasing Hg exposure [58], but not identical with a recent cohort study reporting no significant correlation of neutrophil counts with the Hg concentration [59].

Due to the difficulty of evaluating tissue-resident macrophages, as well as the rarity of dendritic cells in human blood [60], we could only analyze monocyte counts in child peripheral blood. However, a significant alteration in monocyte amount was not found in the present study. Despite that, our finding of reduced lymphocyte count in exposed populations is in accordance with several studies [59, 61, 62]. It is well known that the migration of circulating lymphocytes plays a more prominent role in eliminating pathogenic microorganisms or noxious stimuli in chronic inflammation; hence, the decreased lymphocyte quantity is not conducive to the immune system in response to harmful conditions [63-66]. A decrease in lymphocyte count might hint that the proliferation of lymphocytes is disturbed or suppressed, which could lead to an ineffective immune function of children in e-waste areas.

Heavy metals upset the balance of the pro- and anti-inflammatory processes. Our study

demonstrated that children from the e-waste recycling area have elevated pro-inflammatory IL-1 β and IL-6, but lower anti-inflammatory IL-1RA and IL-13 levels. No difference in IL-8, TNF- α , IL-4, and IL-10 production was found between the two groups, which is not surprising because the release of cytokines may partially be attributed to stimulators, but could be driven by genetics, cytokine half-lives or other unexpected confounders. The half-life of each biomarker is shown in **Table S4** in Supplementary Materials. IL-1 β is mainly secreted by monocytes, tissue macrophages and dendritic cells. It exerts diverse effects on multiple cell types, culminating in broad inflammation, although often being at best only slightly elevated in human inflammatory diseases even under conditions of severe pathology [67, 68]. IL-1RA is expressed by various cells in an inflammatory environment and is regarded as an essential negative feedback mechanism to limit inflammation mediated by IL-1 β [69]. IL-13 is primarily generated by T helper type 2 (Th2) cells and is excessively produced in allergy, asthma and other chronic inflammatory diseases [70, 71]. At present, we cannot identify far-reaching health consequences, such as chronic diseases and malignancies in children from e-waste areas. According to the imbalance of inflammatory cytokines, our finding suggests that children living in the e-waste areas gravitate towards an inflammatory status or at least possess a relatively high inflammation risk.

We noticed that abnormal cytokine expression, including of IL-1 β , IL-6 and IL-1RA, is dependent on heavy metal exposure. Our observation indicates that Pb exposure induces IL-1 β and IL-6 but suppresses IL-1RA production. The association between Pb and IL-1 β can be weakened when adjusting individuals' characteristics and social-behavioral factors, indicating that these factors may contribute to IL-1 β production. In parallel with our results, previous research in both humans and animals has also shown IL-6 is over-expressed following Pb exposure [72-75]. IL-6 is proposed as a predictor for malignancy since elevated IL-6 level is closely associated with tumorigenic progression; in addition, dysregulated IL-6 synthesis plays a pathological role in chronic inflammation and autoimmunity [76-78]. Moreover, Pb, Cd, Hg and As exposure all exhibit inhibitory contribution to IL-1RA synthesis. With regard to the associations between all 4 metals and IL-1RA, they did not exist in model 3, suggesting that they may be hampered by social-behavioral factors. There unfortunately is no supporting evidence for the correlation of their exposure and IL-1RA secretion. In this case, our finding is novel and could be an opportunity for future research. As a consequence of the alterations in

pro-inflammatory IL-1 β , IL-6 and anti-inflammatory IL-1RA, we speculated that individuals exposed to Pb, Cd, Hg and As might sustain a potential threat by immune imbalance and inflammatory conditions.

Exposure to heavy metals could be harmful to Guiyu children. In general, immune function may be enhanced, as a protective mechanism, to respond to danger signals derived from endogenous and exogenous molecules after acute exposure to occupational and environmental pollutants [79, 80]. However, long-term exposure to heavy metals (either low-dose or high-dose) uniformly causes immunosuppression and directly interferes with the sensitization of the immune system to antigens [81-84]. So to some extent, our observation is in agreement with previous findings that exposure to heavy metals prompts several subtle changes in perturbing immune regulation. Continuous heavy metal exposures could inevitably impair immune function, subsequently resulting in the occurrence of the disease. This however needs further health surveillance and assessment.

Several limitations of our study should be stated. Considering the sample size, we explored the impact of heavy metal exposure on cells and cytokines involved in the immune regulatory and inflammatory processes after merging all data from both groups. Therefore, a few unknown confounders will interfere with our established linear models. Many other toxic compounds and pollutants, for example, persistent organic pollutants are also a major concern of the e-waste dismantling area [85, 86]. It is challenging to run all items of tests in this study due to the insufficient volume of blood samples. Some customs and individual habits may affect immune homeostasis and inflammatory activation in children. However, we failed to provide the evidence for the analysis in this regard. Moreover, since inflammation is generally accompanied by tissue injury, monitoring tissue-resident macrophages or mast cells would be very important in assessing different inflammatory periods. Thus, additional studies are sorely needed to better understand the influence of heavy metal exposure on the occurrence and development of inflammation and other diseases in future investigations.

5. Conclusions

This study identified the alterations of immune cells and cytokines in preschool children exposed to heavy metals Pb, Cd, Hg and As. Children living in the e-waste recycling area had higher levels of heavy metals. Decreased blood lymphocyte amount was observed in children from the e-waste recycling area, which might suggest that their immune function is either disturbed or suppressed. Elevated pro-inflammatory IL-1 β and IL-6, and declined anti-inflammatory IL-1RA and IL-13 may indicate that Guiyu children had dysregulated immune response and high inflammation risk. Association analysis reveals that exposure to Pb, Cd, Hg and As is likely to contribute to the alterations in neutrophils, IL-1 β , IL-6 and IL-1RA, prompting adverse effects on immune response and inflammatory regulation. Our finding of the inverse association between IL-1RA and heavy metals is novel and could be an opportunity for future research.

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REFERENCES

- [1] Grant, K., et al., Health consequences of exposure to e-waste: a systematic review. *The lancet global health*, 2013. 1(6): p. e350-e361.
- [2] Zeng, X., et al., Heavy metals in PM_{2.5} and in blood, and children's respiratory symptoms and asthma from an e-waste recycling area. *Environ. Pollut.*, 2016. 210: p. 346-353.
- [3] Chen, A., et al., Developmental neurotoxicants in e-waste: an emerging health concern. *Environ. Health Perspect.*, 2011. 119(4): p. 431-438.
- [4] Huo, X., et al., Elevated blood lead levels of children in Guiyu, an electronic waste recycling town in China. *Environ. Health Perspect.*, 2007. 115(7): p. 1113-1117.
- [5] Guo, Y., et al., Monitoring of lead, cadmium, chromium and nickel in placenta from an e-waste recycling town in China. *Sci. Total Environ.*, 2010. 408(16): p. 3113-3117.
- [6] Zheng, L., et al., Blood lead and cadmium levels and relevant factors among children from an e-waste recycling town in China. *Environ. Res.*, 2008. 108(1): p. 15-20.
- [7] Dai, Y., et al., Elevated lead levels and changes in blood morphology and erythrocyte CR1 in preschool children from an e-waste area. *Sci. Total Environ.*, 2017. 592: p. 51-59.
- [8] Zeng, X., et al., Heavy metal exposure has adverse effects on the growth and development of preschool children. *Environ. Geochem. Health*, 2019. 41(1): p. 309-321.
- [9] Zheng, X., et al., Cardiovascular endothelial inflammation by chronic coexposure to lead (Pb) and polycyclic aromatic hydrocarbons from preschool children in an e-waste recycling area. *Environ. Pollut.*, 2019. 246: p. 587-596.
- [10] Al Bakheet, S.A., et al., Effect of long-term human exposure to environmental heavy metals on the expression of detoxification and DNA repair genes. *Environ. Pollut.*, 2013. 181: p. 226-232.
- [11] Brucker, N., et al., Relationship between blood metals and inflammation in taxi drivers. *Clin. Chim. Acta*, 2015. 444: p. 176-181.
- [12] Jin, Y., et al., Cadmium exposure to murine macrophages decreases their inflammatory responses and increases their oxidative stress. *Chemosphere*, 2016. 144: p. 168-175.
- [13] Iyer, S.S., et al., Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome. *Proceedings of the National Academy of Sciences*, 2009. 106(48): p. 20388-20393.
- [14] Morcillo, P., et al., Heavy metals produce toxicity, oxidative stress and apoptosis in the marine teleost fish SAF-1 cell line. *Chemosphere*, 2016. 144: p. 225-233.
- [15] Tchounwou, P.B., et al., Heavy metal toxicity and the environment, in *Molecular, clinical and environmental toxicology*. 2012, Springer. p. 133-164.
- [16] Wang, S., et al., Molecular mechanisms of metal toxicity and carcinogenesis. *Mol. Cell. Biochem.*, 2001. 222(1-2): p. 3-9.
- [17] Bae, J.H., et al., Chemical regulation of signaling pathways to programmed necrosis. *Arch. Pharmacol. Res.*, 2014. 37(6): p. 689-697.
- [18] Afridi, H.I., et al., Evaluation of status of arsenic, cadmium, lead and zinc levels in biological samples of normal and arthritis patients of age groups (46-60) and (61-75) years. *Clinical laboratory*, 2013. 59(1-2): p. 143.
- [19] Arora, M., et al., Association of environmental cadmium exposure with periodontal disease in US adults. *Environ. Health Perspect.*, 2009. 117(5): p. 739-744.
- [20] Cavaleri, F., Review of Amyotrophic Lateral Sclerosis, Parkinson's and Alzheimer's diseases helps further define pathology of the novel paradigm for Alzheimer's with heavy metals as primary disease cause. *Med. Hypotheses*, 2015. 85(6): p. 779-790.
- [21] Ioranne Monnet-Tschudi, F., et al., Involvement of environmental mercury and lead in the etiology of neurodegenerative diseases. *Rev. Environ. Health*, 2006. 21(2).
- [22] Rowley, B., et al., Mechanisms of heavy metal-induced autoimmunity. *Mol. Immunol.*, 2005. 42(7): p. 833-838.
- [23] Ferrero-Miliani, L., et al., Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1 β generation. *Clin. Exp. Immunol.*, 2007. 147(2): p. 227-235.
- [24] Olszowski, T., et al., Pro-inflammatory properties of cadmium. *Acta Biochim. Pol.*, 2012. 59(4).
- [25] Wessling-Resnick, M., Iron homeostasis and the inflammatory response. *Annu. Rev. Nutr.*, 2010. 30: p. 105-122.
- [26] Giovanni, M., et al., Pro-inflammatory responses of RAW264.7 macrophages when treated with ultralow concentrations of silver, titanium dioxide, and zinc oxide nanoparticles. *J. Hazard. Mater.*, 2015. 297: p. 146-152.
- [27] Hamilton, J.A., Colony-stimulating factors in inflammation and autoimmunity. *Nature Reviews Immunology*, 2008. 8(7): p. 533-544.
- [28] Schenkein, H.A., et al., Inflammatory mechanisms linking periodontal diseases to cardiovascular diseases. *J. Periodontol.*, 2013. 84: p. S51-S69.
- [29] Tseng, J.-C., et al., In vivo imaging of inflammatory phagocytes. *Chem. Biol.*, 2012. 19(9): p. 1199-1209.
- [30] Tseng, J.-C., et al., In vivo imaging method to distinguish acute and chronic inflammation. *JoVE*

- (Journal of Visualized Experiments), 2013(78): p. e50690.
- [31] Wu, Y., et al., Molecular mechanisms underlying chronic inflammation-associated cancers. *Cancer Lett.*, 2014. 345(2): p. 164-173.
 - [32] Deng, X., et al., Chronic alcohol consumption accelerates fibrosis in response to cerulein-induced pancreatitis in rats. *The American journal of pathology*, 2005. 166(1): p. 93-106.
 - [33] Goecke, H., et al., Macrophages infiltrating the tissue in chronic pancreatitis express the chemokine receptor CCR5. *Surgery*, 2000. 128(5): p. 806-814.
 - [34] Habtezion, A., Inflammation in acute and chronic pancreatitis. *Curr. Opin. Gastroenterol.*, 2015. 31(5): p. 395.
 - [35] Ingersoll, M.A., et al., Monocyte trafficking in acute and chronic inflammation. *Trends Immunol.*, 2011. 32(10): p. 470-477.
 - [36] Häusser, G., et al., Monocyte-derived dendritic cells represent a transient stage of differentiation in the myeloid lineage. *Immunobiology*, 1997. 197(5): p. 534-542.
 - [37] Kumar, S., et al., Invited review: origin of monocytes and their differentiation to macrophages and dendritic cells. *J. Endotoxin Res.*, 2006. 12(5): p. 278-284.
 - [38] Ohradanova-Repic, A., et al., Differentiation of human monocytes and derived subsets of macrophages and dendritic cells by the HLDA10 monoclonal antibody panel. *Clinical & translational immunology*, 2016. 5(1): p. e55.
 - [39] Cho, Y., et al., Identification of novel cytokine biomarkers of hexanal exposure associated with pulmonary toxicity. *Environ. Pollut.*, 2017. 229: p. 810-817.
 - [40] Li, R., et al., Mitochondrial damage: an important mechanism of ambient PM_{2.5} exposure-induced acute heart injury in rats. *J. Hazard. Mater.*, 2015. 287: p. 392-401.
 - [41] Cao, J., et al., Increased memory T cell populations in Pb-exposed children from an e-waste-recycling area. *Sci Total Environ*, 2018. 616-617: p. 988-995.
 - [42] Xu, X., et al., Decreased blood hepatitis B surface antibody levels linked to e-waste lead exposure in preschool children. *J. Hazard. Mater.*, 2015. 298: p. 122-128.
 - [43] Zhang, Y., et al., Elevated lead levels and adverse effects on natural killer cells in children from an electronic waste recycling area. *Environ Pollut*, 2016. 213: p. 143-150.
 - [44] Zeng, X., et al., Heavy metals in PM_{2.5} and in blood, and children's respiratory symptoms and asthma from an e-waste recycling area. *Environ Pollut*, 2016. 210: p. 346-353.
 - [45] Lu, S.Y., et al., Trace elements contamination and human health risk assessment in drinking water from Shenzhen, China. *Environ Monit Assess*, 2015. 187(1): p. 4220.
 - [46] Wu, Y., et al., Concentrations and health risk assessment of trace elements in animal-derived food in southern China. *Chemosphere*, 2016. 144: p. 564-570.
 - [47] Lana, R.M., et al., Socioeconomic and demographic characterization of an endemic malaria region in Brazil by multiple correspondence analysis. *Malar J*, 2017. 16(1): p. 397.
 - [48] Xu, L., et al., Hearing loss risk and DNA methylation signatures in preschool children following lead and cadmium exposure from an electronic waste recycling area. *Chemosphere*, 2020. 246: p. 125829.
 - [49] Lau, W.K., et al., Human health risk assessment based on trace metals in suspended air particulates, surface dust, and floor dust from e-waste recycling workshops in Hong Kong, China. *Environ. Sci. Pollut. Res. Int.*, 2014. 21(5): p. 3813-3825.
 - [50] Arbuckle, T.E., et al., Maternal and fetal exposure to cadmium, lead, manganese and mercury: The MIREC study. *Chemosphere*, 2016. 163: p. 270-282.
 - [51] Liu, R., et al., Thyroid Hormone Status in Umbilical Cord Serum Is Positively Associated with Male Anogenital Distance. *J. Clin. Endocrinol. Metab.*, 2016. 101(9): p. 3378-3385.
 - [52] Rzymiski, P., et al., Impact of heavy metals on the female reproductive system. *Ann Agric Environ Med*, 2015. 22(2): p. 259-264.
 - [53] Silver, M.K., et al., Distribution and predictors of 20 toxic and essential metals in the umbilical cord blood of Chinese newborns. *Chemosphere*, 2018. 210: p. 1167-1175.
 - [54] Fournier, B.M., et al., The role of neutrophils during intestinal inflammation. *Mucosal Immunol.*, 2012. 5(4): p. 354-366.
 - [55] Kolaczowska, E., et al., Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.*, 2013. 13(3): p. 159-175.
 - [56] Di Lorenzo, L., et al., Evaluation of peripheral blood neutrophil leucocytes in lead-exposed workers. *Int. Arch. Occup. Environ. Health*, 2006. 79(6): p. 491-498.
 - [57] El-Boshy, M., et al., Studies on the protective effect of the artichoke (*Cynara scolymus*) leaf extract against cadmium toxicity-induced oxidative stress, hepatorenal damage, and immunosuppressive and hematological disorders in rats. *Environ. Sci. Pollut. Res. Int.*, 2017. 24(13): p. 12372-12383.
 - [58] Schaefer, A.M., et al., Associations between mercury and hepatic, renal, endocrine, and hematological parameters in Atlantic bottlenose dolphins (*Tursiops truncatus*) along the eastern coast of Florida and South Carolina. *Arch Environ Contam Toxicol*, 2011. 61(4): p. 688-695.
 - [59] Oulhote, Y., et al., Children's white blood cell counts in relation to developmental exposures to

- methylmercury and persistent organic pollutants. *Reprod. Toxicol.*, 2017. 68: p. 207-214.
- [60] Ohradanova-Repic, A., et al., Differentiation of human monocytes and derived subsets of macrophages and dendritic cells by the HLDA10 monoclonal antibody panel. *Clin Transl Immunology*, 2016. 5(1): p. e55.
- [61] Boscolo, P., et al., Lymphocyte subpopulations, cytokines and trace elements in asymptomatic atopic women exposed to an urban environment. *Life Sci*, 2000. 67(10): p. 1119-1126.
- [62] Mishra, K.P., et al., Effect of lead exposure on lymphocyte subsets and activation markers. *Immunopharmacol. Immunotoxicol.*, 2010. 32(3): p. 446-449.
- [63] Fortier, M., et al., Effects of physiological concentrations of heavy metals both individually and in mixtures on the viability and function of peripheral blood human leukocytes in vitro. *J. Toxicol. Environ. Health A*, 2008. 71(19): p. 1327-1337.
- [64] Sakai, Y., et al., Lymphocyte 'homing' and chronic inflammation. *Pathol Int*, 2015. 65(7): p. 344-354.
- [65] Steffensen, I.L., et al., Cytotoxicity and accumulation of Hg, Ag, Cd, Cu, Pb and Zn in human peripheral T and B lymphocytes and monocytes in vitro. *Gen. Pharmacol.*, 1994. 25(8): p. 1621-1633.
- [66] Valentino, M., et al., Effect of lead on the levels of some immunoregulatory cytokines in occupationally exposed workers. *Hum. Exp. Toxicol.*, 2007. 26(7): p. 551-556.
- [67] Dinarello, C.A., Immunological and inflammatory functions of the interleukin-1 family. *Annu. Rev. Immunol.*, 2009. 27: p. 519-550.
- [68] Lachmann, H.J., et al., In vivo regulation of interleukin 1beta in patients with cryopyrin-associated periodic syndromes. *J Exp Med*, 2009. 206(5): p. 1029-1036.
- [69] Roerink, M.E., et al., Interleukin-1 as a mediator of fatigue in disease: a narrative review. *J. Neuroinflammation*, 2017. 14(1): p. 16.
- [70] Alasandagutti, M.L., et al., Role of IL-13 Genetic Variants in Signalling of Asthma. *Inflammation*, 2017. 40(2): p. 566-577.
- [71] Fujii, U., et al., Effect of a retinoid X receptor partial agonist on airway inflammation and hyperresponsiveness in a murine model of asthma. *Respir. Res.*, 2017. 18(1): p. 23.
- [72] Kayaaltı, Z., et al., Effects of the interleukin-6 (IL-6) polymorphism on toxic metal and trace element levels in placental tissues. *Sci Total Environ*, 2011. 409(23): p. 4929-4933.
- [73] Mishra, K.P., et al., Effect of lead exposure on the immune response of some occupationally exposed individuals. *Toxicology*, 2003. 188(2-3): p. 251-259.
- [74] Struzynska, L., et al., Inflammation-like glial response in lead-exposed immature rat brain. *Toxicol. Sci.*, 2007. 95(1): p. 156-162.
- [75] Wang, H., et al., Protective Effects of Green Tea Polyphenol Against Renal Injury Through ROS-Mediated JNK-MAPK Pathway in Lead Exposed Rats. *Mol Cells*, 2016. 39(6): p. 508-513.
- [76] Landskron, G., et al., Chronic inflammation and cytokines in the tumor microenvironment. *J Immunol Res*, 2014. 2014: p. 149185.
- [77] Tanaka, T., et al., IL-6 in inflammation, immunity, and disease. *Cold Spring Harb. Perspect. Biol.*, 2014. 6(10): p. a016295.
- [78] Taniguchi, K., et al., IL-6 and related cytokines as the critical lymphins between inflammation and cancer. *Semin. Immunol.*, 2014. 26(1): p. 54-74.
- [79] Franklin, B.A., et al., Air pollution and cardiovascular disease. *Curr. Probl. Cardiol.*, 2015. 40(5): p. 207-238.
- [80] Steinvil, A., et al., Short-term exposure to air pollution and inflammation-sensitive biomarkers. *Environ Res*, 2008. 106(1): p. 51-61.
- [81] Habtezion, A., Inflammation in acute and chronic pancreatitis. *Curr. Opin. Gastroenterol.*, 2015. 31(5): p. 395-399.
- [82] Hoffmann, B., et al., Chronic residential exposure to particulate matter air pollution and systemic inflammatory markers. *Environ Health Perspect*, 2009. 117(8): p. 1302-1308.
- [83] Sorvari, J., et al., Heavy metal pollution disturbs immune response in wild ant populations. *Environ Pollut*, 2007. 145(1): p. 324-328.
- [84] Viehmann, A., et al., Long-term residential exposure to urban air pollution, and repeated measures of systemic blood markers of inflammation and coagulation. *Occup. Environ. Med.*, 2015. 72(9): p. 656-663.
- [85] Leung, A.O., et al., Heavy metals concentrations of surface dust from e-waste recycling and its human health implications in southeast China. *Environ Sci Technol*, 2008. 42(7): p. 2674-2680.
- [86] Xu, P., et al., Levels, profiles and source identification of PCDD/Fs in farmland soils of Guiyu, China. *Chemosphere*, 2013. 91(6): p. 824-831.

*Supplementary Materials :***Table S1. Distribution of heavy metals ($\mu\text{g/L}$) in different percentiles.**

Heavy metals	n	Range
Pb	147	3.45 - 142.73
Quartile 1	37	3.45 - 21.84
Quartile 2	37	21.84 - 28.20
Quartile 3	37	28.20 - 39.27
Quartile 4	36	39.27 - 142.73
Cd	147	0.03 - 0.82
Tertile 1	50	0.03 - 0.21
Tertile 2	48	0.21 - 0.34
Tertile 3	49	0.34 - 0.82
Hg	147	0.19 - 3.62
Tertile 1	50	0.19 - 1.07
Tertile 2	48	1.07 - 1.64
Tertile 3	49	1.64 - 3.62
As	147	0.70 - 22.62
Quartile 1	37	0.70 - 3.32
Quartile 2	39	3.32 - 4.77
Quartile 3	35	4.77 - 7.03
Quartile 4	36	7.03 - 22.62

Data from all participants (n=147) were processed into different percentiles.

Table S2. Interaction effects of heavy metals on immune cell counts (n = 147).

	Lymphocyte			Neutrophil			Monocyte		
	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3
Pb	-0.194 (-0.374, -0.013)*	-0.227 (-0.407, -0.046)*	-0.134 (-0.369, 0.102)	0.102 (-0.075, 0.278)	0.103 (-0.074, 0.281)	0.189 (-0.042, 0.420)	-0.041 (-0.192, 0.110)	-0.044 (-0.199, 0.111)	-0.045 (-0.238, 0.147)
Cd	0.030 (-0.113, 0.173)	0.010 (-0.135, 0.155)	-0.027 (-0.192, 0.137)	0.084 (-0.056, 0.224)	0.053 (-0.090, 0.195)	0.098 (-0.063, 0.259)	0.065 (-0.055, 0.184)	0.069 (-0.055, 0.194)	0.130 (0.004, 0.265)*
Hg	-0.037 (-0.214, 0.139)	-0.024 (-0.204, 0.156)	0.039 (-0.157, 0.235)	0.197 (0.024, 0.369)*	0.245 (0.068, 0.422)*	0.233 (0.041, 0.425)*	0.035 (-0.112, 0.183)	0.042 (-0.112, 0.196)	0.032 (-0.128, 0.193)
As	0.160 (-0.027, 0.348)	0.176 (-0.019, 0.371)	0.185 (-0.030, 0.400)	-0.150 (-0.334, 0.034)	-0.193 (-0.385, 0.001)*	-0.253 (-0.463, 0.042)*	-0.021 (-0.178, 0.135)	0.038 (-0.205, 0.129)	-0.150 (-0.326, 0.025)
Pb * Cd * Hg * As	-0.002 (-0.007, 0.003)	-0.002 (-0.007, 0.003)	-0.001 (-0.007, 0.005)	0.001 (-0.004, 0.006)	0.001 (-0.004, 0.006)	0.002 (-0.004, 0.007)	-0.001 (-0.005, 0.004)	-0.001 (-0.005, 0.004)	-0.002 (-0.007, 0.003)

Immune cells: lymphocyte, neutrophil and monocyte; heavy metals: Pb, Cd, Hg and As. Data of heavy metals and immune cell counts were ln-transformed to normalize the distribution. Data in this table are presented as estimates (95% confidence intervals). The estimates (β value) were calculated by multiple linear regression models. Model 1 represents a crude model without any adjustment; model 2 was adjusted for child age, gender and BMI; model 3 was adjusted for child age, gender and BMI, distance of the road from the residence, milk consumption frequency, parents' education level, daily smoking by family members, parents' residence time, frequency of washing hands before meals, child born locally (Guiyu or Haojiang), child e-waste contact, ventilation of house, e-waste pollution within 50 meters (m) of residence, maternal occupation associated with e-waste dismantling, residence used as a workshop. Values of $*p < 0.05$ was considered statistically significant.

Table S3. Interaction effects of heavy metals on immune regulatory and inflammatory cytokines (n = 147).

	IL-1 β			IL-6			IL-8			TNF- α		
	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3
Pb	0.255 (0.013, 0.511)*	0.211 (- 0.046, 0.469)	-0.180 (- 0.136, 0.497)	0.368 (0.108, 0.628)**	0.335 (0.080, 0.590)*	0.331 (- 0.066, 0.666)	-0.053 (- 0.367, 0.261)	-0.069 (- 0.378, 0.240)	0.140 (- 0.253, 0.532)	0.027 (- 0.214, 0.268)	-0.022 (- 0.254, 0.220)	-0.142 (- 0.455, 0.172)
Cd	0.149 (- 0.053, 0.352)	0.163 (- 0.044, 0.370)	-0.184 (- 0.037, 0.406)	-0.008 (- 0.214, 0.198)	0.001 (- 0.204, 0.206)	0.042 (- 0.192, 0.276)	-0.002 (- 0.250, 0.247)	-0.095 (- 0.342, 0.154)	-0.025 (- 0.299, 0.250)	-0.113 (- 0.304, 0.077)	-0.113 (- 0.308, 0.081)	-0.107 (- 0.326, 0.112)
Hg	-0.233 (- 0.483, 0.017)	-0.196 (- 0.453, 0.061)	-0.226 (- 0.489, 0.038)	-0.428 (- 0.682, 0.175)**	-0.344 (- 0.598, 0.089)**	-0.360 (- 0.639, 0.082)*	-0.173 (- 0.480, 0.133)	-0.056 (- 0.364, 0.252)	-0.059 (- 0.386, 0.268)	-0.083 (- 0.318, 0.152)	-0.042 (- 0.283, 0.199)	-0.066 (- 0.327, 0.195)
As	0.037 (- 0.229, 0.304)	-0.026 (- 0.305, 0.253)	-0.055 (- 0.344, 0.234)	0.141 (- 0.129, 0.412)	-0.002 (- 0.278, 0.274)	-0.086 (- 0.391, 0.220)	0.073 (- 0.254, 0.400)	-0.003 (- 0.337, 0.331)	-0.075 (- 0.433, 0.284)	0.106 (- 0.145, 0.357)	0.056 (- 0.206, 0.317)	-0.057 (- 0.343, 0.229)
Pb * Cd * Hg * As	0.001 (- 0.006, 0.008)	0.001 (- 0.007, 0.007)	-0.001 (- 0.009, 0.007)	-0.004 (- 0.011, 0.004)	-0.005 (- 0.013, 0.002)	-0.006 (- 0.015, 0.002)	0.001 (- 0.008, 0.009)	0.001 (- 0.009, 0.009)	0.001 (- 0.008, 0.011)	0.001 (- 0.005, 0.008)	0.001 (- 0.006, 0.008)	-0.002 (- 0.010, 0.005)
IL-10												
IL-4												
Pb	-0.262 (- 0.548, 0.024)	-0.323 (- 0.610, 0.037)*	-0.161 (- 0.536, 0.214)	-0.012 (- 0.155, 0.130)	-0.054 (- 0.194, 0.086)	-0.047 (- 0.228, 0.135)	0.082 (- 0.247, 0.412)	0.074 (- 0.263, 0.410)	0.100 (- 0.330, 0.530)	-0.068 (- 0.257, 0.121)	-0.100 (- 0.291, 0.091)	-0.065 (- 0.309, 0.180)
Cd	-0.095 (- 0.321, 0.132)	-0.097 (- 0.327, 0.132)	-0.107 (- 0.369, 0.155)	0.003 (- 0.110, 0.116)	-0.001 (- 0.114, 0.111)	-0.020 (- 0.147, 0.107)	-0.182 (- 0.443, 0.079)	-0.143 (- 0.413, 0.128)	-0.232 (- 0.533, 0.068)	0.022 (- 0.127, 0.172)	0.022 (- 0.132, 0.175)	-0.003 (- 0.174, 0.168)
Hg	-0.234 (- 0.512, 0.046)	-0.177 (- 0.463, 0.108)	-0.098 (- 0.410, 0.214)	-0.091 (- 0.230, 0.049)	-0.069 (- 0.209, 0.071)	-0.082 (- 0.233, 0.070)	-0.029 (- 0.351, 0.293)	-0.045 (- 0.381, 0.291)	0.104 (- 0.254, 0.462)	-0.172 (- 0.357, 0.012)	-0.169 (- 0.360, 0.021)	-0.135 (- 0.339, 0.069)
As	0.039 (- 0.259, 0.337)	-0.030 (- 0.339, 0.280)	-0.045 (- 0.387, 0.297)	0.060 (- 0.089, 0.208)	0.045 (- 0.107, 0.196)	0.029 (- 0.137, 0.195)	-0.176 (- 0.520, 0.167)	-0.196 (- 0.560, 0.168)	-0.237 (- 0.629, 0.155)	0.153 (- 0.044, 0.349)	0.160 (- 0.046, 0.367)	0.093 (- 0.130, 0.316)
Pb * Cd * Hg * As	-0.003 (- 0.005, 0.005)	-0.004 (- 0.012, 0.004)	-0.003 (- 0.013, 0.006)	0.002 (- 0.002, 0.006)	0.002 (- 0.002, 0.006)	0.002 (- 0.003, 0.006)	-0.004 (- 0.014, 0.005)	-0.005 (- 0.014, 0.005)	-0.005 (- 0.016, 0.005)	0.001 (- 0.004, 0.006)	0.001 (- 0.004, 0.006)	0.001 (- 0.006, 0.006)

Pro-inflammatory cytokines: IL-1 β , IL-6, IL-8 and TNF- α ; anti-inflammatory cytokines: IL-1RA, IL-4, IL-10 and IL-13; IL: interleukin; TNF: tumor necrosis factor; RA: receptor antagonist. Heavy metals: Pb, Cd, Hg and As. Data of heavy metals and pro-inflammatory cytokines were ln-transformed to normalize the distribution. Data in this table are presented as estimates (95% confidence intervals). The estimates (β value) were calculated by linear regression models. Model 1 represents a crude model without any adjustment; model 2 was adjusted for child age, gender and BMI; model 3 was adjusted for child age, gender and BMI. distance of the road from the residence, milk consumption frequency, parents' education level, daily smoking by family members, parents' residence time, frequency of washing hands before meals, child born locally (Guayu or Haojiang), child e-waste contact, ventilation of house, e-waste pollution within 50 meters (m) of residence, maternal occupation associated with e-waste dismantling, residence used as a workshop. Values of * $p < 0.05$ and ** $p < 0.01$ were considered statistically significant.

Table S4. Half-lives of biomarkers.

Biomarker	Half-life in human blood	References
Pb	30 days	<i>Rehman, et al. 2018</i>
Cd	10 to 30 years	<i>Järup, et al. 2009</i>
Hg	70 days	<i>Bernhoft, et al. 2012</i>
As	4 days	<i>Hughes, et al. 2006</i>
neutrophils	5.4 days	<i>Pillay J, et al. 2010</i>
monocytes	71 hours	<i>Patel AA, et al. 2017</i>
lymphocytes	41 days for total B lymphocytes; 63 days for CD4 ⁺ T lymphocytes; 93 days for CD8 ⁺ T lymphocytes	<i>Kaur A, et al. 2008</i>
Interleukin (IL)-1 β	2.5 hours	<i>Hazuda DJ, et al.1988</i>
IL-6	15.5 hours	<i>Kuribayashi T. 2018</i>
IL-8	4 hours	<i>Redl H, et al. 1991</i>
Tumor necrosis factor (TNF)- α	18.2 min	<i>Oliver JC, et al. 1993</i>
IL-1 receptor antagonist (IL-1 RA)	21 mins	<i>Granowitz EV, et al. 1992</i>
IL-4	15 mins	<i>Khodoun M, et al. 2008</i>
IL-10	2.7 to 4.5 hours	<i>Saxena A, et al. 2015</i>
IL-13	3.3 hours	<i>Khodoun M, et al. 2008</i>

References:

1. Rehman K, Fatima F, Waheed I, Akash MSH. Prevalence of exposure of heavy metals and their impact on health consequences. *J Cell Biochem.* 2018;119(1):157-184.
2. Järup L, Akesson A. Current status of cadmium as an environmental health problem. *Toxicol Appl Pharmacol.* 2009;238(3):201-208.
3. Bernhoft RA. Mercury toxicity and treatment: a review of the literature. *J Environ Public Health.* 2012; 2012:460508.
4. Hughes MF. Biomarkers of exposure: a case study with inorganic arsenic. *Environ Health Perspect.* 2006;114(11):1790-1796.
5. Pillay J, den Braber I, Vrsekoop N, et al. In vivo labeling with ²H₂O reveals a human neutrophil lifespan of 5.4 days. *Blood.* 2010;116(4):625-627.
6. Patel AA, Zhang Y, Fullerton JN, et al. The fate and lifespan of human monocyte subsets in steady state and systemic inflammation. *J Exp Med.* 2017;214(7):1913-1923.
7. Kaur A, Di Mascio M, Barabasz A, et al. Dynamics of T- and B-lymphocyte turnover in a natural host of simian immunodeficiency virus. *J Virol.* 2008;82(3):1084-1093.
8. Hazuda DJ, Lee JC, Young PR. The kinetics of interleukin 1 secretion from activated monocytes. Differences between interleukin 1 alpha and interleukin 1 beta. *J Biol Chem.* 1988;263(17):8473-8479.
9. Kuribayashi T. Elimination half-lives of interleukin-6 and cytokine-induced neutrophil chemoattractant-1 synthesized in response to inflammatory stimulation in rats. *Lab Anim Res.* 2018;34(2):80-83.
10. Redl H, Schlag G, Bahrami S, Schade U, Ceska M, Stütz P. Plasma neutrophil-activating peptide-1/interleukin-8 and neutrophil elastase in a primate bacteremia model. *J Infect Dis.* 1991;164(2):383-388.
11. Oliver JC, Bland LA, Oettinger CW, et al. Cytokine kinetics in an in vitro whole blood model following an endotoxin challenge. *Lymphokine Cytokine Res.* 1993;12(2):115-120.
12. Granowitz EV, Porat R, Mier JW, et al. Pharmacokinetics, safety and immunomodulatory effects of human recombinant interleukin-1 receptor antagonist in healthy humans. *Cytokine.* 1992;4(5):353-360.
13. Khodoun M, Lewis CC, Yang JQ, et al. Differences in expression, affinity, and function of soluble (s)IL-4Ralpha and sIL-13Ralpha2 suggest opposite effects on allergic responses [published correction appears in *J Immunol.* 2008 Jan 1;180(1):664. Lewis, Christina [corrected to Lewis, Christina C]]. *J Immunol.* 2007;179(10):6429-6438.
14. Saxena A, Khosraviani S, Noel S, Mohan D, Donner T, Hamad AR. Interleukin-10 paradox: A potent immunoregulatory cytokine that has been difficult to harness for immunotherapy. *Cytokine.* 2015;74(1):27-34.
15. Khodoun M, Lewis CC, Yang JQ, et al. Differences in expression, affinity, and function of soluble (s)IL-4Ralpha and sIL-13Ralpha2 suggest opposite effects on allergic responses [published correction appears in *J Immunol.* 2008 Jan 1;180(1):664. Lewis, Christina [corrected to Lewis, Christina C]]. *J Immunol.* 2007;179(10):6429-6438.

6

TOLL-LIKE RECEPTOR, NF-KB AND P38 MAPK SIGNALLING ACTIVATION IN MONOMAC 6 CELLS EXPOSED TO HEAVY METALS LEAD AND CADMIUM

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ABSTRACT

Heavy metals lead (Pb) and cadmium (Cd) impair host defense to pathogens, while the precise mechanisms are not fully elucidated. This study aimed to demonstrate whether Toll-like receptor (TLR) and NF- κ B activation are induced by Pb and Cd. Using Monomac 6 cells, we evaluated how Pb and Cd induce cell death and cell apoptosis, as well as cell activation and inflammatory cytokine release of monocytes; we also studied the activation of the p38 MAPK pathway in these cells by Pb and Cd. The activation and inhibition assays in TLR reporter cell lines showed that Pb exposure enhanced agonist-induced activation of TLR4, 5 and 8, but inhibited agonist-induced activation of TLR3 and 9. Exposure to the high Cd concentration attenuated agonist-mediated TLR2, 4, 5, 7 and 8 activations; on the other hand, Cd also increased agonist-induced activation of TLR, 4, 5, and 8 at a relatively low concentration. In Monomac 6 cells, Pb and Cd induced cell death and cell apoptosis in the absence and presence of LPS. Pb slightly increased ICAM-1 expression, while both Pb and Cd decreased LPS-induced ICAM-1 expression on Monomac 6 cells. LPS-mediated pro-inflammatory TNF- α production of MonoMac 6 was elevated by Pb, while anti-inflammatory IL-10 was down-regulated by both Pb and Cd exposure. Moreover, Cd exposure was found to elevate phospho-p38 expression. LPS-induced p38 phosphorylation was up-regulated by Pb and Cd, indicating that Pb and Cd exposure could promote LPS-mediated p38 MAPK activation in MonoMac 6. Taken together, our findings revolving around TLRs, NF- κ B and p38 MAPK in MonoMac 6 provide an insight into a possible mechanism that Pb and Cd exposure may disturb immune reactions of monocytes affecting host resistance to pathogens.

1. Introduction

The heavy metals lead (Pb) and cadmium (Cd) are ubiquitous environmental pollutants. Various mechanical studies concerning Pb and Cd toxicity demonstrated that both Pb or Cd could result in decreased cell viability, enhanced oxidative stress and lipid peroxidation, mitochondrial and DNA damage, cohesion loss and cell apoptosis [1-4]. Exposure to Pb and Cd has also been shown to exert detrimental effects on the innate immune cells, even at low concentrations [5, 6]. Studies indicated that Pb negatively affected chemotaxis in neutrophils, and decreased phagocytosis and eicosanoid metabolism in macrophages [7-9]. Cd could induce oxidative stress of macrophages and mast cells in response to infections [10]. In vivo, Cd exposure has been reported to be associated with an increase in neutrophil counts in children from an electronic waste recycling area and monocyte counts in Cd-exposed workers [11, 12].

The mechanism by which Pb and Cd affect the innate immune cells is not precisely known. It has been suggested that these heavy metals affect Toll-like receptor (TLR) signalling. TLRs serve a vital role in host defense combating invading infectious agents. In the vertebrate innate immune system, TLRs sense and recognize diverse damage/danger-associated molecular patterns (DAMPs) generated by the host itself, as well as pathogen-associated molecular patterns (PAMPs) from bacteria, fungi, parasites and viruses [13-15]. To date, 10 TLRs in humans have been well demonstrated to be involved in the process of adaptive immune and inflammatory responses [13, 16, 17]. All TLRs except TLR3 utilize a common myeloid differentiation primary response protein (MyD) 88 mediated by Toll-IL-1 receptor (TIR) domains resulting in the activation of the downstream signalling pathways nuclear factor-kappa B (NF- κ B) and mitogen-activated protein kinase (MAPK), which trigger inflammatory cytokine productions and cell apoptosis [13, 16].

Although various studies have shown the effects of Pb and Cd on TLRs, the effects of Pb or Cd on TLR activation remain unclear. Luna et al. considered TLR4 as a target of environmentally relevant Pb exposure (≤ 5 μ g/dL established by the United States Centers for Disease Control and Prevention) [18]. Studies concerning gene expressions observed that TLR3, TLR5, and TLR9 in the Madin-Darby canine kidney cell line and TLR2 and TLR4 in rat kidney tissue were up-regulated by Cd treatment [19, 20]. Although little is known about the effects of Pb and Cd on TLR activation, the TLR signalling pathway has been associated with other heavy metals,

such as mercury, copper, nickel and zinc. For instance, Pollard et al. showed that mercury elicited NF- κ B activation and pro-inflammatory cytokine expression via endosomal TLR7 and TLR9 trafficking and signalling [21]. Schmidt et al. identified nickel ion as one of the chemical activators of the TLR system, which activated human TLR4 to promote the inflammatory response through NF- κ B-, p38- and IRF3-dependent signal pathways [22]. An in vivo study by Hu et al. revealed that zinc supplementation is beneficial to protect intestinal tissue from inflammation via inhibiting the TLR4-MyD88 signalling pathways [23]. Similar observations made by Jarrousse et al. demonstrated that zinc could inhibit TLR2 expression to alleviate inflammation [24].

In the present study, we hypothesize that Pb and Cd may affect the immune system via TLRs. Therefore, this study aimed to determine whether heavy metals Pb and Cd exposure alters the immune cell activation by interacting with TLRs and the downstream NF- κ B and p38 MAPK signalling. We used a TLR reported cell line (HEK-Blue™-hTLR2, 3, 4, 5, 7, 8 and 9) to study whether Pb and Cd could activate or inhibit TLRs and NF- κ B signalling. We also used the MonoMac 6 cell line to study p38 MAPK activity, cell apoptosis, and inflammatory cytokine production following exposure to Pb and Cd.

2. Materials and Methods

Experimental setup: The human monocytic leukemia reporter cell line (THP1-XBlue™-MD2-CD14) was used to evaluate whether Pb and Cd could activate or inhibit monocytes via TLRs; HEK-Blue™ reporter cells were used to identify which TLR signalling can be specifically activated or inhibited by Pb and Cd. After showing that Pb and Cd affected TLR activation, we next explored the effect of Pb and Cd on MonoMac 6 cells, a monocytic cell line, by studying the effects of Pb and Cd on cell apoptosis and cell activation as measured by ICAM-1 expression and TNF- α and IL-10 production. Finally, we studied the effects of Pb and Cd on p38 MAPK activation in MonoMac 6 cells.

2.1. Lead and Cadmium

Lead chloride (PbCl₂) and cadmium chloride (CdCl₂) were purchased from Sigma-Aldrich, the Netherlands, then diluted in sterile water (Fresenius Kabi, France) and filtered by 0.22 μ M

sterile syringe filters (Sigma-Aldrich, The Netherlands) prior to cell stimulation. Endotoxin measurements for PbCl₂ and CdCl₂ stock solutions were carried out by pierce LAL chromogenic endotoxin quantitation kit (Thermo Fisher Scientific, USA) and no endotoxin contamination was found. Cells including THP1 and HEK-Blue reporter cells, MonoMac 6 were treated with PbCl₂ at the final concentration of 0.18 (equal to 50 μ g/L), 1.8, 18 and 100 μ M, or with CdCl₂ at the concentration of 0.027 (equal to 5 μ g/L), 0.27, 2.7 and 10 μ M.

2.2. Reporter cell stimulation and Quanti-Blue assay

The human monocytic leukemia reporter cell line (THP1-XBlue™-MD2-CD14) (InvivoGen, Toulouse, France) and seven human embryonic kidney (HEK) reporter cell lines (HEK-Blue™-hTLR2, 3, 4, 5, 7, 8 and 9) (InvivoGen, Toulouse, France) were applied to explore the effects of Pb and Cd on TLR signalling in this study. As previously described [25, 26], THP1-XBlue™-MD2-CD14 can produce the human TLRs endogenously and carry an insert for MD2 and CD14 that boosts TLRs/NF- κ B signalling. Not only THP1-XBlue™-MD2-CD14 but also the HEK-Blue cell lines contain a Secreted Embryonic Alkaline Phosphatase (SEAP) construct, which is coupled to the NF- κ B/AP-1 promotor and is capable of expressing SEAP once TLR signalling and NF- κ B are activated. The released SEAP in the supernatant can be measured using Quanti-Blue reagent (InvivoGen, Toulouse, France).

THP1-XBlue™-MD2-CD14 cells were cultured in RPMI 1640 medium (Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated fetal calf serum (FCS, Sigma-Aldrich, The Netherlands), 1.5 g/L sodium bicarbonate (Boom B.V. Meppel, The Netherlands), 100 mg/mL Normocin™ (Invivogen, Toulouse, France), 2 mM L-glutamine, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate and 50 U/mL Penicillin, 50 μ g/mL Streptomycin, all purchased from Sigma Aldrich according to the recommendations of the supplier. To measure the TLR activation in THP1, cells were kept at a concentration of 1.0×10^6 cells/mL as presented in **Table 1** and cultured in 200 μ L per well with different dosages of PbCl₂ or CdCl₂ in a flat-bottom 96-well plate (Corning, Amsterdam, the Netherlands). The negative and positive control was treated with medium and 100 ng/mL LPS (InvivoGen, Toulouse, France), respectively. After 24-hour incubation, the supernatant was taken out to a new 96-well plate and mixed with Quanti-Blue reagent in a ratio of 1:10 and incubated for 1 h at 37°C incubator. The measurement of absorbance at 650 nm was performed on a Bio-Rad Benchmark Plus

microplate reader and data acquisition was obtained by Microplate Manager version 5.2.1 (Bio-Rad Laboratories, USA). The color change for each well was shown as the fold-change of NF- κ B activation normalized to the negative control.

HEK-Blue cell lines were cultured in Dulbecco's modified eagle's medium (DMEM) (Lonza, Basel, Switzerland) containing 10% heat-inactivated FCS, 2 mM L-glutamine, 4.5 g/L glucose, 50 U/mL penicillin and 50 mg/mL streptomycin, all ordered from Sigma Aldrich and 100 mg/mL Normocin (Invivogen, Toulouse, France) according to the recommendations of the supplier. To maintain stable expression of TLR genes, the selection antibiotics in **Table 1** were added in the medium of reporter cell lines. When cells reached approximately 80% confluence, to assess the activation and inhibition of individual TLR signalling, they were diluted at their respective density and seeded in a total of 200 μ L per well in the 96-well flat bottom plate according to manufacturer's instructions (**Table 1**). The negative and positive control was treated with medium and or the corresponding TLR agonists, respectively (all from InvivoGen, see **Table 1**). For activation assays, the procedure was similar to that in THP1 cells, as described above. For inhibition assays, cells were seeded at a relevant density and incubated with different concentrations of PbCl₂ or CdCl₂ for 1 h prior to adding individual TLR agonists. For both the activation and inhibition assay, after 24 h of incubation, the supernatant was diluted 1:10 with Quanti-Blue reagent and incubated for 1 h at 37°C incubator. The absorbance at 650 nm was measured as mentioned before. Data for each well was presented as the fold-change of NF- κ B activation compared to the positive control (inhibition assay) or the negative control (activation assay). Per experiment, each condition has three replications and each experiment was repeated 6 times.

Table 1. Cell densities and agonists applied in the reporter cell line assays.

Cell line	Cell density (cell/mL)	Selection antibiotics	Agonist (positive control)
THP1-MD2-CD14	1.0×10^6	Zeocin (100 μ g/mL)	LPS, 100 ng/mL
HEK-Blue-hTLR2	2.8×10^5	G418 (100 mg/mL)	Escherichia coli K12 lipopolysaccharide
		HEK-Blue (1 μ L/mL)	Pam3CSK4, 100 ng/mL
HEK-Blue-hTLR3	2.8×10^5	Blasticidin (30 μ g/mL)	synthetic tripalmitoylated lipopeptide
		Zeocin (100 μ g/mL)	Poly (I:C) LMW, 5 μ g/mL
HEK-Blue-hTLR4	1.4×10^5	HEK-Blue (1 μ L/mL)	Poly (I:C) low molecular weight
			LPS, 100 ng/mL
HEK-Blue-hTLR5	1.4×10^5	Blasticidin (30 μ g/mL)	Escherichia coli K12 lipopolysaccharide
		Zeocin (100 μ g/mL)	FLA-ST, 10 ng/mL
HEK-Blue-hTLR7	2.2×10^5	Blasticidin (10 μ g/mL)	Salmonella typhimurium derived flagellin
		Zeocin (100 μ g/mL)	CL264, 5 μ g/mL
HEK-Blue-hTLR8	2.2×10^5	Blasticidin (30 μ g/mL)	9-Benzyl-8 hydroxyadenine derivative
		Zeocin (100 μ g/mL)	ssRNA40/LyoVec, 2.5 μ g/mL
HEK-Blue-hTLR9	4.5×10^5	Blasticidin (10 μ g/mL)	Single stranded RNA complexed with LyoVec
		Zeocin (100 μ g/mL)	ODN2006, 0.25 μ M
			Type B CpG oligonucleotide

2.3. MonoMac 6 cell culture

To explore the effects of Pb and Cd on monocyte activation and apoptosis, a human monocytic leukemia cell line, MonoMac 6, was used. Cells were cultured in RPMI1640 (Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, The Netherlands), 2 mM glutamine (Lonza, Basel, Switzerland), 1 mM sodium pyruvate (Lonza, Basel, Switzerland), 50 μ M mercapto-ethanol (Merck, Germany), 60 μ g/mL gentamycin (Lonza, Basel, Switzerland), and 2.2 μ g/mL amphotericin B (Thermo Fisher Scientific, USA) at 37°C in a humidified atmosphere containing 5% CO₂. Cells were diluted with the culture medium at 1:5 ratios in a new flask twice a week.

2.4. Cell viability assay by WST-1

WST-1 assay was conducted according to the manufacturer's protocol. Briefly, MonoMac 6 cells were harvested and were seeded in the 96-well flat-bottom plate (Corning, Amsterdam, the Netherlands) at the cell density of 2.0×10^5 cells/mL. After treatment with various doses of PbCl₂ or CdCl₂ in the presence or absence of 100 ng/mL LPS for 24 h, 10 μ L cell proliferation reagent WST-1 (Thermo Fisher Scientific, USA) was added to each well and kept in the plate

at 37°C for 4 h. The absorbance of each well was read at 440 nm, meanwhile, 620 nm was set as the reference wavelength. The relative cell viability of each group was presented as the percentage calculated by comparison to that of the control group treated with the medium. This experiment was performed in triplicates for each condition and repeated three times.

2.5. FACS analysis of cell apoptosis

MonoMac 6 cells (2.0×10^5 cells/mL) were seeded in a 12-well plate (Corning, Amsterdam, the Netherlands) and incubated with different concentrations of PbCl₂ or CdCl₂ in the presence or absence of 100 ng/mL LPS for 24 h. Cells treated with the medium were considered as the negative control, and 100 ng/mL LPS were served as the positive control. Fluorescein isothiocyanate (FITC)-conjugated AnnexinV (BioLegend, Uithoorn, The Netherlands) and propidium iodide (PI) (Invitrogen, USA) staining were performed for apoptotic test. In brief, cells were harvested in Falcon round-bottom polypropylene tubes (Corning, Amsterdam, the Netherlands) and washed twice with 1 ml cold PBS. After spinning at 4 °C, $300 \times g$ for 5 min, cell pellets in each tube were resuspended in 100 µL binding buffer (0.01M HEPES, 140 mM NaCl, 2.5 mM CaCl₂, PH7.4) with 5 µL Annexin V and 1 µL PI staining solution. The mixture was incubated at room temperature in the dark for 15 min. Afterward 1 mL PBS was added to each tube. After Spinning at 4 °C, $300 \times g$ for 5 min, cell pellet was resuspended with 400 µL PBS containing 2% heat-inactivated FCS. Annexin V and PI staining was determined by using a BD FACSVerse flow cytometer (BD Biosciences, USA). Data analysis for the percentage of live, early apoptotic and late apoptotic (or necrotic) cells in each condition were obtained by Kaluza 2.1 (Beckman Coulter, USA).

2.6. ICAM-1 expression

To assess the ICAM-1 expression on MonoMac 6, cells were treated in the same way as stated above for the apoptotic test. After washing twice with cold PBS, cells in each FACS tube were stained with 5 µL FITC-conjugated ICAM-1 or 5 µL FITC mouse IgG1, κ Isotype control antibody (all from BioLegend) in $1 \times$ PBS containing 2% heat-inactivated FCS at a final volume of 50 µL and incubated at room temperature in the dark for 30 min. The mixture was washed twice with 2 ml of cold PBS by centrifugation at 4 °C, $300 \times g$ for 5 min, then cell pellet was suspended in 300 µL $1 \times$ PBS containing 2% heat-inactivated FCS. Finally, flow cytometric data acquisition was collected immediately by a BD FACSVerse instrument and data analysis was performed by Kaluza 2.1 (Beckman Coulter, USA).

2.7. Quantification of TNF- α and IL-10 by ELISA

To measure cytokine TNF- α and IL-10 production by MonoMac 6 cells following exposure to PbCl₂ or CdCl₂ in the presence or absence of 100 ng/mL LPS for 12 h; thereafter, the supernatants were collected. The 12 hr activation period was chosen, since a pilot experiment showed that this incubation with PbCl₂ or CdCl₂ and LPS showed maximal cytokine production (results not shown). The commercially available ELISA kits from R&D systems (Thermo Fisher Scientific, USA) for human TNF-alpha (Catalog number: DY210) and IL-10 DuoSet (Catalog number: DY217B), were used to detect cytokine concentration via sandwich assay according to the manufacturer's instructions. TNF- α and IL-10 concentrations were calculated based on standard curves and expressed as pg/mL. The values below the limits of the assay's detectability were depicted as 0.

2.8. Western blot analysis for p38 MAPK signalling pathway

MonoMac 6 Cells were challenged with the various dosages of PbCl₂ or CdCl₂ as described above with or without 100 ng/mL LPS and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 0.5 h. A pilot experiment revealed optimal results after stimulation with PbCl₂ or CdCl₂ and LPS for 0.5 h (results not shown). Cells from each set of experiments at 0.5-hour time points were solubilized in ice-cold 1 $\times$ RIPA lysis buffer (Thermo Fisher Scientific, USA) containing protease and phosphatase inhibitor cocktail (Sigma-Aldrich, The Netherlands). After sonication, the lysates were separated by 10-min 14000 $\times$ g centrifugation at 4 °C. Proteins in the supernatant were collected and protein concentrations were measured by using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. An equal amount of 15 μ g heat-denatured total protein was loaded into each well of 15-well sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE with 10% acrylamide). After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF, Millipore, USA) membranes. The membranes were blocked for 1 h with 5% bovine serum albumin (BSA) in 1 $\times$ Tris-buffered saline with 1% Tween 20 (1 $\times$ TBST buffer), then incubated overnight at 4 °C with specific primary antibody for phospho-p38 (pp38 Thr180/Tyr182, rabbit-anti-human, 1:1000; Cell Signalling Technology, USA), p38 (mouse-anti-human, 1:400; Santa Cruz Biotechnology, USA) and β -actin (mouse-anti-human, 1:2500; Santa Cruz Biotechnology, USA). The following day, the membranes were washed with 1 $\times$ TBST buffer three times and incubated with the corresponding peroxidase-conjugated

secondary antibody (anti-rabbit, 1:5000; or anti-mouse, 1:5000; all from Dako, Agilent, Denmark) for 1 h. After adding Supersignal™ west pico plus chemiluminescent substrate (Thermo Fisher Scientific, USA), protein band intensity was quantified by ChemiDoc Imaging and ChemiDoc MP Imaging System (Bio-Rad Laboratories, USA). The data were processed and analyzed by Image J software (bundled with 64-bit Java 1.8.0_112 for windows system, from <https://imagej.nih.gov/ij/>). The target protein expression was normalized to the band intensity of p38 or β -actin on the same membrane. Each experiment was repeated 6 times.

2.9. Statistical analysis

All results were analyzed using Graphpad prism software version 8.0 (GraphPad, San Diego, CA, USA). Normality of data distribution was confirmed using the Kolmogorov-Smirnov test. Normally distributed data were expressed as mean $\pm$ standard error of the mean (SEM) and non-normally distributed data were presented as median with interquartile range. The groups treated with LPS, PbCl₂ or CdCl₂ only were compared with the negative control, while the comparison between groups incubated PbCl₂ or CdCl₂ with LPS and the positive control was performed. Parametrically distributed data were assessed using the one-way repeated measures analysis of variance (rmANOVA) followed by Dunnet's post-test. Nonparametric data were log-transformed and further analyzed using the rmANOVA with Dunnet's post-test. Significant difference level was defined as $P < 0.05$. A $P < 0.1$ was considered to be a statistical trend.

3. Results

3.1. Exposure to PbCl₂ may have an inhibitory effect on TLR8 activation

To explore whether PbCl₂ and CdCl₂ are able to activate or inhibit TLRs/NF- κ B signalling, TLR reporter cell lines were used. We first made sure that no endotoxin components existed in the solutions with PbCl₂ or CdCl₂. We found that that PbCl₂ and CdCl₂ stock solutions were endotoxin-free (data not shown).

To investigate the possible activation and inhibitory actions of metals on TLRs/NF- κ B signalling, THP1-MD2-CD14 and HEK-Blue reporter cell lines expressing TLRs (2, 3, 4, 5, 7, 8 and 9) were exposed to PbCl₂ and CdCl₂ without (**Figure 1**) or with (**Figure 2**) corresponding agonists. As presented in **Figure 1**, PbCl₂ did not activate TLRs in THP1-MD2-CD14 cells or

any of the TLR-expressing HEK reporter cells. CdCl₂ at the concentration of 10 μ M was found to inhibit TLR8 activation (0.69-fold change, $P < 0.01$, **Figure 1G**).

Figure 1. Activation of THP1 and HEK reporter cells carrying individual TLRs. Cells cultured with medium served as the negative control, cells treated with their corresponding TLR agonist served as the positive control. Data are presented as mean $\pm$ SEM (n=6). The negative control was set to 1 and all other data were presented relative to this negative control. Significant difference compared to the negative control were determined by rmANOVA followed by Dunnet's post-test. *** $P < 0.001$, ** $P < 0.01$.

3.2 TLRs/NF- κ B activations were altered in response to PbCl₂ and CdCl₂ with agonists

Next, we evaluated whether PbCl₂ or CdCl₂ affected the responses of TLRs to their agonists (**Figure 2**). In contrast to the previous results that showed no activation of TLRs by PbCl₂ or CdCl₂, they both affected the response of TLRs to their agonists. Although PbCl₂ did not affect the response of TLR2 or TLR7 towards their respective ligands, it did significantly increase the LPS-induced TLR4 activation at the concentrations of 0.18 μ M (1.35-fold change, $P < 0.05$) and 1.8 μ M (1.38-fold change, $P < 0.05$). TLR5 signalling was increased by PbCl₂ (1.18-fold change, $P < 0.05$) at the dosages of 0.18, 1.8 and 18 μ M (1.18, 1.13 and 1.14-fold change, respectively, all $P < 0.05$) when agonist FLA-ST existed. PbCl₂ at the concentration of 10 μ M had a tendency to increase the FLA-ST-induced TLR5 activation ($P < 0.1$). TLR8 activation by its agonist ssRNA40 was enhanced in the presence of 0.18 μ M PbCl₂ (1.82-fold change, $P < 0.001$). However, TLR3 and TLR9 activation by their agonists were suppressed due to the existence of 100 μ M PbCl₂ (0.88 and 0.79-fold change, respectively, both $P < 0.05$).

CdCl₂ decreased TLR2/1 activation by its agonist Pam3CSK4 at the concentration of 2.7 μ M and 10 μ M (0.70 and 0.51-fold change, respectively; both $P < 0.001$). TLR3 activation by its

agonist Poly (I:C) LMW was elevated in the presence of 2.7 μM CdCl_2 (1.42-fold change, $P < 0.001$). LPS-induced TLR4 activation was enhanced by CdCl_2 at 0.27 μM (1.25-fold change, $P < 0.05$), but was inhibited at 2.7 and 10 μM (0.46 and 0.31-fold change, respectively, both $P < 0.001$). Intriguingly, TLR5 activation by its agonist FLA-ST was increased by CdCl_2 at 0.027 and 2.7 μM (1.16 and 1.18-fold change, respectively; both $P < 0.05$), while TLR5 activation was decreased with a higher CdCl_2 dosage (10 μM , 0.64-fold change, $P < 0.001$). TLR7 activation by its agonist CL264 was suppressed in the presence of 10 μM CdCl_2 (0.58-fold change, $P < 0.05$). TLR8 activation by its agonist ssRNA40 was increased by 0.027 and 0.27 μM of CdCl_2 (1.48 and 1.56-fold change, respectively, both $P < 0.05$) but was suppressed by 10 μM of CdCl_2 (0.51-fold change, $P < 0.05$). CdCl_2 did not affect the response of TLR9 to its ligand.

Figure 2. Effects of PbCl_2 and CdCl_2 on HEK reporter cells carrying individual TLRs activated by their respective ligands. Cells were incubated with different doses of metals in combination with their corresponding agonist. Data are presented as mean $\pm$ SEM ($n=6$). The positive control, which is the relevant agonist, was set to 1. Significant difference compared to the positive control were determined by Repeated Measures ANOVA followed by Dunnet's test. Values of $P < 0.05$ were considered significant. *** $P < 0.001$, ** $P < 0.05$, * $P < 0.05$, # $P < 0.1$.

3.3. PbCl_2 and CdCl_2 exposure caused apoptosis in MonoMac 6 cells

As mentioned above, TLR signalling by agonists can be affected in the presence of PbCl_2 and CdCl_2 . We next used MonoMac 6 cells to further investigate the effects of PbCl_2 and CdCl_2 on monocytes. We focused on TLR4 since this TLR was one of the TLRs mostly affected by the presence of PbCl_2 and CdCl_2 . Therefore, we used LPS to stimulate MonoMac 6 cells via TLR4.

We studied toxicity, apoptosis, activation, cytokine production and activation of the p38 MAPK signalling pathway.

The WST-1 cell proliferation assay was performed to study the viability of MonoMac 6 cells challenged with PbCl₂ and CdCl₂. **Table 2** shows that viability decreased little as a result of treatment with either PbCl₂ or CdCl₂.

Table 2. MonoMac 6 Cell viability following treatment with PbCl₂ and CdCl₂ with or without LPS.

Compounds	n	mean $\pm$ SEM (%)
Negative control (medium)	3	100
Positive control (100 ng/mL LPS)	3	90.24 $\pm$ 0.83
PbCl ₂ (0.18 μ M)	3	89.54 $\pm$ 2.98
PbCl ₂ (1.8 μ M)	3	89.87 $\pm$ 3.23
PbCl ₂ (18 μ M)	3	88.34 $\pm$ 3.38
PbCl ₂ (100 μ M)	3	88.72 $\pm$ 2.06
PbCl ₂ (0.18 μ M) + LPS (100 ng/mL)	3	86.80 $\pm$ 1.92
PbCl ₂ (1.8 μ M) + LPS (100 ng/mL)	3	81.30 $\pm$ 3.01
PbCl ₂ (18 μ M) + LPS (100 ng/mL)	3	78.56 $\pm$ 4.47
PbCl ₂ (100 μ M) + LPS (100 ng/mL)	3	77.10 $\pm$ 4.11
CdCl ₂ (0.027 μ M)	3	91.48 $\pm$ 1.63
CdCl ₂ (0.27 μ M)	3	86.95 $\pm$ 0.81
CdCl ₂ (2.7 μ M)	3	86.80 $\pm$ 5.42
CdCl ₂ (10 μ M)	3	85.73 $\pm$ 6.04
CdCl ₂ (0.027 μ M) + LPS (100 ng/mL)	3	89.25 $\pm$ 2.29
CdCl ₂ (0.27 μ M) + LPS (100 ng/mL)	3	83.46 $\pm$ 3.34
CdCl ₂ (2.7 μ M) + LPS (100 ng/mL)	3	83.54 $\pm$ 3.16
CdCl ₂ (10 μ M) + LPS (100 ng/mL)	3	81.80 $\pm$ 4.30

Since the WST-1 assay showed an effect of PbCl₂ and CdCl₂ on cell viability, to further assess the toxicity of PbCl₂ and CdCl₂ on MonoMac 6 cells, we studied the induction of apoptosis of MonoMac 6 cells following treatment with PbCl₂ or CdCl₂ in the absence or presence of LPS. After 24-hour treatment (**Figure 3**), all 4 dosages of PbCl₂ slightly, but significantly decreased the percentage of living cells (0.18 μ M: 85.4%, 1.8 μ M: 85.3%, 18 μ M: 83.6%, 100 μ M: 82.0%) compared with the negative control (86.7%) (all $P < 0.01$). This was associated with higher percentages of both early and late apoptosis in MonoMac 6 cells, especially at the concentrations of 0.18 (early: 20.5%, late: 6.80%), 18 (early: 11.1%, late: 4.33%) and 100 μ M

(early: 11.8%, late: 4.95%). Furthermore, all 4 dosages of PbCl₂ aggravated both the early and late apoptosis of MonoMac cells in the presence of LPS (all $P < 0.001$, **Figure 3D** and **3F**).

Likewise, CdCl₂ treatment affected cell survival of MonoMac 6. Due to the presence of 2.7 and 10 μM of CdCl₂, the percentage of living cells (84.7% and 77.6%, respectively, versus the negative control: 86.7%) was but significantly decreased (both $P < 0.001$, **Figure 3C**). All 4 dosages of CdCl₂ helped decrease living cell percentage induced by LPS (all $P < 0.001$). The early apoptotic percentage was found increased in MonoMac 6 treated with 0.027 and 10 μM of CdCl₂ (25.6% and 11.9%, respectively, versus the negative control: 9.6%) (both $P < 0.001$). Similar findings were determined that CdCl₂ at 0.027, 2.7 and 10 μM (9.82%, 4.24% and 6.36%, respectively, versus the negative control: 3.34%) induced an elevation in late apoptosis (all $P < 0.01$, **Figure 3G**). Moreover, all 4 dosages of CdCl₂ were observed to promote the pro-apoptotic effects (both early and late apoptotic) of LPS on MonoMac 6 (all $P < 0.001$).

Figure 3. Apoptotic test of MonoMac 6 cells treated with different doses of PbCl₂ and CdCl₂ in the absence or presence of LPS. (A) Gating strategy to select living, early and later apoptotic cells in MonoMac 6 cells. Cells were plated at a density of 2×10^5 cells/mL in a 12-well plate for 24 h. Conditions in each time were performed in triplicate. FACS data were obtained by the BD FACSVerse flow cytometer after Annexin V-FITC/PI staining. (B) Results were expressed as mean $\pm$ SEM from 6 independent experiments and analyzed by using One-Way Repeated Measures ANOVA followed by Dunnett's post-test. Versus control without LPS: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; Versus control with LPS: ### $P < 0.001$.

3.4. PbCl₂ and CdCl₂ increased LPS-induced cytokine production in MonoMac 6 cells

We further evaluate whether PbCl₂ and CdCl₂ incubation resulted in the activation of MonoMac 6 cells, as measured by the expression of ICAM-1 (an activation marker of monocytes), and production of TNF- α and IL-10.

ICAM-1, also known as CD54, is an adhesion molecule in immune-mediated and inflammatory processes and functions as a co-stimulatory signal that is functionally implicated in trans-endothelial trafficking of leukocytes and T cells priming [27]. Our results showed Monocyte ICAM-1 is upregulated upon activation with PbCl₂ at 0.18 and 18 μ M (4.81% and 4.58%, respectively, versus the negative control 3.25%, both $P < 0.05$, **Figure 4B**). Besides, PbCl₂ at 18 and 100 μ M had a tendency to reduce ICAM-1 expression induced by LPS (both $P < 0.1$). The effect of CdCl₂ on ICAM-1 expression was not found. Nevertheless, LPS induced ICAM-1 expression (57.7%) was inhibited by CdCl₂ at the concentration of 2.7 and 10 μ M (52.6% and 50.6%, respectively, both $P < 0.01$) ($P < 0.01$, **Figure 4C**).

Figure 4. ICAM-1 expression on MonoMac 6 cells challenged with PbCl₂ and CdCl₂ in the absence or presence of LPS. (A) Gating strategy ICAM-1 positive cells in MonoMac 6 cells. Cells were stained with FITC-conjugated antibody; the evaluation of ICAM-1 positive cell percentage was performed by flow cytometry. ICAM-1 positive population in each sample was gated compared to the isotype control (set to 1%). (B) Data are presented as mean $\pm$ SEM ($n = 6$). Significant differences compared to the negative control were determined by using One-Way Repeated Measures ANOVA followed by Dunnett's post-test. Versus control without LPS: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; Versus control with LPS: ### $P < 0.001$, ## $P < 0.01$, # $P < 0.1$.

Since in a pilot study, PbCl₂ and CdCl₂ in the lower concentrations with or without LPS did not affect the production of TNF- α and IL-10 (data not shown), in the present experiment we only exposed cells to the highest concentration of metals with or without LPS in order to evaluate whether PbCl₂ or CdCl₂ were able to affect the cytokine response of MonoMac 6 cells to LPS. Although PbCl₂ itself did not alter the production of the pro-inflammatory cytokine TNF- α , it increased LPS-stimulated TNF- α expression ($P < 0.05$, **Figure 5**). CdCl₂ did also not affect TNF- α production nor did it affect the LPS-induced TNF- α production. The anti-inflammatory cytokine IL-10 was not affected by either PbCl₂ or CdCl₂ alone. However, the LPS-induced IL-10 production was significantly down-regulated by both PbCl₂ and CdCl₂ (both $P < 0.001$).

Figure 5. TNF- α and IL-10 production by MonoMac 6 cells. MonoMac 6 cells were treated with 100 μ M PbCl₂ and 10 μ M CdCl₂ alone or combined with 100 ng/mL LPS (the positive control) for 12 h. Untreated MonoMac 6 cells were served as the negative control. The values below the limits of the assay's detectability were concerned as 0. Data are presented as mean $\pm$ SEM (n = 6). Significant differences compared to the corresponding control were determined by using One-Way Repeated Measures ANOVA followed by Dunnet's post-test. Versus control without LPS: *** $P < 0.001$; Versus control with LPS: ### $P < 0.001$, ## $P < 0.01$.

3.5. PbCl₂ and CdCl₂ activated LPS-induced p38 MAPK signalling pathway

We showed that the heavy metals PbCl₂ and CdCl₂ alter TLR4 activation in the presence of LPS. We next studied whether this was associated with activation of p38 MAPK. As shown in **Figure 6**, the expression of p38 (p38/ β -actin) was not altered after treatment with PbCl₂ and CdCl₂ in the presence or absence of LPS. The production of phospho-p38 (pp38/p38), which indicates the activation of the p38 MAPK, was not affected by PbCl₂ alone. However, an elevation in phospho-p38 expression by MonoMac 6 cells was found due to the existence of 10 μ M CdCl₂ (1.79-fold change, $P < 0.05$, **Figure 6E**). Moreover, both PbCl₂ (except for 0.18 μ M) and CdCl₂ (all concentrations) increased LPS-induced phospho-p38 expression as compared

with incubation with LPS alone (1.91-fold change shown in **Figure 6D** and 1.63-fold change shown in **Figure 6E**, $P < 0.05$).

Figure 6. Effects of PbCl₂ and CdCl₂ on the p38 MAPK and pp38 MAPK expression in MonoMac 6 cells. Cells were incubated for 0.5 h with different dosages of metals alone or in combination with LPS. Data are presented as median with interquartile range ($n = 6$). Data were log-transformed and analyzed using Repeated Measures ANOVA followed by Dunnet's post-test. Versus control without LPS: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; Versus control with LPS: # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$.

4. Discussion

In this study, we evaluated the potential effects of Pb and Cd on monocyte TLR activation and downstream NF- κ B and p38 MAPK signalling pathways. By using THP-1, HEK TLR reporter cells and MonoMac cells, our observations showed that Pb and Cd could significantly enhance or reduce agonist-mediated TLRs/NF- κ B and p38 MAPK actions. Our further findings indicate that Pb and Cd treatment affected MonoMac 6 cells in their cell viability, apoptosis, and expression of ICAM-1, production of TNF- α and IL-10 in the presence of the TLR4 agonist LPS. Our results thus demonstrated that Pb and Cd may affect immune cells via TLRs.

By using THP1-MD2-CD14 and the stable HEK reporter cell lines expressing TLR2, 3, 4, 5, 7, 8, and 9, we showed an effect of Pb and Cd on immune cells via TLRs. TLRs are expressed by all innate immune cells, including monocytes, macrophages, neutrophils, dendritic cells, mast cells, NK cells, epithelial and endothelial cells [28-32], and are responsible for cellular recognition of PAMPs and DAMPs [33]. Upon TLRs' activation, an immune response is induced to clear the pathogens [34, 35]. According to our observations, Pb and Cd alone did

not activate TLRs/NF- κ B signalling, however, they affect the response to the respective ligand. Our findings revealed that Pb could increase the agonist-induced activation of TLR4, 5 and 8; however, Pb was found to attenuate TLR3 and TLR9 activation by their agonist. High exposure to Cd negatively affected TLR2, 4, 5 and 7 activation induced by their agonists, while this may increase TLR3 activation. On the other hand, the lower dose of CdCl₂ increased TLR4, 5 and 8 activations.

Not many studies have been performed on the effect of either Pb or Cd on TLR signalling activation. Our data for TLR4 activation by Pb may not be in line with previous research reporting the immunomodulatory effect of Pb on macrophages and showing that Pb at environmentally relevant levels (lower or equal to 5 μ g/dL) interferes with the binding of LPS to TLR4, suggesting Pb can disrupt LPS-induced TLR4 signalling [18]. The difference between our study and the study of Luna et al. may be the difference in Pb concentrations. Also, the study of Elshaer et al. appeared not to be in line with our study since they showed in an *in vivo* study in rats that renal TLR4/NF- κ B was activated by Cd supplementation to mediate deleterious effects in renal injury [20]. Our study, however, was an *in vitro* study with TLR reporter cell lines. One study showed that Cd may affect TLR expression: in spite of differential concentration and cell line, a study by Capellini et al. demonstrated that Cd exposure lowers TLR3 and 5 gene expression in Madin-Darby canine kidney cells [19]; Hence, alteration of TLRs mRNA expression by Pb and Cd might be an explanation for their effects on TLR. Further studies are needed to substantiate this hypothesis.

Although few papers report on the effects of Pb or Cd on TLR signalling, various papers report on the effect of other metals on TLR signalling. Rachmawati et al. demonstrated that ionized gold can mimic viral dsRNA in triggering TLR3 in keratinocytes; other investigations verified that cobalt, nickel and zinc ions are involved in TLR4 signalling, contributing to the release of the pro-inflammatory mediators NO, IL-6 and IL-8 [35-39]. Additionally, the binding of nickel ion was reported to ligate with two histidine residues of the TLR4 molecule [22, 35]. Thus, because of the similar physical properties of metal divalent cations, we suggest that Pb and Cd ions also bind to two histidine residues of TLRs in order to affect TLR function.

Since we observed that Pb and Cd were able to affect TLRs/NF- κ B signalling, we next wanted to verify whether incubation of monocytes (using the MonoMac 6 cell line) results in activation

of monocytes, as measured by effects of Pb and Cd on MonoMac 6 apoptosis, ICAM-1 expression, production of cytokines and p38/pp38 MAPK expression. MonoMac 6 possesses the characteristic of human mature blood monocytes, including expression of CD14, TLRs and cytokines, and phagocytotic ability, therefore, this cell line has been applied for demonstrating the actions of monocytes in vitro studies [40]. Since the prominent effects of Pb and Cd were observed on TLR4, we choose to further study the effects of Pb and Cd on this TLR on MonoMac 6 cells. Both Pb and Cd have been known as poisonous metals with cytopathological toxicity, for example, promoting ROS generation, apoptosis, and DNA damage [41, 42]. In accordance with this knowledge, our results showed that Pb and Cd incubation of MonoMac cells inhibited cell viability, and increased both early and late apoptosis. This is consistent with previous studies suggesting that exposure to Pb and Cd affected cell survival [42-45]. LPS, also known as endotoxin, is a component of Gram-negative bacterial cell wall and recognized by the TLR 4 in association with MD-2 and CD14 [46, 47]. It has been shown to activate monocytes to elicit a series of responses, including phosphorylation of p38, and secretion of TNF- α and IL-10 [48, 49]. In the present study, our findings suggest that exposure to either Pb or Cd causes an increase in pp38 phosphorylation and TNF- α expression but a decrease in IL-10 production in the presence of LPS. Although different cells and LPS dosage were used, a former study [50] reported that Pb increased TNF- α expression of human peripheral blood mononuclear cells stimulated with 1 ng/mL of LPS, which is partially in line with our results. Nevertheless, LPS-induced ICAM-1 expression was significantly decreased when MonoMac 6 cells were treated with Cd at 10 μ M, which may be attributed to cell death by the collaborative pro-apoptotic effect of Cd and LPS.

It is known that TLR induced not only a subsequent response of NF- κ B but also p38 MAPK activation [34, 51]. The p38 MAPK is responsive to a variety of cellular stimuli, such as inflammatory cytokines and LPS, and is important for the regulation of immune responses, including cell differentiation, survival, and apoptosis inflammatory cytokines production [51, 52]. Although the results of the activation of p38 MAPK in **Figure 6** show Pb and Cd alone do not evoke a significant increase in the activation of p38 MAPK, they both increase the phosphorylation of LPS-induced p38 MAPK. Based on this finding, the MonoMac 6 cell survival, apoptosis, and activation affected by Pb and Cd can be associated with not only TLRs induced NF- κ B activation, but also with p38 MAPK activation.

Previous studies in other cell lines supported that Pb and Cd can induce p38 MAPK activation. For instance, Cd-induced toxicity promotes cell death of adult subventricular neural stem cells and cerebral cortical astrocytes via the p38 MAPK pathway [53, 54]; Pb was reported to induce significant activation of p38 MAPK, which eventually inhibits the proliferation and viability of rat C6 glioma cells [55]. An earlier study carried out in human monocytic THP-1 cells observed that Pb exposure can upregulate TNF- α mRNA expression and protein secretion via the activation of MAPKs [56]. However, our study showed TNF- α production might not be affected by Pb stimulation in the absence of LPS. It's also known that Cd stimulation could increase ICAM-1 expression mediated by NF- κ B activation in mouse brain microvessel endothelial cells [57]; nevertheless, the same cell line applied in another study demonstrated that the elevation of ICAM-1 production is associated with Cd-induced p38 MAPK activation [58]. Although its role in MonoMac 6 cells challenged with metals remains unclear, according to our data, it is evident that p38 MAPK is affected by Pb and Cd and is probably involved in the activation process of MonoMac 6.

5. Conclusions

This study provides evidence of the effect of the heavy metals Pb and Cd on TLRs, NF- κ B, and p38 MAPK signalling. Our findings suggest that Pb and Cd alone only slightly induce monocyte activation. However, they do interfere with TLR signalling with their ligands. Both Pb and Cd seem to affect monocytes TLR activation by their ligand and affect NF- κ B and p38 MAPK activation affecting apoptosis, cell activation and cytokine production. Therefore, the involvement of TLR and the downstream NF- κ B and p38 MAPK in metal-induced immunoregulatory actions of MonoMac 6 is one possible mechanism by which Pb and Cd exposure affect immune responses and thus host defense to pathogens.

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REFERENCES

- [1] Gargouri, M., et al., Cytoprotective and antioxidant effects of the edible halophyte *Sarcocornia perennis* L. (swampfire) against lead-induced toxicity in renal cells. *Ecotoxicol Environ Saf*, 2013. 95: p. 44-51.
- [2] Rashid, K., et al., An update on oxidative stress-mediated organ pathophysiology. *Food Chem. Toxicol.*, 2013. 62: p. 584-600.
- [3] Wu, X., et al., A review of toxicity and mechanisms of individual and mixtures of heavy metals in the environment. *Environ. Sci. Pollut. Res. Int.*, 2016. 23(9): p. 8244-8259.
- [4] Yang, J.M., et al., Cadmium-induced damage to primary cultures of rat Leydig cells. *Reprod. Toxicol.*, 2003. 17(5): p. 553-560.
- [5] Jin, Y., et al., Cadmium exposure to murine macrophages decreases their inflammatory responses and increases their oxidative stress. *Chemosphere*, 2016. 144: p. 168-175.
- [6] Marth, E., et al., The effect of heavy metals on the immune system at low concentrations. *Int. J. Occup. Med. Environ. Health*, 2001. 14(4): p. 375-386.
- [7] Di Lorenzo, L., et al., Evaluation of peripheral blood neutrophil leucocytes in lead-exposed workers. *Int. Arch. Occup. Environ. Health*, 2006. 79(6): p. 491-498.
- [8] Mitra, P., et al., Clinical and molecular aspects of lead toxicity: An update. *Crit. Rev. Clin. Lab. Sci.*, 2017. 54(7-8): p. 506-528.
- [9] Theron, A.J., et al., Harmful interactions of non-essential heavy metals with cells of the innate immune system. 2012.
- [10] García-Mendoza, D., et al., Cell-specific immune-modulation of cadmium on murine macrophages and mast cell lines in vitro. *J. Appl. Toxicol.*, 2019. 39(7): p. 992-1001.
- [11] Karakaya, A., et al., An immunological study on workers occupationally exposed to cadmium. *Hum. Exp. Toxicol.*, 1994. 13(2): p. 73-75.
- [12] Zhang, Y., et al., Alteration of the number and percentage of innate immune cells in preschool children from an e-waste recycling area. *Ecotoxicol Environ Saf*, 2017. 145: p. 615-622.
- [13] Murphy, M., et al., Pellino-1 Positively Regulates Toll-like Receptor (TLR) 2 and TLR4 Signalling and Is Suppressed upon Induction of Endotoxin Tolerance. *J Biol Chem*, 2015. 290(31): p. 19218-19232.
- [14] Wada, J., et al., Innate immunity in diabetes and diabetic nephropathy. *Nat Rev Nephrol*, 2016. 12(1): p. 13-26.
- [15] Yoshino, H., et al., Involvement of reactive oxygen species in ionizing radiation-induced upregulation of cell surface Toll-like receptor 2 and 4 expression in human monocytic cells. *J Radiat Res*, 2017. 58(5): p. 626-635.
- [16] Parker, L.C., et al., Toll-like receptor (TLR)2 and TLR4 agonists regulate CCR expression in human monocytic cells. *J Immunol*, 2004. 172(8): p. 4977-4986.
- [17] Sugitharini, V., et al., TLR-mediated inflammatory response to neonatal pathogens and co-infection in neonatal immune cells. *Cytokine*, 2014. 69(2): p. 211-217.
- [18] Luna, A.L., et al., TLR4 is a target of environmentally relevant concentration of lead. *Toxicol. Lett.*, 2012. 214(3): p. 301-306.
- [19] Capellini, F.M., et al., Characterization of MDCK cells and evaluation of their ability to respond to infectious and non-infectious stressors. *Cytotechnology*, 2020. 72(1): p. 97-109.
- [20] Elshaer, S.S., et al., Relevance of megalin receptor injury with nuclear factor-kappa B upregulation in acute kidney injury induced in rats. *J Biochem Mol Toxicol*, 2018. 32(1).
- [21] Pollard, K.M., et al., Induction of Systemic Autoimmunity by a Xenobiotic Requires Endosomal TLR Trafficking and Signalling from the Late Endosome and Endolysosome but Not Type I IFN. *J Immunol*, 2017. 199(11): p. 3739-3747.
- [22] Schmidt, M., et al., Crucial role for human Toll-like receptor 4 in the development of contact allergy to nickel. *Nat. Immunol.*, 2010. 11(9): p. 814-819.
- [23] Hu, C.H., et al., Zinc oxide influences intestinal integrity, the expressions of genes associated with inflammation and TLR4-myeloid differentiation factor 88 signalling pathways in weanling pigs. *Innate Immun.*, 2014. 20(5): p. 478-486.
- [24] Jarrousse, V., et al., Zinc salts inhibit in vitro Toll-like receptor 2 surface expression by keratinocytes. *Eur. J. Dermatol.*, 2007. 17(6): p. 492-496.
- [25] Cheng, L., et al., Human milk oligosaccharides and its acid hydrolysate LNT2 show immunomodulatory effects via TLRs in a dose and structure-dependent way. *J. Funct. Foods*, 2019. 59: p. 174-184.
- [26] Lépine, A., et al., Synbiotic Effects of the Dietary Fiber Long-Chain Inulin and Probiotic *Lactobacillus acidophilus* W37 Can be Caused by Direct, Synergistic Stimulation of Immune Toll-Like Receptors and Dendritic Cells. *Mol. Nutr. Food Res.*, 2018. 62(15): p. e1800251.
- [27] Figenschau, S.L., et al., ICAM1 expression is induced by proinflammatory cytokines and associated with TLS formation in aggressive breast cancer subtypes. *Sci. Rep.*, 2018. 8(1): p. 11720.
- [28] Della Chiesa, M., et al., Human NK cell response to pathogens. *Semin. Immunol.*, 2014. 26(2): p. 152-160.
- [29] Jarrossay, D., et al., Specialization and complementarity in microbial molecule recognition by

- human myeloid and plasmacytoid dendritic cells. *Eur J Immunol*, 2001. 31(11): p. 3388-3393.
- [30] Marone, G., et al., Pharmacological modulation of human mast cells and basophils. *Clin. Exp. Allergy*, 2002. 32(12): p. 1682-1689.
 - [31] Sabroe, I., et al., Toll-like receptor (TLR)2 and TLR4 in human peripheral blood granulocytes: a critical role for monocytes in leukocyte lipopolysaccharide responses. *J Immunol*, 2002. 168(9): p. 4701-4710.
 - [32] Supajatura, V., et al., Differential responses of mast cell Toll-like receptors 2 and 4 in allergy and innate immunity. *J. Clin. Invest.*, 2002. 109(10): p. 1351-1359.
 - [33] Kawasaki, T., et al., Toll-like receptor signalling pathways. *Front. Immunol.*, 2014. 5: p. 461.
 - [34] Kawai, T., et al., The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.*, 2010. 11(5): p. 373-384.
 - [35] Rachmawati, D., et al., Transition metal sensing by Toll-like receptor-4: next to nickel, cobalt and palladium are potent human dendritic cell stimulators. *Contact Dermatitis*, 2013. 68(6): p. 331-338.
 - [36] Rachmawati, D., et al., Dental metal-induced innate reactivity in keratinocytes. *Toxicol In Vitro*, 2015. 30(1 Pt B): p. 325-330.
 - [37] Raghavan, B., et al., Metal allergens nickel and cobalt facilitate TLR4 homodimerization independently of MD2. *EMBO Rep*, 2012. 13(12): p. 1109-1115.
 - [38] Saito, M., et al., Molecular Mechanisms of Nickel Allergy. *Int. J. Mol. Sci.*, 2016. 17(2).
 - [39] Vijay, K., Toll-like receptors in immunity and inflammatory diseases: Past, present, and future. *Int. Immunopharmacol.*, 2018. 59: p. 391-412.
 - [40] Neustock, P., et al., Cytokine production of the human monocytic cell line Mono Mac 6 in comparison to mature monocytes in peripheral blood mononuclear cells. *Immunobiology*, 1993. 188(3): p. 293-302.
 - [41] Trabelsi, F., et al., Genotoxic effects of cadmium in human head and neck cell line SQ20B. *Environ. Sci. Pollut. Res. Int.*, 2016. 23(16): p. 16127-16136.
 - [42] Slijvic Husejnovic, M., et al., Assessment of Pb, Cd and Hg soil contamination and its potential to cause cytotoxic and genotoxic effects in human cell lines (CaCo-2 and HaCaT). *Environ Geochem Health*, 2018. 40(4): p. 1557-1572.
 - [43] Honda, A., et al., Effects of air pollution-related heavy metals on the viability and inflammatory responses of human airway epithelial cells. *Int. J. Toxicol.*, 2015. 34(2): p. 195-203.
 - [44] Morcillo, P., et al., Heavy metals produce toxicity, oxidative stress and apoptosis in the marine teleost fish SAF-1 cell line. *Chemosphere*, 2016. 144: p. 225-233.
 - [45] Xue, S., et al., Cadmium, lead, and arsenic contamination in paddy soils of a mining area and their exposure effects on human HEPG2 and keratinocyte cell-lines. *Environ Res*, 2017. 156: p. 23-30.
 - [46] Fonceca, A.M., et al., Accumulation mode particles and LPS exposure induce TLR-4 dependent and independent inflammatory responses in the lung. *Respir. Res.*, 2018. 19(1): p. 15.
 - [47] Park, B.S., et al., The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature*, 2009. 458(7242): p. 1191-1195.
 - [48] Plevin, R.E., et al., The Role of Lipopolysaccharide Structure in Monocyte Activation and Cytokine Secretion. *Shock*, 2016. 45(1): p. 22-27.
 - [49] Tucureanu, M.M., et al., Lipopolysaccharide-induced inflammation in monocytes/macrophages is blocked by liposomal delivery of G(i)-protein inhibitor. *Int J Nanomedicine*, 2018. 13: p. 63-76.
 - [50] Guo, T.L., et al., The heavy metal lead modulates the expression of both TNF-alpha and TNF-alpha receptors in lipopolysaccharide-activated human peripheral blood mononuclear cells. *J. Leukoc. Biol.*, 1996. 59(6): p. 932-939.
 - [51] Li, H., et al., Isotrifoliol inhibits pro-inflammatory mediators by suppression of TLR/NF-kB and TLR/MAPK signalling in LPS-induced RAW264.7 cells. *Int. Immunopharmacol.*, 2017. 45: p. 110-119.
 - [52] Kim, E.K., et al., Pathological roles of MAPK signalling pathways in human diseases. *Biochim Biophys Acta*, 2010. 1802(4): p. 396-405.
 - [53] Jiang, J.H., et al., Calcium Signalling Involvement in Cadmium-Induced Astrocyte Cytotoxicity and Cell Death Through Activation of MAPK and PI3K/Akt Signalling Pathways. *Neurochem Res*, 2015. 40(9): p. 1929-1944.
 - [54] Wang, H., et al., Cadmium impairs the survival and proliferation of cultured adult subventricular neural stem cells through activation of the JNK and p38 MAP kinases. *Toxicology*, 2017. 380: p. 30-37.
 - [55] Posser, T., et al., Exposure of C6 glioma cells to Pb(II) increases the phosphorylation of p38(MAPK) and JNK1/2 but not of ERK1/2. *Arch Toxicol*, 2007. 81(6): p. 407-414.
 - [56] Khan, M.I., et al., Ubiquitous hazardous metal lead induces TNF- α in human phagocytic THP-1 cells: primary role of ERK 1/2. *J. Hazard. Mater.*, 2011. 189(1-2): p. 255-264.
 - [57] Jeong, E.M., et al., Cadmium stimulates the expression of ICAM-1 via NF-kappaB activation in cerebrovascular endothelial cells. *Biochem Biophys Res Commun*, 2004. 320(3): p. 887-892.
 - [58] Seok, S.M., et al., COX-2 is associated with cadmium-induced ICAM-1 expression in cerebrovascular endothelial cells. *Toxicol. Lett.*, 2006. 165(3): p. 212-220.

7

EFFECTS OF LEAD AND CADMIUM ON NF-KB ACTIVITY/MITOCHONDRIAL DYNAMICS AND THE ROLE FOR THE SURVIVAL AND ACTIVATION OF JURKAT CELLS

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ABSTRACT

Heavy metals lead and cadmium are widespread environmental contaminants capable of impeding immune function. Nuclear factor-kappa B (NF- κ B) transcription factors and mitochondria are known as key targets for various stimuli, but their roles in mediating the survival, proliferation, and activation in T cells in response to heavy metals have been incompletely demonstrated. Here, Jurkat, an immortalized human T cell line, was treated with series of dosages of lead chloride (PbCl₂) and cadmium chloride (CdCl₂) in the presence or absence of phorbol 12-myristate 13-acetate/calcium ionophore (PMA/Ca²⁺ ionophore). PbCl₂ and CdCl₂ suppressed the cell viability of Jurkat cells without PMA/Ca²⁺ ionophore activation. PbCl₂ and CdCl₂ were also found to increase cell apoptosis and reduce IL-2 production in PMA/Ca²⁺ ionophore-stimulated Jurkat cells. PbCl₂ induced an elevation in p65 and I κ B α in non-activated Jurkat cells, while PbCl₂ decreased p65 phosphorylation in activated Jurkat cells. CdCl₂ increased both p65 and phosphorylated p65 levels in non-activated Jurkat cells. PbCl₂ and CdCl₂ enhanced mitochondrial DNA copy number in Jurkat cells without PMA/Ca²⁺ ionophore activation. CdCl₂ downregulated mitochondrial fusion *MFN1* mRNA expression in non-activated Jurkat cells but upregulated *OPA1* in activated Jurkat cells. Suppressed transcription levels of mitochondria-encoded electron transport chain genes: *mtND2*, *mtCYB* and *mtATP8*, accompanied by decreased expression of nuclear-encoded mitochondrial regulator genes: *TFAM* and *NRF1*, were associated with either PbCl₂ or CdCl₂ treatment. Our findings indicate that PbCl₂ and CdCl₂ tend to pose an adverse impact on the survival, proliferation and activation of T cells in vitro. We proposed a potential mechanism whereby PbCl₂ and CdCl₂ affected T cells by disrupting NF- κ B activity and mitochondrial biogenesis. In addition, the toxic effects of PbCl₂ and CdCl₂ may be associated with the activation state of T cells.

1. Introduction

The heavy metals lead and cadmium are widespread immunotoxic contaminants. Exposure to lead and cadmium has been linked with an impaired immune system of individuals due to their potential to suppress or stimulate the immune system [1, 2]. As an essential component of the adaptive immune system, T lymphocytes (or T cells) help defend the body against pathogens, allergens, and tumours [3]. Much of the existing evidence demonstrates that lead and cadmium are capable of modulating T lymphocyte functions, such as cell death, proliferation, and cytokine production [2, 4]. A population study showed that the T helper (Th) lymphocyte count in peripheral blood of lead-exposed workers was significantly reduced compared to healthy controls, suggesting that lead may detrimentally affect T cell survival [5]. Another study carried out in preschool children from an e-waste recycling area demonstrated that blood lead levels showed a positive correlation with the CD4⁺ central memory T cell percentage [6]. Lead has also been shown to disrupt the balance between Th1 and Th2 by reducing Th1 and enhancing Th2 cell proliferation [7]. Similarly, a significantly greater cadmium-induced reduction in Th1-derived cytokines (IL-2 and IFN- γ) was observed as compared with Th2-derived cytokine (IL-4) in BALB/c mice. Cadmium has been shown to induce apoptosis in T cells [8, 9], both in the CD4⁺ and CD8⁺ T subsets [4]. This may be due to the effects of cadmium on interleukin (IL)-2 production. IL-2 is the major growth factor essential for T cell proliferation and survival. IL-2 production could be inhibited by lead treatment; however, in activated T cells, IL-2 release was promoted as a result of exposure to cadmium [2].

Nuclear factor-kappa B (NF- κ B) is well documented to be a crucial player to allow cells to adapt and respond to environmental stress [10, 11]. The mammalian NF- κ B transcription factor family is comprised of 5 members: p65 (RelA), RelB, c-Rel, p105/p50 (NF- κ B1), and p100/52 (NF- κ B2) that associate with each other to form distinct transcriptionally complexes [12]. Among them, p65 is the best-studied family member that, together with p50, forms the most common heterodimer in the NF- κ B signalling pathway in most cell types [13]. I κ B α is the primary inhibitory I κ B proteins which bound to nearly all of the NF- κ B family of transcription factors leading to the inhibition of nuclear localization and transcriptional activation [14]. Studies support the notion that NF- κ B activation is responsible for the transcription of specific sets of target genes associated with inflammatory responses, cell proliferation, apoptotic or anti-apoptotic regulators, and neoplastic transformation [10, 12].

NF- κ B signalling cooperates with mitochondria in response to stressors [11, 15, 16]. Mitochondria are membrane-bound organelles in the eukaryotic cytoplasm that served as the powerhouse of the cell because they generate large quantities of cellular energy in the form of adenosine triphosphate (ATP) through oxidative phosphorylation [17]. Besides, mitochondria are known to be essential for cellular function, including ROS production and consumption, cellular metabolism, calcium homeostasis, and apoptotic regulation [18]. Mitochondrial function depends on the amount and quality of mitochondria, which are largely controlled by a balance between fission and fusion processes as well as nuclear signalling-mediated nuclear and mitochondrial transcription [17-20].

So far only a few studies have addressed the effects of lead and cadmium on T cell survival apoptosis in vitro, while no in vitro studies have addressed the impact of lead and cadmium on NF- κ B and mitochondrial function. Therefore, in the present study, we evaluated the effects of lead and cadmium on T cell survival and function, as well as NF- κ B activity and mitochondrial biogenesis in T cells in vitro. For this, Jurkat cells, an immortalized T cell line, were cultured with PbCl₂ and CdCl₂ in the absence or presence of the non-specific T cell activator, phorbol 12-myristate 13-acetate (PMA)/calcium ionophore and cell survival, apoptosis, IL-2 production, NF- κ B activity and mitochondrial function were evaluated.

2. Materials and Methods

Experimental design: In this study, we studied the effects of PbCl₂ and CdCl₂ treatment on resting and activated T cells. Therefore, Jurkat cells were incubated with PbCl₂ and CdCl₂ in the presence or absence of phorbol 12-myristate 13-acetate and calcium ionophore A23187. We used the WST-1 assay to evaluate the viability and proliferation of Jurkat cells; flow cytometric analysis was carried out to check cell survival, apoptosis and IL-2 production of Jurkat following PbCl₂ and CdCl₂ treatment in activated and resting cells. We also evaluated the activation status of NF- κ B and mitochondrial dynamics in these Jurkat cells following treatment with PbCl₂ and CdCl₂ by western blot and quantitative reverse transcription PCR (qRT-PCR), respectively.

2.1. Cell Culture and Treatment

Jurkat cells were obtained from American Type Culture Collection (clone E6-1, ATCC TIB-152, France) and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, The Netherlands), 2 mM glutamine (Lonza, Switzerland), 1 mM sodium pyruvate (Lonza, Switzerland), 50 μ M mercapto-ethanol (Merck, Germany), 60 μ g/mL gentamycin (Lonza, Switzerland), and 2.2 μ g/mL amphotericin B (Thermo Fisher Scientific, USA) in a humidified atmosphere containing 5% CO₂ at 37 °C. The culture was diluted at the split ratio of 1:15 and passaged twice a week.

Cells (1×10^6 cells/ml) were exposed to lead chloride (PbCl₂) (Sigma-Aldrich, The Netherlands) at the final concentration of 0.18 (equal to 50 μ g/L), 1.8, 18 and 100 μ M, or with cadmium chloride (CdCl₂) (Sigma-Aldrich, The Netherlands) at the concentration of 0.027 (equal to 5 μ g/L), 0.27, 2.7 and 10 μ M in the presence or absence of phorbol 12-myristate 13-acetate (PMA, 10 nM) /calcium ionophore A23187 (Ca²⁺ ionophore, 500 nM) (both from Sigma-Aldrich, The Netherlands). The lowest concentrations of PbCl₂ and CdCl₂ were chosen as concentrations similar to the safety limit of lead and calcium in blood issued by the United States Centers for Disease Control and Prevention. As a negative control, cells were treated with medium only. Cells incubated with PMA (10 nM)/Ca²⁺ ionophore (500 nM) only were used as the positive control. Pilot experiments were performed to verify the appropriate exposure time of Jurkat cells to stimulus depending on different parameters tested in this study (data not shown). All experiments in the present study were performed 6 times separately.

2.2. Cell viability assay

Jurkat cell viability was measured by water-soluble tetrazolium salt-1 (WST-1) assay according to the manufacturer's protocol (Roche, Switzerland). Jurkat cells (1×10^5 cells/100 μ l) were seeded in 96-well plates (Corning, the Netherlands) and treated with the various concentrations of PbCl₂ and CdCl₂ with or without PMA/Ca²⁺ ionophore. After incubation for 24 hours, 10 μ l WST-1 reagent solution was added to each well and plates were incubated for an extra 4 hours at 37 °C. The absorbance at 440 nm and 620 nm (the reference wavelength) was analyzed by using a Bio-Rad Benchmark Plus microplate reader (Bio-Rad Laboratories, USA). The relative value of viable cells was expressed as a percentage of the controls, i.e. cells only incubated with the medium.

2.3. Cell death analysis

The Annexin V and PI staining was carried out to monitor apoptosis. Jurkat cells (1×10^6 cells/ml) were cultured with the various concentrations of PbCl₂ and CdCl₂ with or without PMA/Ca²⁺ ionophore in 12-well plates (Corning, the Netherlands). After 24-hour incubation, cells were collected in Falcon round-bottom polypropylene tubes (Corning, the Netherlands) and washed twice with 1 ml of $1 \times$ cold phosphate-buffered saline (PBS) (Lonza, Switzerland). Cell pellets were suspended in 100 μ L Annexin V binding buffer (0.01M HEPES, 140 mM NaCl, 2.5 mM CaCl₂, PH7.4) containing 5 μ L fluorescein isothiocyanate (FITC)-conjugated AnnexinV (BioLegend, The Netherlands) and 1 μ L propidium iodide (PI) (Invitrogen, USA) staining solution for 15 minutes at room temperature. Afterward, cells were washed once with PBS and resuspended in PBS containing 2% FBS until flow cytometric analysis immediately. Flow cytometry data collected from 10,000 cells were obtained on a FacsVerse cytometer (BD Biosciences) and analyzed in a dot-plot manner on logical/biexponential scales by Kaluza 2.1 (Beckman Coulter, USA). The gating strategy for selecting cells, i.e. living, early, and late apoptotic, is presented in *Figure 2*.

2.4. IL-2 expression

Intracellular IL-2 was detected by flow cytometry. Jurkat cells (1×10^6 cells/ml) were exposed to the various concentrations of PbCl₂ and CdCl₂ with or without PMA (10 nM)/Ca²⁺ ionophore (500 nM) in 24-well plates (Corning, the Netherlands). After 19 hour-incubation, 10 μ g/ml Brefeldin A (Sigma-Aldrich, The Netherlands) was added to each well and incubated with cells at 37 °C for another 5 hours. Treated cells were washed once with ice-cold PBS containing 2% heat-inactivated FBS and then incubated with fluorescence-activated cell sorting (FACS) lysing buffer according to the manufacture's protocol (BD Biosciences) for 30 minutes at room temperature. After centrifugation at 4 °C, $300 \times g$ for 5 minutes, cell pellets were incubated with permeabilization buffer (Invitrogen, USA) for 10 minutes on ice. The permeabilized cells were washed once and incubated in 50 μ L permeabilization buffer containing 5 μ L FITC-conjugated anti-human IL-2 antibody (BioLegend, The Netherlands) for 30 minutes at room temperature. Samples were washed once with permeabilization buffer and resuspended in $1 \times$ PBS containing 0.5% paraformaldehyde prior to acquisition on the BD FACSVerse instrument.

Ten thousand events were acquired for each sample and data analysis was performed by Kaluza 2.1 (Beckman Coulter, USA).

2.5. Evaluation of nuclear factor kappa B (NF- κ B) activation

The expression of NF- κ B proteins, including phosphorylated-p65, p65 and NF- κ B inhibitor alpha (I κ B α) in Jurkat cells were measured by western blot. Jurkat cells (1×10^6 cells/ml) treated with the various concentrations of PbCl₂ and CdCl₂ in the absence or presence of PMA/Ca²⁺ ionophore were cultured at 37°C. Pilot experiments were performed to test the incubation time and 2 hours was shown to be optimal (data not shown). After incubation, cells were washed twice with ice-cold PBS. Cells were collected in 100 μ L radioimmunoprecipitation assay buffer (RIPA buffer) (Thermo Fisher Scientific, USA) with 1% protease and phosphatase inhibitor cocktail (Sigma-Aldrich, The Netherlands) and lysed by sonication. The total protein concentration of each sample was determined by using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Equal amounts of protein (10 μ g) were separated in 10% polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The membranes were probed with primary antibodies: rabbit anti-human phosphorylated-p65 (Ser536) (1:1000), mouse anti-human p65(1:1000), mouse anti-human I κ B α (1:1000) and mouse anti-human internal control β -actin (1:3000) (all from Cell Signalling Technology, USA). Immunoreactive proteins were detected with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG secondary antibody (1: 5000) (both from Dako, Agilent, Denmark) and analyzed by ChemiDoc Imaging and ChemiDoc MP Imaging System (Bio-Rad Laboratories, USA). Quantitation of bands was conducted by Image J software (bundled with 64-bit Java 1.8.0_112 for windows system). The target protein expression was normalized to the band intensity of p65 or β -actin on the same membrane.

2.6. Messenger RNA (mRNA) expression

Cells at a density of 1×10^6 cells/ml were seeded into 12-well plates (Corning, the Netherlands) and treated with the various concentrations of PbCl₂ and CdCl₂ in the absence or presence of PMA/Ca²⁺ ionophore. Pilot experiments were performed to test the incubation time and 6 hours was shown to be optimal incubation time (data not shown). Total RNA and DNA (for mitochondrial DNA copy number analysis) were isolated from cells using the Trizol reagent

(Thermo Fisher Scientific, USA) following the manufacturer's manual. Briefly, cells were collected in a 1.5-ml Eppendorf tube after twice washing with $1 \times$ PBS, then incubated with a 500 μ L of TRIzol® reagent at room temperature for 5 minutes. Next, 0.1 ml chloroform (Sigma-Aldrich, The Netherlands) was added, and the sample was incubated for 5 minutes at room temperature. This was followed by 15-minute centrifugation at $12,000 \times g$ at 4°C . Once the three phases were separated, the aqueous phase and the interphase were used for subsequent RNA and DNA isolation, respectively. For RNA, the separated aqueous phase was incubated with isopropanol (Sigma-Aldrich) at room temperature for 5 minutes. After centrifugation, the RNA pellet was washed with 75% ethanol (Sigma-Aldrich). Followed by centrifuging, the RNA pellet was dried in a dry bath and then resuspended in Nuclease-Free water (Thermo Fisher Scientific).

The concentration and purity of total RNA extraction were measured by a NanoDrop 1000 UV-vis spectrophotometer (Thermo Fisher Scientific). RNA samples with 260/280 and 260/230 ratios of ≥ 1.90 were used for further analysis. A total of 500 ng of RNA from each sample was used for reverse transcriptase cDNA synthesis. Briefly, RNA was mixed with 300 ng of random hexamer primers and 10 mM dNTP and incubated at 65°C for 10 minutes. Afterward, a mixture containing 4 μ L $5 \times$ First-strand buffer, 2 μ L 0.1 mM DTT, 1 μ L RNaseOUT recombinant ribonuclease inhibitor and 1 μ L superscript II (all from Thermo Fisher Scientific) was added to have a final volume of 20 μ L for each sample. Reactions were carried out at 25°C for 10 minutes, 42°C for 50 minutes and 70°C for 15 minutes on a T100 Thermal Cycler (Bio-Rad Laboratories, USA).

The mRNA expression levels of genes associated with mitochondrial fission and fusion process, mitochondrial function regulators and genes encoded in the mitochondrial genome (**Table I**) were detected by quantitative reverse transcription PCR (qRT-PCR) method and performed in a Viia 7 Real-time PCR system (Applied Biosystems, USA). The cDNA product was 25-time diluted with RNase-free water and then 5 μ L of each dilution was mixed with FastStart Universal SYBR Green Master (Roche, Switzerland) and corresponding forward and reverse primers (**Table I**) reaching a total volume of 10 μ L. Reaction conditions were set up as follows: denaturation step, 95°C for 10 minutes; amplification step, 40 cycles at 95°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds; and melting curve step, 95°C for 15 seconds, 60°C for 1 minute, 95°C for 15 seconds. The annealing temperature and efficiency of primers were

verified using a 5-point standard curve obtained from a pool of total cDNA diluted 5, 10, 20, 40 and 80 times. The acceptable efficiency of each primer is 90-110%. The expression level of each gene was normalized to the housekeeping genes (HKG): β -actin and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and was calculated using the following method: $2^{-(Ct \text{ gene of interest} - Ct \text{ HKG})}$.

2.7. Mitochondrial DNA copy number

Mitochondrial DNA copy number or mitochondrial/nuclear DNA ratio was measured by qRT-PCR method performed as described above. Total DNA including mitochondrial and genomic were extracted from cells that were used for total RNA isolation. Followed by removing the upper aqueous phase, the interphase was separated and incubated with a back extraction buffer (4M Guanidine Thiocyanate, 50 mM Sodium Citrate, 1M Tris, all from Sigma-Aldrich) for 5 minutes at 55 °C. After centrifugation, the upper aqueous phase was separated and then mixed with acrylamide carrier and isopropanol (Sigma-Aldrich) to precipitate the DNA. The DNA pellet was washed twice with 75% ethanol and then was solubilized in 1x TE buffer (Sigma-Aldrich). DNA concentration and purity was measured by a NanoDrop 1000 UV-vis spectrophotometer (Thermo Fisher Scientific). A total of 500 ng DNA was incubated with RNase A (Thermo Fisher Scientific) for 30 minutes at 37°C prior to amplification. The amplification of *D-loop*, a stably expressed gene in mitochondria, and β -2-microglobulin (*B2M*), a nuclear single-copy gene, were evaluated to quantify mitochondrial/nuclear DNA ratio. Their primers' efficiency was measured by a 5-point standard curve from a pooled total DNA of measured samples. The results were calculated using the following method: $2^{-(Ct \text{ B2M} \times \text{primer efficiency} - Ct \text{ D-loop} \times \text{primer efficiency})}$.

2.8. Statistical analysis

Graphpad software was used to perform statistical analysis (version 8.0a, GraphPad InStat, USA). The normality of data distribution was confirmed using the Kolmogorov-Smirnov test. Data that are normally distributed are shown as mean $\pm$ SEM, data not normally distributed are shown as median with interquartile range. Not normally distributed data were log-transformed before data analysis. The groups treated with PMA/ Ca^{2+} ionophore, PbCl_2 or CdCl_2 only were compared with the negative control, while the comparison between groups incubated PbCl_2 or CdCl_2 with PMA/ Ca^{2+} ionophore and the positive control was performed. Statistical difference

was assessed using the one-way repeated measures analysis of variance (rmANOVA) followed by Dunnet's post-test. Significant difference level was defined as $P < 0.05$.

Table 1. Primer sequences for qRT-PCR.

Gene	Forward Sequence (5' - 3')	Reverse Sequence (5' - 3')
<i>B2M</i>	GCTGGGTAGCTCTAAACAATGTATTCA	CCATGTACTAACAAATGTCTAAAATGGT
<i>β-Actin</i>	GCACCACACCTTCTACAATG	TGCTTGCTGATCCACATCTG
<i>D-loop</i>	TCACCCTATTAACCACTCACGG	ATACTGCGACATAGGGTGCTC
<i>DRP1</i>	CTGCCTCAAATCGTCGTAAGT	GAGGTCTCCGGGTGACAATTC
<i>FIS1</i>	GTCCAAGAGCACGCAGTTTG	ATGCCTTTACGGATGTCATCATT
<i>GAPDH</i>	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
<i>MFN1</i>	TGGCTAAGAAGGCGATTACTGC	TCTCCGAGATAGCACCTCAC
<i>MFN2</i>	CTCTCGATGCAACTCTATCGTC	TCCTGTACGTGTCTTCAAGGAA
<i>mt12S</i>	CTGCTCGCCAGAACACTACG	TGAGCAAGAGGTGGTGAGGT
<i>mt16S</i>	GTATGAATGGCTCCACGAGG	GGTCTTCTCGTCTTGCTGTG
<i>mtATP8</i>	TAAATACTACCGTATGGCCAC	GTGATGAGGAATAGTGAAGGAG
<i>mtCO1</i>	CGATGCATACACCACATGAA	AGCGAAGGCTTCTCAAATCA
<i>mtCYB</i>	AATTCTCCGATCCGTCCCTA	GGAGGATGGGGATTATTGCT
<i>mtND2</i>	GTAAGCCTTCTCCTCACTCTC	TTAATCCACCTCAACTGCCT
<i>NRF1</i>	GGGAGCTACAGTCACTATGG	TCCAGTAAGTGCTCCGAC
<i>OPA1</i>	TGTGAGGTCTGCCAGTCTTTA	TGTCCTTAATTGGGGTCGTTG
<i>TFAM</i>	CCGAGGTGGTTTTCATCTGT	TCCGCCCTATAAGCATCTTG

Abbreviations: *B2M*: beta-2-microglobulin; *β-Actin*: beta-Actin; *D-loop*: displacement loop; *DRP1*: dynamin-related protein-1; *FIS1*: fission 1; *GAPDH*: Glyceraldehyde-3-Phosphate Dehydrogenase; *MFN1*: mitofusin-1; *MFN2*: mitofusin-2; *mt12S*: 12S ribosomal unit; *mt16S*: 16S ribosomal unit; *mtATP8*: ATP synthase gene unit (complex V); *mtCO1*: cytochrome c oxidase subunit (complex IV); *mtCYB*: cytochrome b (complex III); *mtND2*: NADPH dehydrogenase subunit 2 (complex I); *NRF1*: nuclear respiratory factor 1; *OPA1*: optic atrophy-1; *TFAM*: mitochondrial transcription factor A.

3. Results

3.1. PbCl₂ and CdCl₂ challenge inhibited Jurkat cell viability

Our results of the WST-1 assay showed that Jurkat cell viability was slightly, but significantly reduced by PbCl₂ exposure for 24 hours at the concentrations of 18 and 100 μM (92.6% and 93.5%, respectively) when compared with non-treated control (100%) (**Figure 1A**, both $P < 0.05$). Cells treated with 10 μM of CdCl₂ alone (93.3%) also had lower viability than the negative control (**Figure 1B**, $P < 0.05$).

Cells treated with PMA/Ca²⁺ ionophore showed a decreased viability (62.9%) as compared with control cells ($P < 0.001$). PbCl₂ did not affect the effect of PMA/Ca²⁺ ionophore on Jurkat cell viability (**Figure 1A**). CdCl₂ exposure (10 μ M) in the presence of PMA/Ca²⁺ ionophore significantly decreased living cell percentage of Jurkat (52.9%) compared with cells treated with PMA/Ca²⁺ ionophore alone (62.9%) (**Figure 1B**, $P < 0.05$).

Figure 1. Assessment of Jurkat cell viability following treatment with PbCl₂ and CdCl₂ with or without PMA/Ca²⁺ ionophore. Cells were incubated for 24 hours with different concentrations of metals alone or in combination with PMA/Ca²⁺ ionophore (10 nM/500 nM). The percentage of cell viability in the negative control group treated with medium only was set to 100%. The relative cell viability of each group was presented as a percentage and median with interquartile range that was calculated by comparison to that of the negative control group from six independent experiments. After log-transformation, data analysis was determined by using rmANOVA followed by Dunnet's post-test. Versus the control without PMA/Ca²⁺ ionophore: *** $P < 0.001$, * $P < 0.05$; versus the control with PMA/Ca²⁺ ionophore: # $P < 0.05$.

3.2. PbCl₂ and CdCl₂ exposure increased PMA/Ca²⁺ ionophore-induced apoptosis

As indicated above, Jurkat cell viability was affected by PbCl₂ and CdCl₂ with or without PMA/Ca²⁺ ionophore. We next performed FACS analysis to evaluate whether PbCl₂ and CdCl₂ induced apoptosis in Jurkat cells. As shown in **Figure 2B** and **2C**, both PbCl₂ and CdCl₂ did not significantly alter living cell percentage, while PMA/Ca²⁺ ionophore was found to decrease the percentage of living cells (71.8%) as compared with non-treated Jurkat cells (96.3%) ($P < 0.001$); moreover, both PbCl₂ (1.8 μ M: 67.8%, $P < 0.05$; 18 μ M: 67.9%, $P < 0.01$) and CdCl₂ (2.7 μ M: 66.9%, $P < 0.001$; 10 μ M: 61.5%, $P < 0.001$) further decreased the percentage of living cells induced by PMA/Ca²⁺ ionophore.

Neither PbCl₂ or CdCl₂ alone showed a significant influence on early and late apoptosis in Jurkat cell. However, PMA/Ca²⁺ ionophore treatment increased both early and late apoptosis (13.7% and 12.8%, respectively) as compared with the negative control (early: 2.3%, late: 1.4%)

(**Figure 2D-2G**, both $P < 0.001$). Early apoptosis induced by PMA/Ca²⁺ ionophore was increased by PbCl₂ (100 μ M; 16.1%, $P < 0.01$, **Figure 2D**) and CdCl₂ (10 μ M; 16.3%, **Figure 2E**, $P < 0.05$). CdCl₂ at 2.7 and 10 μ M in the presence of PMA/Ca²⁺ ionophore (16.7% and 19.8%, respectively) also increased late apoptosis as compared with PMA/Ca²⁺ ionophore alone (**Figure 2G**, both $P < 0.001$).

Figure 2. Apoptosis of Jurkat cells treated with different concentrations of PbCl₂ and CdCl₂ in the absence or presence of PMA/Ca²⁺ ionophore (10 nM/500 nM). (A) Gating strategy to select living, early and late apoptotic cells in Jurkat cells. Cells were gated on FSC vs. SSC removing the small debris near the origin with single cells gated on an FSC-A vs. FSC-H dot-plot. This was followed by a dot-plot of FITC-Annexin V vs. PI with a quadrant placed marking off living cells in the double negative quadrant (lower left), early apoptotic cells were FITC-Annexin V⁺/PI⁻ (lower right) and lastly with late apoptotic cells labeled with FITC-Annexin V⁺/PI⁺ (upper right) indicating late apoptotic cells. (B-G) Percentages of living cells, early and late apoptosis in Jurkat cells. Data are presented as median with interquartile range (n = 6). After log-transformation, data analysis was determined by using rmANOVA followed by Dunnet's post-test. Versus the control without PMA/Ca²⁺ ionophore: *** $P < 0.001$; Versus the control with PMA/Ca²⁺ ionophore: ### $P < 0.001$, # $P < 0.05$.

3.3. CdCl₂ lowered intracellular IL-2 expression of Jurkat cells induced by PMA/Ca²⁺ ionophore treatment

IL-2 is the major growth factor of activated T lymphocytes, which not only is responsible for T cell proliferation but also inhibits cell apoptosis in these cells [21]. Therefore, we measured intracellular IL-2 in Jurkat cells after incubation with PbCl₂ or CdCl₂ with or without PMA/Ca²⁺ ionophore. In the present study, neither PbCl₂ nor CdCl₂ alone significantly altered IL-2 production by Jurkat cells. Cells treated with PMA/Ca²⁺ ionophore (16.7%) increased IL-2 production compared with non-treated cells (3.0%) (**Figure 3B** or **3C**, $P < 0.001$). PbCl₂ at 1.8 μ M (14.4%) inhibited IL-2 production in PMA/Ca²⁺ ionophore stimulated cells (**Figure 3B**, $P < 0.05$). We also observed a significant reduction in IL-2 expression by Jurkat cells with stimulation of CdCl₂ (all four concentrations) plus PMA/Ca²⁺ ionophore (14.4%, 13.8%, 13.5% and 11.0%, respectively) compared with that in PMA/Ca²⁺ ionophore-treated cells (**Figure 3C**, all $P < 0.01$).

Figure 3. Intracellular IL-2 expression in Jurkat cells. Cells were incubated for 24 hours with PbCl₂ and CdCl₂ alone or in combination with PMA/Ca²⁺ ionophore (10 nM/500 nM). (A) Gating strategy of IL-2 positive cells. Cells were stained with FITC-conjugated antibody; the evaluation of IL-2 positive cell percentage was performed by flow cytometry. IL-2 positive population in each sample was gated compared to the blank (without staining, set to 1%). (B-C) Data are presented as mean $\pm$ SEM (n = 6). Data analysis was determined by using rmANOVA followed by Dunnet's post-test. Versus the control without PMA/Ca²⁺ ionophore: *** $P < 0.001$. Versus the control with PMA/Ca²⁺ ionophore: ### $P < 0.001$, ## $P < 0.01$, # $P < 0.05$.

3.4. PbCl₂ and CdCl₂ disturbed NF- κ B activation in Jurkat cells

NF- κ B activation is involved in cell survival, proliferation and apoptosis in many cell types [21, 22]. Besides, the induction of IL-2 expression following T cell stimulation relies on NF- κ B activation [12, 22]. In our study, the protein expression of transcription factor p65, an important member of NF- κ B family, and its phosphorylation level were analyzed. An increase of total p65 protein level in Jurkat cells was found after incubation with PbCl₂ at the concentrations of 1.8, 18 and 100 μ M (**Figure 4B**, 1.69, 1.87 and 1.72 fold-change, respectively) or with CdCl₂ at 0.027, 0.27 and 2.7 μ M (**Figure 4C**, 1.53, 1.58 and 1.66 fold-change, respectively) (all $P < 0.05$). PMA/Ca²⁺ ionophore did not affect p65 expression.

With regard to the phosphorylation status of p65 in Jurkat cells, this was not significantly altered by PbCl₂ alone (**Figure 4D**). CdCl₂ was found to elevate p65 phosphorylation (0.027 and 10 μ M: 1.28 and 1.24 fold-change, respectively) (**Figure 4E**, both $P < 0.05$), and this elevation was also found in PMA/Ca²⁺ ionophore-treated cells (1.46 fold-change shown in **Figure 4D**, or 1.43 fold-change in **Figure 4E**, $P < 0.01$). Phosphorylated p65 expression in Jurkat cells induced by PMA/Ca²⁺ ionophore was reduced by PbCl₂ (0.18 μ M: 1.13 fold-change; 18 μ M: 1.04 fold-change) (**Figure 4D**, all $P < 0.01$). CdCl₂ did not influence the effect of PMA/Ca²⁺ ionophore on phosphorylated p65.

In resting cells, NF- κ B complexes are inactive in the cytoplasm in a complex with members of the inhibitor of the NF- κ B (I κ B) family [12]. I κ B α is the best-studied member of the I κ B family and is critical for nuclear translocation and DNA binding of NF- κ B p65/p50 [12, 23]. PbCl₂ (18 and 100 μ M: 1.34 and 1.33 fold-change, respectively, **Figure 4F**, both $P < 0.05$) increased I κ B α levels. CdCl₂ did not significantly change I κ B α production (**Figure 4G**). A significant reduction of I κ B α expression was found in PMA/Ca²⁺ ionophore-treated cells (0.79 fold-change in **Figure 4F** or 0.72 fold-change in **Figure 4G**, $P < 0.01$), which was not altered by PbCl₂ or CdCl₂.

Figure 4. Expression of the NF- κ B unit p65, phosphorylated p65 (pp65) and the NF- κ B inhibitor I κ Ba in Jurkat T cells. Cells were incubated for 2 hours with different concentrations of PbCl₂ and CdCl₂ alone or in combination with PMA/Ca²⁺ ionophore (10 nM/500 nM). Data of pp65 and I κ Ba expression are presented as median with interquartile range, while data of p65 expression are presented as mean $\pm$ SEM (n = 6). The negative control was set to 1 and all other data were presented relative to this negative control. Data analysis was determined by using rmANOVA followed by Dunnet's post-test. Versus the control without PMA/Ca²⁺ ionophore: ***P* < 0.01, **P* < 0.05; versus the control with PMA/Ca²⁺ ionophore: ###*P* < 0.001, ##*P* < 0.01.

3.5. PbCl₂ and CdCl₂ increased mitochondrial DNA content in Jurkat cells

Mitochondria have been recognized as playing a central role in activating apoptosis and subsequent IL-2 induction in T cells [24, 25]. To gain insight into the possible role of PbCl₂ and CdCl₂ in mitochondrial function, we investigated alterations in mitochondria of Jurkat cells stimulated with PbCl₂ and CdCl₂ with or without PMA/Ca²⁺ ionophore. We firstly evaluated mitochondrial DNA (mtDNA) copy number, as an accessible proxy for the mtDNA content to reflect mitochondrial dysfunction [26]. According to our findings, PbCl₂ treatment significantly increased mitochondrial DNA copy number at the concentrations 0.18, 18 and 100 μ M (2.85, 1.28 and 2.16 fold-change, respectively, **Figure 5A**, both *P* < 0.05); CdCl₂ in the concentrations of 0.027, 2.7 and 10 μ M also increased mitochondrial DNA copy number in Jurkat cells (2.19, 2.22 and 1.94 fold-change, respectively, **Figure 5B**, all *P* < 0.05). PMA/Ca²⁺ ionophore increased DNA copy number by 2.56 fold-change (**Figure 5A or 5B**, *P* < 0.001). Neither PbCl₂ nor CdCl₂ affected DNA copy numbers after incubation with PMA/Ca²⁺ ionophore.

Figure 5. Mitochondrial DNA content in Jurkat cells. Cells were incubated for 6 hours with different concentrations of PbCl₂ and CdCl₂ alone or in combination with PMA/Ca²⁺ ionophore (10 nM/500 nM). Data are presented as median with interquartile range (n = 6). The negative control was set to 1 and all other data were presented relative to this negative control. After log-transformation, data analysis was determined by using rmANOVA followed by Dunnet's post-test. Versus the control without PMA/Ca²⁺ ionophore: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

3.6. CdCl₂ only slightly affected mitochondrial fusion gene expression in Jurkat cells

To maintain a functional balance when cells experience environment changes, mitochondria exist within a network of dynamical fusion and fission that is involved in apoptosis, differentiation and various stress responses [27, 28]. Mitochondrial fusion requires OPA1, MFN1 and MFN2, while the fission process is mediated by DRP1 and FIS1 [29, 30]. PbCl₂ did influence the mRNA levels of both fusion and fission genes. CdCl₂ at 0.027 μM was found to decrease *MFN1* mRNA level (0.74 fold-change) compared with non-treated cells (**Figure 6D**, $P < 0.05$). No significant effects of PMA/Ca²⁺ ionophore on mitochondrial fusion and fission gene expression in Jurkat cells were found. However, the fusion gene *OPA1* was significantly upregulated in Jurkat cells after coincubation with 10 μM of CdCl₂ and PMA/Ca²⁺ ionophore (1.49 fold-change) compared with cells exposed to only PMA/Ca²⁺ ionophore (1.11 fold-change) (**Figure 6B**, $P < 0.05$).

alter *NRF1* gene expression. However, the expression of *NRF1* in PMA/ Ca^{2+} ionophore-treated cells was reduced by 15.0% due to the presence of 0.18 μM PbCl_2 (**Figure 7C**, $P < 0.05$).

Figure 7. mRNA expression of nuclear-encoded mitochondrial regulators in Jurkat cells. Cells were incubated for 6 hours with different concentrations of PbCl_2 and CdCl_2 alone or in combination with PMA/ Ca^{2+} ionophore (10 nM/500 nM). Data are presented as median with interquartile range ($n = 6$). The negative control was set to 1 and all other data were presented relative to this negative control. After log-transformation, data analysis was determined by using *rmANOVA* followed by Dunnet's post-test. Versus the control without PMA/ Ca^{2+} ionophore: ** $P < 0.01$, * $P < 0.05$; versus control with PMA/ Ca^{2+} ionophore: # $P < 0.05$.

3.8. PbCl_2 and CdCl_2 suppressed mitochondrial DNA transcription in Jurkat cells

We next evaluated whether mitochondrial DNA transcription was affected. The mRNA expression of genes encoded in the mitochondrial genome, including the respiratory chain component *mtND2* (complex I), *mtCYB* (complex III), *mtCOI* (complex IV) and *mtATP8* (complex V), and ribosomal unit *mt12S* and *mt16S*, were measured in Jurkat cells after incubation with PbCl_2 or CdCl_2 with or without PMA/ Ca^{2+} ionophore. Our results showed that, except for the highest concentration, PbCl_2 significantly decreased the mRNA level of *mtND2* in Jurkat cells by 14.0%, 10.0% and 14.5%, respectively, compared to the untreated cells (**Figure 8A**, all $P < 0.05$). At the concentrations of 18 and 100 μM , PbCl_2 inhibited *mtCYB* mRNA by 11.5% and 16.0%, as well as *mtATP8* mRNA by 10.5% and 12.5%, respectively (**Figure 8C** and **8G**, all $P < 0.05$). CdCl_2 treatment (at 0.027 and 0.27 μM) decreased the expression of *mtND2* mRNA by 9.0% and 13.0%, respectively (**Figure 8B**, both $P < 0.05$).

Except for the lowest concentration, CdCl₂ was also found to inhibited *mtCYB mRNA* by 16.0%, 12.5% and 8.0% respectively (**Figure 8D**, all $P < 0.05$). Moreover, when its concentration was 0.027 and 2.7μM, CdCl₂ reduced *mtATP8* gene expression by 11.5% and 14.5%, respectively (**Figure 8H**, both $P < 0.05$).

Except for *mtND2*, PMA/Ca²⁺ ionophore significantly decreased the expression of the 5 other mitochondrial genes in Jurkat cells (**Figure 8E-8L**, all $P < 0.01$). PbCl₂ enhanced the suppressive effect of PMA/Ca²⁺ ionophore on *mt12S* (at 0.18, 1.8 and 18 μM, **Figure 8I**, all $P < 0.05$) and *mt16S* (at 0.18 μM, **Figure 8K**, $P < 0.05$) gene expression in Jurkat cells. CdCl₂ did not influence the decreased gene expression induced by PMA/Ca²⁺ ionophore.

Figure 8. Mitochondrial encoded gene expression in Jurkat cells. Cells were incubated for 6 hours with different concentrations of PbCl₂ and CdCl₂ alone or in combination with PMA/Ca²⁺ ionophore (10 nM/500 nM). Data are presented as median with interquartile range (n = 6). The negative control was set to 1 and all other data were presented relative to this negative control. After log-transformation, data analysis was determined by using rmANOVA followed by Dunnet's post-test. Versus the control without PMA/Ca²⁺ ionophore: ****P* < 0.001, ***P* < 0.01, **P* < 0.05; versus the control with PMA/Ca²⁺ ionophore: ##*P* < 0.01, #*P* < 0.05.

4. Discussion

In the present study, we evaluated the cytotoxic effects of PbCl₂ and CdCl₂ on a human T cell line, Jurkat cells. Our findings showed that PbCl₂ and CdCl₂ slightly affected Jurkat cell viability and apoptosis. However, the effects of PbCl₂ and CdCl₂ on viability and apoptosis were more prominent when cells were also activated by PMA/Ca²⁺ ionophore. We further demonstrated the impacts of PbCl₂ and CdCl₂ on Jurkat viability and apoptosis were associated with a decline of IL-2 production after stimulation with PMA/Ca²⁺ ionophore. The activation of the NF-κB pathway was also altered by PbCl₂ and CdCl₂ in Jurkat cells with or without activation. An elevation in mitochondrial DNA content by PbCl₂ and CdCl₂ was observed, which suggests that PbCl₂ and CdCl₂ treatment might affect Jurkat mitochondrial function. Only minor effects were found on fusion and fission gene expression. PbCl₂ and CdCl₂ were shown to downregulate the mRNA expression of mitochondrial regulator genes: *TFAM* and *NRF1*. We also observed PbCl₂ and CdCl₂ might influence mitochondrial DNA transcription in Jurkat cells by reducing mRNA levels of *mtND2*, *mtCYB* and *mtATP8*. Altogether these findings in NF-κB activation and mitochondria by PbCl₂ and CdCl₂ may be a potential mechanism of how PbCl₂ and CdCl₂ affect T cells in terms of cell viability, survival, and function, especially under activation states.

Jurkat is a well-characterized CD4⁺ T cell line that is widely used for T-cell research in vitro [33, 34]. T cells are known as a major component of the adaptive immune system responsible for controlling multiple aspects of immune responses, while their functional role highly depends on cell survival and apoptosis [3, 35]. In this study, we showed a slight, but significant decline in Jurkat cell viability after exposure to PbCl₂ and CdCl₂, which is in accordance with previous studies [36, 37]. The reduced cell viability is not reflected by an increased apoptosis rate in these cells following incubation with PbCl₂ and CdCl₂. This may indicate that apoptotic cell death might not be the mechanism involved in the downregulation of cell viability by PbCl₂ and CdCl₂. Unfortunately, we did not measure the proliferation rate of the cells. It might be suggested that PbCl₂ and CdCl₂ may decrease the proliferation of Jurkat cells. It has been reported that both lead and cadmium could promote apoptosis in most cells, which is not in line with our finding. This conflicting finding may be due to the fact that we used a different cell type but also to a different exposure dose and duration of exposure [38-40]. For instance, lead treatment was shown to induce apoptosis in cerebellar neurons even at a minor concentration

as 1 μ M; nevertheless, this phenomenon is not observed in peripheral blood mononuclear cells even at a very high concentration of 500 μ M [41, 42]. The apoptotic effect of PbCl₂ and CdCl₂ may thus indeed be cell-dependent. Interestingly, PbCl₂ (100 μ M) and CdCl₂ (10 μ M) significantly enhanced cell apoptosis in PMA/Ca²⁺ ionophore-treated Jurkat cells, suggesting that PbCl₂ and CdCl₂ tend to promote apoptosis events of activated Jurkat cells. It has been demonstrated before that the process of apoptosis in naïve T cells and activated T cells regulated by pro- and anti-apoptotic molecules (such as the BCL-2 family) are remarkably different [35], which may partially explain our results of a different pro-apoptotic effect of PbCl₂ and CdCl₂ on non-activated and activated Jurkat cells.

Activated T cells synthesize IL-2, which in turn drives the proliferation, survival, and differentiation of T cells [43]. We observed PbCl₂ and CdCl₂ alone did not affect IL-2 synthesis in non-activated Jurkat cells. This was different in other studies: *in vivo* studies have previously shown that lead treatment significantly inhibited IL-2 expression in chicken, while cadmium exposure caused a decreased production of IL-2 in mice splenocyte [44, 45]. *In vitro* studies regarding T cells demonstrated that lead (1 μ M) resulted in a suppressed IL-2 secretion by Jurkat cells [1], while cadmium (50 μ M) downregulated IL-2 production by primary T cells derived from BALB/c mice [4]. In spite of this, we noticed that IL-2 production in PMA/Ca²⁺ ionophore-activated Jurkat cells was significantly declined by PbCl₂ and CdCl₂, which indicates that the effect of PbCl₂ and CdCl₂ on IL-2 synthesis may be related to the activation state of Jurkat cells.

The NF- κ B pathway has emerged as an important contributor to T cell development and function [46-48]. Among NF- κ B subunits, p65 has received the most attention in studies of NF- κ B phosphorylation, in which serine 536 is one of the best-understood phosphorylation target within this subunit [49]. We show that PbCl₂ and CdCl₂ seem to have different effects on p65 activation. While both PbCl₂ and CdCl₂ increased the protein expression of p65, only CdCl₂ slightly but significantly increased p65 phosphorylation and thus activation. This difference in effect could be due to their different effects on I κ B α . It was previously demonstrated that p65 is sequestered in the cytoplasm in a complex with an inhibitor subunit I κ B α , whose overexpression is associated with inhibited NF- κ B activity [12, 49]. Therefore, our finding of elevated I κ B α expression in PbCl₂-treated cells and not in CdCl₂-treated cells may explain why increased phosphorylation of p65 was only observed after PbCl₂ treatment. Again we

demonstrate a difference in effect of PbCl₂ and CdCl₂ on activated and resting cells. It is worth noting that the effect of CdCl₂ in non-activated Jurkat cells was not associated with alterations in apoptosis rate and IL-2 production. We suggest CdCl₂ may rely on other mechanisms, i.e. increasing the depletion of intracellular glutathione or inducing oxidative stress [50-52], to directly or indirectly influence the association between the NF-κB pathway and apoptosis rate and IL-2 production in Jurkat cells.

Mitochondria are critical targets for the accumulation of cationic metals, such as lead, cadmium, mercury, and manganese [53, 54]. In this study, our comparative analysis on mtDNA copy number demonstrated that PbCl₂ and CdCl₂ enhanced mitochondrial content only in non-activated Jurkat cells. A previous study demonstrated that increased mtDNA copy number is protective for cells under stressful conditions [55]. Our findings, therefore, may indicate that Jurkat mitochondria react to the stressful condition of PbCl₂ and CdCl₂ stimulation. As activation with PMA/Ca²⁺ ionophore per se also increased mtDNA copy number, it may be suggested that PbCl₂ and CdCl₂ were not able to further increase mtDNA copy number.

To obtain a good insight into the effect of PbCl₂ and CdCl₂ on mtDNA transcriptional activity, we assessed the gene expression of mtDNA and mitochondrial regulators. The replication and transcription of mtDNA are regulated in part by TFAM and NRF1, which are respectively encoded by *TFAM* and *NRF1* in the nuclear genome [31, 32]. Our observations showed a reduction in mRNA level of *TFAM* and *NRF1* in Jurkat cells by PbCl₂ or CdCl₂, mainly in non-activated cells, implying that both PbCl₂ and CdCl₂ may affect mitochondrial biogenesis. This finding seems to be inconsistent with the fact that PbCl₂ and CdCl₂ increased mtDNA copy number. It may therefore be that increased mtDNA replication leads to negative feedback regulation of *TFAM* and *NRF1* mRNA levels [56]. Further studies, however, need to show that a reduction in the mRNA levels of *TFAM* and *NRF1* reflects a decrease in protein levels.

Our present study also revealed that PbCl₂ or CdCl₂ declined the transcription level of mtDNA, such as *mtND2*, *mtCYB* and *mtATP8* in non-activated Jurkat cells. This suggests that PbCl₂ and CdCl₂ are most likely to inhibit Jurkat mitochondrial transcription and function, particularly in the aspect of mitochondrial respiration and the ATP synthesizing capacity, since these genes encode proteins being components of complexes I, III and V of the electron transport chain [57, 58]. Although unfortunately the protein levels of these above-noted genes were not evaluated

in this study, our results are in line with previous experiments showing heavy metals can disrupt mitochondrial dynamics by inhibiting the mitochondrial electron transport chain [59, 60]. For instance, lead and cadmium bind to complex III to block the mitochondrial electron transport chain, consequently elevating ROS formation [61, 62].

In summary, by using an immortalized Jurkat cell line, our findings demonstrated that exposure to PbCl₂ and CdCl₂ tend to pose an adverse impact on the survival, proliferation and activation of T cells in vitro. This study also shows that the toxic effect of PbCl₂ and CdCl₂ may be associated with the activation state of T cells. Most importantly, we proposed a potential mechanism whereby PbCl₂ or CdCl₂ may affect T cells by disturbing NF- κ B activity and mitochondrial biogenesis. In further research, additional parameters, such as DNA binding activity of NF- κ B transcription factors, ATP generation and ROS production, should be evaluated to decipher the changes in NF- κ B and mitochondrial function in other cell types under numerous experimental settings.

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REFERENCES

- [1] Jorissen, A., et al., Impact of lead and mercuric ions on the interleukin-2-dependent proliferation and survival of T cells. *Arch Toxicol*, 2013. 87(2): p. 249-258.
- [2] Colombo, M., et al., Differential effects of mercury, lead, and cadmium on IL-2 production by Jurkat T cells. *Clin. Immunol.*, 2004. 111(3): p. 311-322.
- [3] Kumar, B.V., et al., Human T Cell Development, Localization, and Function throughout Life. *Immunity*, 2018. 48(2): p. 202-213.
- [4] Pathak, N., et al., Impact of cadmium in T lymphocyte subsets and cytokine expression: differential regulation by oxidative stress and apoptosis. *BioMetals*, 2008. 21(2): p. 179-187.
- [5] Undeger, U., et al., Immune alterations in lead-exposed workers. *Toxicology*, 1996. 109(2-3): p. 167-172.
- [6] Cao, J., et al., Increased memory T cell populations in Pb-exposed children from an e-waste-recycling area. *Sci Total Environ*, 2018. 616-617: p. 988-995.
- [7] Heo, Y., et al., Differential effects of lead and cAMP on development and activities of Th1- and Th2-lymphocytes. *Toxicol. Sci.*, 1998. 43(2): p. 172-185.
- [8] Tsangaris, G.T., et al., Cadmium induces apoptosis differentially on immune system cell lines. *Toxicology*, 1998. 128(2): p. 143-150.
- [9] el Azzouzi, B., et al., Cadmium induces apoptosis in a human T cell line. *Toxicology*, 1994. 88(1-3): p. 127-139.
- [10] Oeckinghaus, A., et al., The NF-kappaB family of transcription factors and its regulation. *Cold Spring Harb. Perspect. Biol.*, 2009. 1(4): p. a000034.
- [11] Albensi, B.C., What Is Nuclear Factor Kappa B (NF-kB) Doing in and to the Mitochondrion? *Front Cell Dev Biol*, 2019. 7: p. 154.
- [12] Hayden, M.S., et al., NF-kB in immunobiology. *Cell Res.*, 2011. 21(2): p. 223-244.
- [13] Giuliani, C., et al., The Role of the Transcription Factor Nuclear Factor-kappa B in Thyroid Autoimmunity and Cancer. *Front. Endocrinol. (Lausanne)*, 2018. 9: p. 471.
- [14] Ferreira, D.U., et al., Molecular mechanisms of system control of NF-kappaB signalling by IkappaBalpha. *Biochemistry*, 2010. 49(8): p. 1560-1567.
- [15] Laforge, M., et al., NF-kB pathway controls mitochondrial dynamics. *Cell Death Differ.*, 2016. 23(1): p. 89-98.
- [16] Zhou, H., et al., Mitochondrial function and nuclear factor-kappaB-mediated signalling in radiation-induced bystander effects. *Cancer Res.*, 2008. 68(7): p. 2233-2240.
- [17] Brand, M.D., et al., The role of mitochondrial function and cellular bioenergetics in ageing and disease. *Br. J. Dermatol.*, 2013. 169 Suppl 2(0 2): p. 1-8.
- [18] Osellame, L.D., et al., Cellular and molecular mechanisms of mitochondrial function. *Best Pract. Res. Clin. Endocrinol. Metab.*, 2012. 26(6): p. 711-723.
- [19] Palmer, C.S., et al., The regulation of mitochondrial morphology: intricate mechanisms and dynamic machinery. *Cell. Signal.*, 2011. 23(10): p. 1534-1545.
- [20] Quan, Y., et al., Mitochondrial ROS-Modulated mtDNA: A Potential Target for Cardiac Aging. *Oxid. Med. Cell. Longev.*, 2020. 2020: p. 9423593.
- [21] Iacobelli, M., et al., IL-2-mediated cell cycle progression and inhibition of apoptosis does not require NF-kappa B or activating protein-1 activation in primary human T cells. *J Immunol*, 1999. 162(6): p. 3308-3315.
- [22] Oh, H., et al., NF-kB: roles and regulation in different CD4(+) T-cell subsets. *Immunol Rev*, 2013. 252(1): p. 41-51.
- [23] Lopez-Granados, E., et al., A novel mutation in NFKBIA/IKBA results in a degradation-resistant N-truncated protein and is associated with ectodermal dysplasia with immunodeficiency. *Hum. Mutat.*, 2008. 29(6): p. 861-868.
- [24] Faris, R., et al., Mitochondrial Glycerol-3-Phosphate Acyltransferase-Dependent Phospholipid Synthesis Modulates Phospholipid Mass and IL-2 Production in Jurkat T Cells. *Lipids*, 2016. 51(3): p. 291-301.
- [25] Sena, L.A., et al., Mitochondria are required for antigen-specific T cell activation through reactive oxygen species signalling. *Immunity*, 2013. 38(2): p. 225-236.
- [26] Fazzini, F., et al., Plasmid-normalized quantification of relative mitochondrial DNA copy number. *Sci. Rep.*, 2018. 8(1): p. 15347.
- [27] Murphy, E., et al., Mitochondrial Function, Biology, and Role in Disease: A Scientific Statement From the American Heart Association. *Circ. Res.*, 2016. 118(12): p. 1960-1991.
- [28] Aryaman, J., et al., Mitochondrial Network State Scales mtDNA Genetic Dynamics. *Genetics*, 2019. 212(4): p. 1429-1443.
- [29] Mishra, P., et al., Mitochondrial dynamics and inheritance during cell division, development and disease. *Nat. Rev. Mol. Cell Biol.*, 2014. 15(10): p. 634-646.
- [30] Liesa, M., et al., Mitochondrial dynamics in the regulation of nutrient utilization and energy expenditure. *Cell Metab.*, 2013. 17(4): p. 491-506.
- [31] Lezza, A.M., Mitochondrial transcription factor A (TFAM): one actor for different roles. *Frontiers in Biology*, 2012. 7(1): p. 30-39.
- [32] Kiyama, T., et al., Essential roles of mitochondrial biogenesis regulator Nrf1 in retinal development and homeostasis. *Mol. Neurodegener.*, 2018. 13(1): p. 56.

- [33] Bani, L., et al., Unstimulated human CD4 lymphocytes express a cytoplasmic immature form of the common cytokine receptor gamma-chain. *J Immunol*, 2001. 167(1): p. 344-349.
- [34] Valle-Reyes, S., et al., Differential Activity of Voltage- and Ca(2+)-Dependent Potassium Channels in Leukemic T Cell Lines: Jurkat Cells Represent an Exceptional Case. *Front. Physiol.*, 2018. 9: p. 499.
- [35] Zhang, N., et al., The role of apoptosis in the development and function of T lymphocytes. *Cell Res.*, 2005. 15(10): p. 749-769.
- [36] Nemmiche, S., et al., Cadmium chloride-induced oxidative stress and DNA damage in the human Jurkat T cell line is not linked to intracellular trace elements depletion. *Toxicol In Vitro*, 2011. 25(1): p. 191-198.
- [37] Dautović, E., et al., Lead and cadmium induced cytotoxic and genotoxic effects on HL-60 and Jurkat cell lines. *Genetics & Applications*, 2019. 3(1): p. 57-64.
- [38] Tchounwou, P.B., et al., Heavy metal toxicity and the environment. *Exp Suppl*, 2012. 101: p. 133-164.
- [39] Jaishankar, M., et al., Toxicity, mechanism and health effects of some heavy metals. *Interdiscip. Toxicol.*, 2014. 7(2): p. 60-72.
- [40] Morcillo, P., et al., Heavy metals produce toxicity, oxidative stress and apoptosis in the marine teleost fish SAF-1 cell line. *Chemosphere*, 2016. 144: p. 225-233.
- [41] Oberto, A., et al., Lead (Pb+2) promotes apoptosis in newborn rat cerebellar neurons: pathological implications. *J. Pharmacol. Exp. Ther.*, 1996. 279(1): p. 435-442.
- [42] de la Fuente, H., et al., Effect of arsenic, cadmium and lead on the induction of apoptosis of normal human mononuclear cells. *Clin Exp Immunol*, 2002. 129(1): p. 69-77.
- [43] Ross, S.H., et al., Signalling and Function of Interleukin-2 in T Lymphocytes. *Annu. Rev. Immunol.*, 2018. 36: p. 411-433.
- [44] Hanson, M.L., et al., Prenatal cadmium exposure alters postnatal immune cell development and function. *Toxicol Appl Pharmacol*, 2012. 261(2): p. 196-203.
- [45] Han, Y., et al., Antagonistic effects of selenium on lead-induced autophagy by influencing mitochondrial dynamics in the spleen of chickens. *Oncotarget*, 2017. 8(20): p. 33725-33735.
- [46] Pahl, H.L., Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene*, 1999. 18(49): p. 6853-6866.
- [47] Gerondakis, S., et al., Roles of the NF-kappaB pathway in lymphocyte development and function. *Cold Spring Harb. Perspect. Biol.*, 2010. 2(5): p. a000182.
- [48] Barkett, M., et al., Control of apoptosis by Rel/NF-kappaB transcription factors. *Oncogene*, 1999. 18(49): p. 6910-6924.
- [49] Christian, F., et al., The Regulation of NF-kB Subunits by Phosphorylation. *Cells*, 2016. 5(1).
- [50] Pathak, N., et al., Impact of cadmium in T lymphocyte subsets and cytokine expression: differential regulation by oxidative stress and apoptosis. *Biometals*, 2008.21(2): p.179-187.
- [51] Lizotte, J., et al., Expression of macrophage migration inhibitory factor by osteoblastic cells: protection against cadmium toxicity. *Toxicol Lett*. 2012. 215(3): p.167-173.
- [52] Wu, X., et al., Santamarine Inhibits NF-kB Activation and Induces Mitochondrial Apoptosis in A549 Lung Adenocarcinoma Cells via Oxidative Stress. *Biomed Res Int*, 2017. 2017: p. 4734127.
- [53] Cannino, G., et al., Cadmium and mitochondria. *Mitochondrion*, 2009. 9(6): p. 377-384.
- [54] Pecze, L., et al., Divalent heavy metal cations block the TRPV1 Ca(2+) channel. *Biol. Trace Elem. Res.*, 2013. 151(3): p. 451-461.
- [55] Sadakierska-Chudy, A., et al., The Alterations in Mitochondrial DNA Copy Number and Nuclear-Encoded Mitochondrial Genes in Rat Brain Structures after Cocaine Self-Administration. *Mol Neurobiol*, 2017. 54(9): p. 7460-7470.
- [56] Ekstrand, M. I., et al., Mitochondrial transcription factor A regulates mtDNA copy number in mammals. *Hum Mol Genet*, 2004.13(9): p. 935-944.
- [57] Moilanen, J.S., et al., Lineage-specific selection in human mtDNA: lack of polymorphisms in a segment of MTND5 gene in haplogroup J. *Mol Biol Evol.*, 2003. 20(12): p. 2132-2142.
- [58] Rumsey, W.L., et al., Effects of airborne toxicants on pulmonary function and mitochondrial DNA damage in rodent lungs. *Mutagenesis*, 2017. 32(3): p. 343-353.
- [59] Engwa, G.A., et al., Mechanism and health effects of heavy metal toxicity in humans, in *Poisoning in the Modern World-New Tricks for an Old Dog?* 2019, IntechOpen.
- [60] Bansal, P., et al., Impact of lead and cadmium on enzyme of citric acid cycle in germinating pea seeds. *Biol. Plant.*, 2002. 45(1): p. 125-127.
- [61] Wang, Y., et al., Cadmium inhibits the electron transfer chain and induces reactive oxygen species. *Free Radic. Biol. Med.*, 2004. 36(11): p. 1434-1443.
- [62] Ma, L., et al., Toxicity of Pb(2+) on rat liver mitochondria induced by oxidative stress and mitochondrial permeability transition. *Toxicol Res (Camb)*, 2017. 6(6): p. 822-830.

8

GENERAL DISCUSSION

Heavy metals are naturally occurring compounds, however, anthropogenic activities introduce them in large, toxic, quantities in the surroundings [1, 2]. With the worldwide increase in the use of heavy metals in numerous applications, such as industrial, agricultural, domestic, and technological, we are all exposed, either voluntarily or involuntarily, to these heavy metals [3, 4]. It has been increasingly acknowledged that heavy metals pose a major threat to human health because of their toxicity, environmental persistence, and bioaccumulative potential [1-3].

Exposure of children to heavy metals should receive attention, as they are more susceptible to heavy metal exposure compared to adults [4, 5]. The vulnerability of children to environmental pollution of heavy metals is largely due to the following reasons: multiple exposure routes (e.g., breastfeeding, placental exposures, hand-to-mouth habits, and object-to-mouth activities), higher demand for food and ventilation per minute in relation to body size, lower toxin metabolic rate; more contacts with secondary pollution from the soil via the near-surface air layer due to their short stature, restricted movement as they live, play outdoors and attend kindergarten in the same residential area [5, 6]. Thus the exposure of children to heavy metals is higher than the exposure of adults [5, 6]. Next to this increased exposure, children are also more sensitive to heavy metal exposure. In early childhood, the immune, respiratory and central nervous systems are still in the developing stage, during which exposure to hazardous heavy metals imposes adverse impacts causing impaired functional consequences and permanent structural changes [4, 6]. In this thesis, we studied the effects of heavy metal exposure on children's health, with a special focus on the immune system.

Children living in the e-waste recycling area face a high risk for heavy metal exposure

Although adverse implications of heavy metals on children's health have been extensively emphasized by international bodies such as the WHO for a long time, the exposure of children to heavy metals still is a problem [7, 8]. The intensive uncontrolled processing of e-waste in Guiyu has caused large amounts of heavy metals to be present in the surrounding air, dust, soils, sediments, and plants, which pose local residents' children at risk of exposure unavoidably. According to our findings in **Chapters 2, 3, 4 and 5**, children living in Guiyu had higher blood levels of heavy metals, including lead, cadmium, mercury and arsenic. This is probably due to

the serious environmental pollution of heavy metals derived from the e-waste dismantling processes. As reported previously, nearly 60–80% of families in Guiyu are engaged in e-waste informal recycling activities conducted by small scale family-run workshops [9]. No or less regulation of processing and deposition of the e-waste contributes to heavy metal contamination in their living environment. Thus, children of Guiyu residents can unavoidably become exposed to heavy metals through many routes, such as the ingestion of contaminated food and water, inhalation of heavy metal-bound dust and atmospheric suspended particulates, and dermal contact of hazardous materials. Although adults are obviously also exposed to heavy metals, as indicated above, children have a higher risk of heavy metal exposure than adults due to pica and play behavior as well as an increased sensitivity to heavy metal toxicity [5, 6]. It has been reported that there has been a surge in cases of medical problems, such as leukemia, skin infections, respiratory and stomach diseases in Guiyu children [10]. Our group has previously observed higher blood chromium and manganese respectively associated with more cough and wheeze in children of Guiyu, which suggests that living in an e-waste exposed area may result in elevated levels of heavy metals accompanied by the accelerated prevalence of respiratory symptoms and asthma [11].

Our studies in this thesis showed that the levels of heavy metals in Guiyu children were lower than those reported in our previous studies. Taking blood lead level in preschool children of Guiyu as an example, the reported value has been gradually decreased from 13.2 $\mu\text{g}/\text{dl}$ of 2004 [12], and 10.3 $\mu\text{g}/\text{dl}$ of 2011 (**Chapter 2**) to 6.0 $\mu\text{g}/\text{dl}$ of 2014 (**Chapter 3**). This is primarily attributed to the environmental protection laws and regulations promulgated by the Chinese government to tackle e-waste pollution throughout the country. However, the impacts of heavy metals still remain in the local surrounding of Guiyu owing to a 30-year history of informal and uncontrolled e-waste dismantling activities, leading to relatively bigger body loading exposure in children in this area. As a consequence, we observed approximately 80% of the children from Guiyu with a blood lead level exceeding the safety limit of 50 $\mu\text{g}/\text{l}$ issued by the U.S. CDC [13], indicating that concern is still required to monitor heavy metal exposure and child's health risk in this area.

To find out the contributed and protective factors for high exposure of children to heavy metals is very important. Although the severe e-waste-induced environmental contamination of the local environment has lasted for decades, lowering the exposure chances from the point of the

child's habits, diets, and contaminant contact sources and duration would be helpful for children living in Guiyu. In our population studies (**Chapters 2, 3 and 5**) we found various child factors to be associated with high levels of heavy metals in blood, including high frequency of contact with e-waste, longer dwelling time of parents in Guiyu, the residence used as an e-waste workshop, parents' occupation associated with e-waste dismantling, and distance between the residence and e-waste deposition within 50 meters were found. As mentioned previously, most e-waste dismantling activities in Guiyu are performed in or around the residence, leading to a large accumulation of heavy metals on the surfaces of the surroundings as well as atmospheric suspended particulates [12, 14]. It is evident that children living in such surroundings would be highly exposed to heavy metals. Prior studies have demonstrated that high levels of heavy metals are present in the blood and milk of mothers with a long-term residence in an e-waste recycling area, which contributes to increased exposure of their children to heavy metals via the placenta or breastfeeding [15-17]. Therefore, the maternal residence time in Guiyu was positively associated with the loading burden of heavy metals in their children, especially those who were born and raised locally. If parents were engaged in e-waste recycling work, it also may put their children at a high risk of heavy metal exposure when in close contact after working. It is worth noting that smoking or passive smoking is also a source of non-occupational lead and cadmium exposure for humans [18, 19]. Although our study (**Chapter 2**) did not show a significant association between a family member who is smoking daily and a child's blood levels of heavy metals, our group has previously reported that parental smoking may promote an increase in child blood lead and cadmium levels [12]. Also, the education levels of the parents were associated with heavy metal levels in the blood, albeit negatively (**Chapter 5**). This can be explained by the fact that parents with a higher educational level may have more awareness of how to prevent their children from e-waste contamination. This suggestion is supported by the fact that high frequency of milk consumption, intake of bean products and supplements of calcium, iron, zinc and vitamin C, hand washing before meals, and house ventilation, were associated with low blood levels of lead, arsenic and cadmium in children (**Chapters 2 and 5**). Milk is an ideal source of macroelements (calcium and potassium) and microelements (copper, iron, zinc and selenium), which are known to play a crucial role in the protection of a human body against the negative effect of toxic free radicals [20]. Thus, milk consumption is considered as a protective factor for exposure to lead as well as other toxic

metals [12]. Keeping ventilation and handwashing is also an effective method for children to decrease the contact opportunity of exposure to heavy metals.

Population studies provide evidence for the toxic effects of heavy metal exposure on both innate and adaptive immune systems

This thesis contains four chapters of population studies describing the effects of heavy metals on both innate and adaptive immune systems of children (**Chapters 2, 3, 4 and 5, Figure 1**). Over the last decades, the immunotoxicity of heavy metals has been well documented in in vitro or animal studies, but few have been done in humans, especially in children from an e-waste recycling area.

In **Chapter 2**, we demonstrated that the alterations in the number and percentage of innate immune cells, such as monocytes, eosinophils, neutrophils, basophils and natural killer (NK) cells, were linked to higher blood lead and cadmium levels in children. Our results showed that blood lead level was positively correlated with monocyte percentage in child's peripheral blood. Although prior studies suggested that exposure to lead and cadmium can suppress the viability of monocytes in bullfrog tadpoles as well as in vitro [21, 22], it may be different in population studies in humans. The animal and in vitro studies are short-term studies. In vivo, heavy metals may also have an effect on the precursors of monocytes. This needs to be the subject of future studies, since no data are available yet on the effects of heavy metals on immune cell precursors. Therefore, the changes in monocytes in the human body may be a reflection of long-term exposure to heavy metals. Elevated neutrophil percentages were also found in children with higher blood lead and cadmium levels. An increase in these innate immune cells usually suggests the presence of inflammation [23, 24]. Indeed, lead and cadmium are able to provoke inflammatory response [25, 26]. We also noticed a trend towards increased eosinophil and basophile counts as a result of enhanced blood lead concentration when it ranged from 9.60 µg/dl to 12.04 µg/dl and from 7.69 µg/dl to 9.60 µg/dl, respectively. Animal studies provided supporting evidence that acute and chronic feeding of lead nitrate resulted in the elevation of basophils and eosinophils [27, 28]. Nevertheless, prior studies in humans showed conflicting results that lead exposure has a positive or negative correlation with eosinophil count [29-31].

The conflicting results may be attributed to the variation in populations, duration and level of exposure, and analysis method.

NK cells are a unique subset of lymphocytes essential for regulating both innate and adaptive immune responses. Two subsets exist: CD56^{dim} subset, co-expressing CD16 are cytotoxic cells, while CD56^{bright} subset are abundant cytokine producers [32]. With respect to the cytotoxic action of heavy metals on NK cells in children, our findings of both **Chapters 2 and 3** showed a significant reduction in the percentage of NK cells in Guiyu children as compared with the reference group. However, an association between blood lead level and total NK cell percentage using univariate linear regression analysis was not found in **Chapter 2** and **Chapter 3**. As noted in **Chapter 3**, the percentages of both subpopulations of NK cells were significantly lower in Guiyu children. Moreover, high blood lead level may be one of the causes of a reduced percentage of CD56^{bright} NK cells, since the blood level of lead negatively correlated with the population of CD56^{bright} NK cells in children by a univariate linear regression analysis. Based on these findings, it is reasonable to suggest that exposure to lead may inhibit the development and proliferation of NK cells in children. This chapter also demonstrated that elevated IL-1 β and decreased IL-27 expression probably linked the adverse consequence in the development and function of NK cells to lead exposure to some extent.

Not only innate immune cells but also lymphocytes appeared to be affected by heavy metal exposure. Our findings in **Chapters 3 and 5** showed that children living in Guiyu had a reduced lymphocyte count as compared with the reference group, suggesting that the proliferation, development and apoptosis of lymphocytes in Guiyu children might be affected. Although no associations between lead and cadmium exposure and lymphocyte count were observed in these two chapters. The observations in **Chapter 4** showed a decreased IL-4 production and an elevated IFN- γ /IL-4 ratio in Guiyu children, suggesting that T cells' function might be also affected. In line with this, our in vitro study (**Chapter 7**) conducted with a T lymphocyte cell line demonstrated that lead and cadmium may indeed influence the cell viability and function of T lymphocytes.

B lymphocytes play a large role in the humoral immune response defending against pathogens [33]. We also conducted an investigation on how heavy metals affect B cells of Guiyu children in **Chapter 4** of this thesis. Although in this chapter, we did not measure B cell counts in

children from the e-waste recycling area, we assessed the survival and development of B cells in children with high blood lead and cadmium levels. We observed that the apoptosis rate of B cells in children from the e-waste area was lower than the reference group. Moreover, multiple linear regression analysis showing the relationship between blood lead and cadmium levels and B cell apoptosis indicated that lead and/or cadmium may suppress B cell apoptosis in children. This seems to be in contradiction to in vitro and animal studies, which mainly show that lead and cadmium tend to promote apoptosis in many cell types [34, 35], including our own studies in **Chapters 6 and 7**. However, studies evaluating the effect of lead or cadmium on B cell apoptosis are scarce in humans. Future studies should focus on this, since this cell type seems to respond differently to lead and cadmium than most other cell types. Noteworthy, we observed a suppression in caspase-9 activity and an increase in the NF- κ B transcription factor *c-Rel* mRNA level in B cells of children in the e-waste area, indicating that these factors may play a mechanistic role in the decreased apoptosis rates in e-waste area children. It has been reported that lead can increase B cell activation/proliferation [35], which is consistent with our finding. From this point of view, heavy metals may exert an adverse effect on the development and functioning of both B cells or T cells, disrupting both humoral and cell-mediated immunity.

Based on the above-described findings, heavy metals appear to have adverse effects on the immune system in children. By using a multiple linear regression analysis, our work of **Chapter 5** further demonstrated that exposure to multiple heavy metals, including lead, cadmium, mercury and arsenic, appears to result in aberrant immune homeostasis and inflammatory activation in children. Different from other chapters, more potential confounders, including characteristics and habits of children, and parents' information, were considered in this chapter in order to find out more profound associations of heavy metal exposure with immune parameters. In addition to their respective effects, the interaction effects of lead, cadmium, mercury and arsenic on immune cells and cytokines were also assessed by a centering methodology in the multiple linear regression models. As important players in mediating inflammatory responses as well as in regulating immune response, measuring pro-inflammatory interleukin (IL)-1 β , IL-6, IL-8, tumor necrosis factor (TNF)- α and anti-inflammatory cytokines IL-1 receptor antagonist (IL-1 RA), IL-4, IL-10 and IL-13 could help in showing the alterations in immune homeostasis [36-39]. As shown in **Chapter 5**, high concentrations of IL-1 β and IL-6 but low IL-1RA and IL-13 were observed with high blood levels of lead, cadmium, mercury,

and arsenic in Guiyu children. Prior data from the epidemiological, animal, and in vitro studies have shown that all these four heavy metals possess pro-inflammatory activities characterized by increasing the production of ROS, TNF- α , IL-6, and IL-8 but reducing IL-10 and IL-1RA [40-43], which is in parallel with our findings. Although we cannot identify far-reaching health consequences in children living in Guiyu at present, our findings showing an immune imbalance and inflammatory state may indicate that children exposed to toxic heavy metals might have a potential risk for developing immune associated diseases in the future. Taking into consideration that prolonged inflammation may contribute to a wide range of chronic diseases, including cancer, diabetes, and cardiovascular, neurodegenerative and autoimmune diseases [44-46], more attention is needed for a better understanding of the exact impacts of heavy metal exposure on children's health in the long term in future investigations.

Figure 1. A summary of the effect of heavy metal derived from e-waste disposal on the immune system of Guiyu children. Created with BioRender.com.

In vitro studies demonstrate possible molecular mechanisms of how heavy metals affect immune response

In the second part of the thesis (**Chapters 6 and 7**), we aimed to evaluate the underlying mechanism of the immunotoxicity of heavy metals in vitro models. The effects of heavy metals on the immune response have been studied in this thesis, as well as by other researchers [47-49]. Existing in vitro studies have shown that heavy metals could inhibit cell viability, enhance oxidative stress and lipid peroxidation, mitochondrial and DNA damage, cohesion loss and cell apoptosis in many cell types [47-49]. However, the precise mechanism of how heavy metal affect the immune cells is not yet fully understood and therefore requires more effort to elucidate. In **Chapters 6 and 7** of this thesis, the human MonoMac 6 and Jurkat cell line was applied to demonstrate the effects of the heavy metals lead and cadmium on monocytes and T cells, respectively.

We first wanted to explore a possible mechanism by which the heavy metals lead and cadmium could affect immune cells. We studied whether lead and cadmium affect TLR activation, since it has been known that many immune cells, such as monocytes, and T lymphocytes, express Toll-like receptors (TLRs) [50-52]. TLR activation can initiate downstream signalling pathway, including NF- κ B and MAPK, consequently triggering host defensive responses and orchestrating immune responses [50, 53]. In **Chapter 6** we used THP1-MD2-CD14 and various HEK-Blue reporter cell lines (stably expressing either TLR 2, 3, 4, 5, 7, 8, and 9, together with NF- κ B inducible secreted alkaline phosphatase) to evaluate whether lead or cadmium could activate or inhibit TLRs. We demonstrated that lead and cadmium may exert toxic effects on immune cells via TLRs and the NF- κ B pathway (**Figure 2**). Although both lead and cadmium hardly activated TLRs by themselves, lead was found to promote the activation of TLR4, 5 and 8 induced by their corresponding agonists. Moreover, lead attenuated TLR3 and TLR9 activation by their agonists. High doses of cadmium negatively affected TLR2, 4, 5 and 7 activation by their agonists, while high cadmium increased TLR3 activation by its agonist. However, the lower dose of cadmium can elevate TLR4, 5 and 8 activations in the presence of their agonists. Our findings provide evidence that exposure to lead and cadmium may affect the response of the immune system against pathogens by affecting activation of TLRs. Although

reports regarding the effects of lead and cadmium on TLR4 signalling are scanty, previous studies showed that other metals, i.e. nickel, can ligate with two histidine residues of the TLR4 molecule [63, 64]. Potentially, lead and cadmium may also bind to TLR4 histidine residues to influence TLR4 and its downstream signalling, since lead and cadmium have similar physical properties of divalent metal cations compared with nickel. Alternatively, lead and cadmium may also bind to LPS, altering the interaction between LPS and TLR4, however, this has not been studied yet.

Figure 2. A summary of the effect of heavy metals on TLRs. (A) Lead action on TLRs in the presence of agonists; (B) Cadmium action on TLRs in the presence of agonists. The red dotted line represents a synergistic effect, the black dotted line represents an antagonistic effect. low: low concentration; high: high concentration. Created with BioRender.com.

Monocytes play important roles in the immune defense, inflammation and tissue remodeling by phagocytosis, antigen processing and presentation, and cytokine synthesis [54, 55]. As described in **Chapter 2**, the number of monocytes in peripheral blood was increased in children with high blood levels of lead and cadmium. Besides, a positive association between blood lead level and monocyte percentage was noted, indicating that exposure to lead may influence the survival of monocytes themselves or their precursors. MonoMac 6 is a monocyte cell line with many similar properties to monocyte, such as CD14 expression, phagocytic ability and cytokine

production, which therefore is considered as a good model for mature blood monocytes [56]. In our study in **Chapter 6**, we used this cell line to explore the effects of lead and cadmium on monocytes in vitro (**Figure 3**). Since TLR4 is prominently expressed on monocytes and since our study on the effects of lead and cadmium are obvious for TLR4, we focussed on the effects of lead and cadmium on this receptor in the MonoMac 6 cells. Our findings showed that lead and cadmium suppressed cell viability and induced apoptosis in MonoMac 6 cells, which is in accordance with many previous in vitro studies with other cell models [57-59]. We further observed the expression of ICAM-1, an activation marker of monocytes, is upregulated by lead treatment, while both lead and cadmium slightly decreased ICAM-1 expression after stimulation with the TLR4 agonist LPS. Therefore, it seems that lead and cadmium can alter the function of monocytes in vitro, but that the effects may be depended on the activation status of the monocytes. Another functional marker for monocytes, i.e., cytokine production, was also affected by lead and cadmium. Although lead and cadmium did not affect TNF- α and IL-10 per se, it was shown that LPS-induced pro-inflammatory TNF- α production was elevated, while anti-inflammatory IL-10 production was inhibited by lead and/or cadmium. This is in agreement with our findings in our vivo studies in **Chapter 5**, suggesting that lead and cadmium have the ability to induce pro-inflammatory effects. These effects may be induced via TLRs, since monocytes express TLRs, such as TLR4.

The role of lead and cadmium in mediating the TLR4 downstream MAPK pathway has also been demonstrated in **Chapter 6**. Our data showed that cadmium but not lead alone did elevate p38 phosphorylation in MonoMac 6 cells, which is not completely consistent with previous studies in other cell lines [60-62], in which both lead and cadmium can induce p38 MAPK activation. This difference may be associated with the different lead concentrations, or different cell models. However, we also observed both lead and cadmium further activate MAPK signalling pathway by increasing phosphorylation of p38 in MonoMac 6 after LPS stimulation, indicating that the effect of lead and cadmium is different in LPS stimulated vs unstimulated MonoMac 6 cells. Our findings in Jurkat on the impact of lead and cadmium on mitochondria in **Chapter 7** suggest that lead and cadmium predominantly affect mitochondria in unstimulated cells rather than that in stimulated cells. These differences could explain why lead and cadmium induced different effects on stimulated and unstimulated MonoMac 6 cells.

Future investigations should therefore also focus on the effects of lead and cadmium in mitochondria in monocytes.

Figure 3. Heavy metals affect the monocytic cell model via TLR4 and the downstream NF-κB and MAPK signalling pathway; A and C represent non-activated MonoMac 6 model; B and D represent LPS-activated MonoMac 6 model. TLR4 localizes to the cell surface and is an important sensor for LPS. The stimulation of TLR4 by LPS initiates the intracellular NF-κB and MAPK signalling pathways and inflammatory cytokine release, which plays an important role in pathogen recognition and activation of innate immunity. Created with BioRender.com.

T cells are known as a major component of the adaptive immune system and are responsible for governing multiple aspects of immune responses. We conducted an in vitro study in Jurkat

cells to investigate how lead and cadmium affect T cells with respect to cell viability, survival and activation (**Chapter 7**) (*Figure 4*). In line with previous studies [65, 66], our data showed a slight, but significant reduction in Jurkat cell viability as a result of lead and cadmium treatment. Our *in vivo* studies (**Chapters 3 and 5**) also provided evidence that lymphocyte counts were reduced in children with lead and cadmium exposure, although the changes in T cells were not specifically studied. Although it has been extensively reported that lead and cadmium-induced apoptosis in most cell types [57-59], lead and cadmium per se did not increase apoptosis of Jurkat cells in our study. Mitogenic activation of resting lymphocytes induces phosphoinositide hydrolysis of the cell membrane leading to the generation of inositol trisphosphate and diacylglycerol, which act as second messengers to liberate intracellular calcium stores and to activate protein kinase C (PKC), respectively. In *in vitro* model studies, these events can be mimicked with agents, i.e., PMA/Ca²⁺ ionophore [67]. PMA can activate PKC, whose activation triggers cellular signals that lead to the activation of the transcription factor NF- κ B in various cell types [67, 68, 69]. Ca²⁺ ionophore treatment elevates intracellular calcium level to bypass the transmembrane signalling inducing the lymphocytes to proliferate and activate [67]. Interestingly, we observed that lead and cadmium promoted apoptosis in Jurkat cells induced by PMA/Ca²⁺ ionophore. IL-2 is an autocrine growth factor that drives the proliferation, survival, and differentiation of T cells [70]. In parallel with the observation that lead and cadmium did not induce apoptosis in Jurkat cells, lead and cadmium per se also did not increase the production of IL-2 in these cells. However, IL-2 production in PMA/Ca²⁺ ionophore-stimulated cells was inhibited by lead and cadmium. Therefore, the decreased IL-2 production induced by lead and cadmium after PMA/Ca²⁺ ionophore stimulation may be (partly) responsible for decreased cell viability and increased apoptosis induced by lead and cadmium in activated Jurkat cells. Lead and cadmium thus also affected PMA/Ca²⁺ ionophore-activated cells and non-activated cells differently.

We expanded our understanding of how lead and cadmium affect Jurkat cells by evaluating NF- κ B activity. Jurkat cells also express most TLRs (TLR1, 2, 3, 4, 5, 7 and 9) [71], offering us a hint that the effects of lead and cadmium on Jurkat cells may be via these TLRs. Although we did not test this in our experiments, this suggestion is supported by our observations in **Chapter 6** that lead and cadmium affect NF- κ B activation, one of the intracellular signalling pathways induced by TLRs. Whether this NF- κ B activation is indeed induced via TLRs needs further

investigation. In **Chapter 7**, lead was found to induce an elevation in p65 and I κ B α in non-activated Jurkat cells, indicating that lead may not elicit NF- κ B activation in non-activated Jurkat cells. Lead induced a decrease in p65 phosphorylation in activated Jurkat cells, therefore it inhibits NF- κ B activation in activated Jurkat cells. Cadmium increased NF- κ B activation by increasing both p65 and p65 phosphorylation in non-activated Jurkat cells, while it does not affect NF- κ B activation in activated cells. NF- κ B may thus act as one of the key targets for heavy metal-induced toxic actions in Jurkat cells. This is in line with a previous paper [72], however, the molecular mechanisms involved in how lead and cadmium impact NF- κ B activation remains unclear. As lead and cadmium can readily bind to sulfhydryl groups and displace zinc and calcium ions from proteins and enzymes, converting them structurally to an inactive form and completely altering their activity [73, 74], we speculated that they are also likely to change NF- κ B p65 and I κ B α in this way. Given the pivotal role of NF- κ B transcription factors in mediating cell activation, proliferation, differentiation and apoptosis, more studies into the effects of lead and cadmium and other heavy metals on the NF- κ B family and their binding activity with DNA would be helpful to reveal the precise mechanism of the immunotoxicity of heavy metals.

Apart from TLRs and the downstream NF- κ B signalling, lead and cadmium can enter cells via ionic mechanisms, for example, via calcium channels [75]. Prior research has demonstrated that intracellular mitochondria are responsive to these heavy metals [76]. Thus, there may be another mechanism by which lead and cadmium may induce toxic effects on Jurkat cells: by disturbing mitochondrial biogenesis (**Chapter 7**). Our study found an increase in mtDNA copy number of Jurkat cells in response to lead and cadmium, suggesting that Jurkat mitochondria enhance biogenesis to adapt to a lead and cadmium challenge. Indeed, it has previously been shown that an increased mtDNA copy number is protective for cells under stressful conditions [77]. We also assessed the effect of lead and cadmium on mtDNA replication and transcription, which are regulated in part by TFAM and NRF1 [78, 79]. Our results showed a reduction in mRNA level of mitochondrial regulators *TFAM* and *NRF1* in non-activated Jurkat cells treated with lead and cadmium, with little and no effect of lead and cadmium in activated cells. It was known that genes in the mitochondrial genome, including *mtND2* (complex I), *mtCYB* (complex III), *mtCOI* (complex IV) and *mtATP8* (complex V) encode polypeptides for subunits of enzyme complexes of the oxidative phosphorylation system [80]. We observed that lead and cadmium

reduced the transcription level of *mtND2*, *mtCYB* and *mtATP8* in non-activated Jurkat cells. This therefore indicates that lead and cadmium may play an inhibitory role in regulating mitochondrial biogenesis and mitochondrial function (i.e. mitochondrial respiration and ATP synthesizing capacity) of non-activated Jurkat cells.

Figure 4. Schematic overview of NF-κB and mitochondrial biogenesis in a T lymphocytic cell model (Jurkat cells). (A) lead treatment; (B) lead plus PMA/Ca²⁺ ionophore treatment; (C) cadmium treatment; (D) cadmium plus PMA/Ca²⁺ ionophore treatment. The effects of lead and cadmium on Jurkat cells are probably via TLRs and ionic channels, which respectively impact NF-κB signalling pathway and mitochondrial biogenesis, accordingly changing the activation, viability and survival of Jurkat cells. Mitochondria play a pivotal role in Ca²⁺ signalling and Ca²⁺ homeostasis and are sensitive to the substitution of lead and cadmium for calcium. Moreover, their effects on Jurkat cells may be associated with cell activation state. Created with BioRender.com.

Conclusions and future perspectives

Taken altogether, this thesis including population and in vitro studies has provided a series of experiments to explore the effects of heavy metals on immune cells in vivo and in vitro.

The risk of exposure to and body loading of heavy metals in Guiyu children remains high, reflecting that unregulated e-waste recycling activities in Guiyu largely pose local children at risk. The local residents should pay more attention to this issue and awareness should be strengthened to protect their children from heavy metals and e-waste pollution. Some factors, such as frequency of contact with e-waste, dwelling time in Guiyu, the residence used as an e-waste workshop, the distance between the residence and e-waste deposition, and parents engaged in e-waste dismantling, were observed to contribute to heavy metal exposure, which could be clues of how to lower the exposure risk of heavy metals.

Importantly, the effects of heavy metals on the immune cells of Guiyu children were evaluated (**Chapters 2, 3, 4 and 5**). We suggest that the development and function of immune cells, such as monocytes, NK cells, B cells, neutrophils, eosinophils and basophils, were affected by heavy metals. This part of the work provides a series of evidence proving the immunotoxic effects of heavy metals on children, especially those who reside in an e-waste recycling area. Although we did not see the adverse consequence resulted from aberrant immune alterations yet, further risk assessment of health problems in children could be necessary to conduct. It is also essential to carry out studies to investigate the association between heavy metal exposure and health problems in adults who have lived in Guiyu for their whole life.

Guiyu populations also suffer from exposure to other environmental chemicals resulting from e-waste dismantling, such as persistent organic pollutants (POPs), which also have immunotoxicity [81-83]. The synergistic or antagonistic interactions between heavy metals and POPs on the immune cells may be important in vivo factors, which we did not study yet. Therefore, further analysis of the interaction between heavy metals and POPs in vitro and in vivo in mediating the immune response are necessary to explore their exact toxic effects on the immune system in humans. Moreover, our results in vitro only reflect the short-term effects of heavy metals on immune cell models, long-term exposure experimental studies, for instance, in animals, are needed in order to obtain a better understanding of the role of heavy metals on the immune response in vivo.

We demonstrated that the toxic action of heavy metals on the immune cells may be via TLRs and their downstream activation of NF- κ B and MAPK. It is noteworthy to mention that the exact effects of heavy metals on the immune cells potentially depend on the activation status of cells. This could also be concluded from our findings of TLR activation and inhibition experiments with HEK-Blue reporter cell lines (**Chapter 6**) showing that TLR activation is only affected by lead and cadmium in the presence of the ligands. Exploring how lead and cadmium interplay with functional domains of TLRs in the absence or presence of their ligands could profoundly clarify the specific role of lead and cadmium in activated immune cells via TLRs. However, the effects of lead and cadmium may not only relate to TLRs but these heavy metals can also enter the cells via, for instance, calcium channels and thus affect intracellular organelles, such as mitochondria. Our studies showed that lead and cadmium increased mtDNA content but may inhibit mtDNA replication and transcription in non-activated Jurkat cells. However, further insight into the effects of heavy metals on mitochondrial function and biogenesis is important for our understating of the effects of heavy metals on immune cells. Our *in vitro* work expands the understanding of how heavy metals affect the immune cells and brings new insights for exploring heavy metal toxicities in other cell types. Besides, our *in vitro* studies leave us a hypothesis for the *in vivo* effects of lead and cadmium that heavy metals may not induce immunological diseases in animals and humans in a direct manner, but may exaggerate diseases once they are caused by other inducers. Further investigations, for instance, in animal models, are necessary in order to substantiate this hypothesis.

Additionally, the findings of TLR activation and inhibition experiments provide us some significant information that not only the role of TLR4, but also the role of various other TLRs (including TLR1, 2, 3, 5, 6, 7, 8, 9 and 10) and their intracellular adaptor molecules (such as MyD88, IRF1, IRF3, IRF7, TRAF6 and TRAF3) [50] should be explored in the future research. Also, other immune receptors, including T cell receptor, B cell receptor, C-type lectin receptor, NOD-like receptor, and RIG-I-like receptor, play a vital role in the infection and immunity of human body [84-86]. To extend our work on how heavy metals affect these receptors and their crosstalk with TLRs, particularly in the presence of the ligands or activators, will obtain more comprehensive knowledge to understand the toxic effects of heavy metals on the immune response.

REFERENCES

- [1] Masindi, V., et al., Environmental contamination by heavy metals. *Heavy metals*, 2018. 10: p. 115-132.
- [2] Ali, H., et al., Environmental chemistry and ecotoxicology of hazardous heavy metals: environmental persistence, toxicity, and bioaccumulation. *Journal of chemistry*, 2019. 2019.
- [3] Tchounwou, P.B., et al., Heavy metal toxicity and the environment, in *Molecular, clinical and environmental toxicology*. 2012, Springer. p. 133-164.
- [4] Al Osman, M., et al., Exposure routes and health effects of heavy metals on children. *BioMetals*, 2019. 32(4): p. 563-573.
- [5] Tepanosyan, G., et al., Heavy metals pollution levels and children health risk assessment of Yerevan kindergartens soils. *Ecotoxicol Environ Saf*, 2017. 142: p. 257-265.
- [6] Zeng, X., et al., Children with health impairments by heavy metals in an e-waste recycling area. *Chemosphere*, 2016. 148: p. 408-415.
- [7] Järup, L., Hazards of heavy metal contamination. *Br Med Bull*, 2003. 68(1): p. 167-182.
- [8] Szkup-Jablonska, M., et al., Effects of blood lead and cadmium levels on the functioning of children with behaviour disorders in the family environment. *Ann Agric Environ Med*, 2012. 19(2).
- [9] Huo, X., et al., Elevated blood lead levels of children in Guiyu, an electronic waste recycling town in China. *Environ Health Perspect*, 2007. 115(7): p. 1113-1117.
- [10] Leung, A., et al., Environmental contamination from electronic waste recycling at Guiyu, southeast China. *J Mater Cycles Waste Manage*, 2006. 8(1): p. 21-33.
- [11] Zeng, X., et al., Heavy metals in PM_{2.5} and in blood, and children's respiratory symptoms and asthma from an e-waste recycling area. *Environ Pollut*, 2016. 210: p. 346-353.
- [12] Zheng, L., et al., Blood lead and cadmium levels and relevant factors among children from an e-waste recycling town in China. *Environ Res*, 2008. 108(1): p. 15-20.
- [13] Betts, K.S., CDC updates guidelines for children's lead exposure. 2012, National Institute of Environmental Health Sciences.
- [14] Guo, P., et al., Blood lead levels and associated factors among children in Guiyu of China: a population-based study. *PLoS One*, 2014. 9(8): p. e105470.
- [15] Guo, Y., et al., Monitoring of lead, cadmium, chromium and nickel in placenta from an e-waste recycling town in China. *Sci Total Environ*, 2010. 408(16): p. 3113-3117.
- [16] Arbuckle, T.E., et al., Maternal and fetal exposure to cadmium, lead, manganese and mercury: the MIREC study. *Chemosphere*, 2016. 163: p. 270-282.
- [17] Silver, M.K., et al., Distribution and predictors of 20 toxic and essential metals in the umbilical cord blood of Chinese newborns. *Chemosphere*, 2018. 210: p. 1167-1175.
- [18] Lutz, P.M., et al., Environmental cigarette smoke exposure modulates IgE levels of Pb-exposed children. *Toxicology*, 2012. 291(1-3): p. 43-50.
- [19] Behera, S.N., et al., Human health risk associated with exposure to toxic elements in mainstream and sidestream cigarette smoke. *Sci Total Environ*, 2014. 472: p. 947-956.
- [20] BIGUCU, E., et al., The Effect of Environmental Factors on Heavy Metal and Mineral Compositions of Raw Milk and Water Samples. 2016.
- [21] Carvalho, C., et al., Blood cell responses and metallothionein in the liver, kidney and muscles of bullfrog tadpoles, *Lithobates catesbeianus*, following exposure to different metals. *Environ Pollut*, 2017. 221: p. 445-452.
- [22] Steffensen, I., et al., Cytotoxicity and accumulation of Hg, Ag, Cd, Cu, Pb and Zn in human peripheral T and B lymphocytes and monocytes in vitro. *General Pharmacology: The Vascular System*, 1994. 25(8): p. 1621-1633.
- [23] Nowarski, R., et al., Innate immune cells in inflammation and cancer. *Cancer immunology research*, 2013. 1(2): p. 77-84.
- [24] Newton, K., et al., Signaling in innate immunity and inflammation. *Cold Spring Harb Perspect Biol*, 2012. 4(3): p. a006049.
- [25] Pineda-Zavaleta, A.P., et al., Nitric oxide and superoxide anion production in monocytes from children exposed to arsenic and lead in region Lagunera, Mexico. *Toxicol. Appl. Pharmacol*, 2004. 198(3): p. 283-290.
- [26] Jin, Y., et al., Cadmium exposure to murine macrophages decreases their inflammatory responses and increases their oxidative stress. *Chemosphere*, 2016. 144: p. 168-175.
- [27] Shah, S.L., et al., Alterations in the immunological parameters of Tench (*Tinca tinca* L. 1758) after acute and chronic exposure to lethal and sublethal treatments with mercury, cadmium and lead. *Turkish Journal of Veterinary and Animal Sciences*, 2005. 29: p. 1163-1168.
- [28] Al-Ali, S.J.K., et al., Effect of acute and chronic lead nitrate exposure on some physiological parameters in experimental mice. *Basrah Journal of Veterinary Research*, 2007. 6(2): p. 78-88.

- [29] Karmaus, W., et al., Immune function biomarkers in children exposed to lead and organochlorine compounds: a cross-sectional study. *Environ Health*, 2005. 4(1): p. 5.
- [30] Ukaejiofo, E., et al., Haematological assessment of occupational exposure to lead handlers in Enugu urban, Enugu State, Nigeria. *Niger J Clin Pract*, 2009. 12(1).
- [31] Wells, E.M., et al., The relationship of blood lead with immunoglobulin E, eosinophils, and asthma among children: NHANES 2005–2006. *Int J Hyg Environ Health*, 2014. 217(2-3): p. 196-204.
- [32] Poli, A., et al., CD56bright natural killer (NK) cells: an important NK cell subset. *Immunology*, 2009. 126(4): p. 458-465.
- [33] Tsai, D.-Y., et al., Regulatory mechanisms of B cell responses and the implication in B cell-related diseases. *J Biomed Sci*, 2019. 26(1): p. 64.
- [34] Skipper, A., et al., Cadmium chloride induces DNA damage and apoptosis of human liver carcinoma cells via oxidative stress. *Int J Env Res Public Health*, 2016. 13(1): p. 88.
- [35] Kasten-Jolly, J., et al., Impact of developmental lead exposure on splenic factors. *Toxicol Appl Pharmacol*, 2010. 247(2): p. 105-115.
- [36] Roerink, M.E., et al., Interleukin-1 as a mediator of fatigue in disease: a narrative review. *J Neuroinflammation*, 2017. 14(1): p. 16.
- [37] Cho, Y., et al., Identification of novel cytokine biomarkers of hexanal exposure associated with pulmonary toxicity. *Environ Pollut*, 2017. 229: p. 810-817.
- [38] Fujii, U., et al., Effect of a retinoid X receptor partial agonist on airway inflammation and hyperresponsiveness in a murine model of asthma. *Respir Res*, 2017. 18(1): p. 1-10.
- [39] Landskron, G., et al., Chronic inflammation and cytokines in the tumor microenvironment. *J Immunol Res*, 2014. 2014: p. 149185.
- [40] Carey, J.B., et al., Immune modulation by cadmium and lead in the acute reporter antigen-popliteal lymph node assay. *Toxicol Sci*, 2006. 91(1): p. 113-122.
- [41] Valentino, M., et al., Effect of lead on the levels of some immunoregulatory cytokines in occupationally exposed workers. *Hum Exp Toxicol*, 2007. 26(7): p. 551-556.
- [42] Zhang, W., et al., Effects of formoterol and ipratropium bromide on repeated cadmium inhalation-induced pulmonary inflammation and emphysema in rats. *Eur. J. Pharmacol.*, 2010. 647(1-3): p. 178-187.
- [43] Maria, B., et al., Cytokine production by human peripheral blood mononuclear cells after exposure to heavy metals. *J Health Sci*, 2000. 46(5): p. 358-362.
- [44] Hamilton, J.A., Colony-stimulating factors in inflammation and autoimmunity. *Nat Rev Immunol*, 2008. 8(7): p. 533-544.
- [45] Schenkein, H.A., et al., Inflammatory mechanisms linking periodontal diseases to cardiovascular diseases. *J Clin Periodontol*, 2013. 40 Suppl 14(0 14): p. S51-69.
- [46] Wu, Y., et al., Molecular mechanisms underlying chronic inflammation-associated cancers. *Cancer Lett*, 2014. 345(2): p. 164-173.
- [47] Gargouri, M., et al., Cytoprotective and antioxidant effects of the edible halophyte *Sarcocornia perennis* L. (swampfire) against lead-induced toxicity in renal cells. *Ecotoxicol. Environ. Saf.*, 2013. 95: p. 44-51.
- [48] Rashid, K., et al., An update on oxidative stress-mediated organ pathophysiology. *Food Chem Toxicol*, 2013. 62: p. 584-600.
- [49] Wu, X., et al., A review of toxicity and mechanisms of individual and mixtures of heavy metals in the environment. *Environ Sci Pollut Res Int*, 2016. 23(9): p. 8244-8259.
- [50] Kawasaki, T., et al., Toll-like receptor signaling pathways. *Front Immunol*, 2014. 5: p. 461.
- [51] Kabelitz, D., Expression and function of Toll-like receptors in T lymphocytes. *Curr Opin Immunol*, 2007. 19(1): p. 39-45.
- [52] Dasari, P., et al., Expression of toll-like receptors on B lymphocytes. *Cell. Immunol.*, 2005. 236(1-2): p. 140-145.
- [53] Murphy, M., et al., Pellino-1 Positively Regulates Toll-like Receptor (TLR) 2 and TLR4 Signaling and Is Suppressed upon Induction of Endotoxin Tolerance. *J. Biol. Chem.*, 2015. 290(31): p. 19218-19232.
- [54] Kumar, S., et al., Origin of monocytes and their differentiation to macrophages and dendritic cells. *J Endotoxin Res*, 2006. 12(5): p. 278-284.
- [55] Geissmann, F., et al., Development of monocytes, macrophages, and dendritic cells. *Science*, 2010. 327(5966): p. 656-661.
- [56] Neustock, P., et al., Cytokine production of the human monocytic cell line Mono Mac 6 in comparison to mature monocytes in peripheral blood mononuclear cells. *Immunobiology*, 1993. 188(3): p. 293-302.
- [57] Honda, A., et al., Effects of air pollution-related heavy metals on the viability and inflammatory responses of human airway epithelial cells. *Int J Toxicol*, 2015. 34(2): p. 195-203.
- [58] Morcillo, P., et al., Heavy metals produce toxicity, oxidative stress and apoptosis in the marine teleost fish SAF-1 cell line. *Chemosphere*, 2016. 144: p. 225-233.
- [59] Sljivic Husejnovic, M., et al., Assessment of Pb, Cd and Hg soil contamination and its potential to cause cytotoxic and genotoxic effects in human cell lines

- (CaCo-2 and HaCaT). *Environ. Geochem. Health.*, 2018. 40(4): p. 1557-1572.
- [60] Posser, T., et al., Exposure of C6 glioma cells to Pb(II) increases the phosphorylation of p38(MAPK) and JNK1/2 but not of ERK1/2. *Arch. Toxicol.*, 2007. 81(6): p. 407-414.
 - [61] Khan, M.I., et al., Ubiquitous hazardous metal lead induces TNF- α in human phagocytic THP-1 cells: primary role of ERK 1/2. *J Hazard Mater*, 2011. 189(1-2): p. 255-264.
 - [62] Seok, S.M., et al., COX-2 is associated with cadmium-induced ICAM-1 expression in cerebrovascular endothelial cells. *Toxicol Lett.*, 2006. 165(3): p. 212-220.
 - [63] Rachmawati, D., et al., Transition metal sensing by Toll-like receptor-4: next to nickel, cobalt and palladium are potent human dendritic cell stimulators. *Contact dermatitis*, 2013. 68(6): p. 331-338.
 - [64] Schmidt, M., et al., Crucial role for human Toll-like receptor 4 in the development of contact allergy to nickel. *Nat. Immunol.*, 2010. 11(9): p. 814-819
 - [65] Nemmiche, S., et al., Cadmium chloride-induced oxidative stress and DNA damage in the human Jurkat T cell line is not linked to intracellular trace elements depletion. *Toxicol. In Vitro*, 2011. 25(1): p. 191-198.
 - [66] Dautović, E., et al., Lead and cadmium induced cytotoxic and genotoxic effects on HL-60 and Jurkat cell lines. *Genetics & Applications*, 2019. 3(1): p. 57-64.
 - [67] Planelles, D., et al., Differential effects of the calcium ionophore A23187 and the phorbol ester PMA on lymphocyte proliferation. *Agents Actions*, 1992. 35(3-4): p. 238-244.
 - [68] Zheng, J., et al., Protein kinase C- α (PKC α) modulates cell apoptosis by stimulating nuclear translocation of NF-kappa-B p65 in urothelial cell carcinoma of the bladder. *BMC Cancer*, 2017. 17(1):432
 - [69] Kannegieter, N. M., et al., Analysis of NFATc1 amplification in T cells for pharmacodynamic monitoring of tacrolimus in kidney transplant recipients. *Plos one*, 2018. 13(7): e0201113.
 - [70] Ross, S.H., et al., Signaling and Function of Interleukin-2 in T Lymphocytes. *Annu. Rev. Immunol.*, 2018. 36: p. 411-433.
 - [71] Dai, J., et al., Regulatory T cells and Toll-like receptors: what is the missing link? *Int Immunopharmacol*, 2009. 9(5): p. 528-533.
 - [72] Chen, F., et al., Signaling from toxic metals to NF-kappaB and beyond: not just a matter of reactive oxygen species. *Environ Health Perspect*, 2002. 110 (Suppl 5): p. 807-811.
 - [73] Engwa G.A., et al., Mechanism and health effects of heavy metal toxicity in humans. *Poisoning in the modern world-new tricks for an old dog*, 2019, 10.
 - [74] Chrestensen, C.A., et al., Acute cadmium exposure inactivates thioltransferase (Glutaredoxin), inhibits intracellular reduction of protein-gluthionyl-mixed disulfides, and initiates apoptosis. *J. Biol. Chem.*, 2000. 275(34): p. 26556-26565.
 - [75] Pecze, L., et al., Divalent heavy metal cations block the TRPV1 Ca(2+) channel. *Biol. Trace Elem. Res.*, 2013. 151(3): p. 451-461.
 - [76] Cannino, G., et al., Cadmium and mitochondria. *Mitochondrion*, 2009. 9(6): p. 377-384.
 - [77] Castellani, C.A., et al., Thinking outside the nucleus: Mitochondrial DNA copy number in health and disease. *Mitochondrion*, 2020. 53: p. 214-223.
 - [78] Lezza, A.M., Mitochondrial transcription factor A (TFAM): one actor for different roles. *Frontiers in Biology*, 2012. 7(1): p. 30-39.
 - [79] Kiyama, T., et al., Essential roles of mitochondrial biogenesis regulator Nrf1 in retinal development and homeostasis. *Mol. Neurodegener*, 2018. 13(1): p. 56.
 - [80] Taanman, J.W., The mitochondrial genome: structure, transcription, translation and replication. *Biochim. Biophys. Acta.*, 1999. 1410(2): p. 103-123.
 - [81] Zheng, X., et al., Cardiovascular endothelial inflammation by chronic coexposure to lead (Pb) and polycyclic aromatic hydrocarbons from preschool children in an e-waste recycling area. *Environ. Pollut.*, 2019. 246: p. 587-596.
 - [82] Cheng, Z., et al., Elevated expression of AhR and NLRP3 link polycyclic aromatic hydrocarbon exposure to cytokine storm in preschool children. *Environ. Int.*, 2020. 139: p. 105720.
 - [83] Dai, Y., et al., Alterations in platelet indices link polycyclic aromatic hydrocarbons toxicity to low-grade inflammation in preschool children. *Environ. Int.*, 2019. 131: p. 105043.
 - [84] Sharma, N., et al., Src kinases central to T-cell receptor signaling regulate TLR-activated innate immune responses from human T cells. *Innate Immun.*, 2016. 22(3): p. 238-244.
 - [85] Barr, T.A., et al., TLR and B cell receptor signals to B cells differentially program primary and memory Th1 responses to *Salmonella enterica*. *J. Immunol.*, 2010. 185(5): p. 2783-2789.
 - [86] Kawai, T., et al., Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity*, 2011. 34(5), p. 637-650.

APPENDICES

Summary

Nederlandse samenvatting

Acknowledgements

Curriculum vitae

Publication list

SUMMARY

E-waste has become a major issue as it continues to generate hazardous chemicals and contaminants that not only pose a serious threat to the environment but also compromises human health. Heavy metals generated during the e-waste dismantling process can persistently remain in the environment, food, and water, putting people who live within a specific distance of e-waste recycling areas at an increased risk of developing severe irreversible health conditions. The immune system of children is still in the developing stages and is more susceptible to threats from toxic heavy metals as compared with the immune system of adults. However, data on how heavy metal exposure affects the immune system in children, especially those who live in an e-waste-exposed area, is relatively scarce. As one of the largest e-waste recycling areas in China and worldwide, Guiyu has received much attention. Our population studies (**Chapters 2, 3, 4 and 5**) in this thesis investigated exposure to heavy metals (lead, cadmium, mercury and arsenic) and their effects on the immune system of children living in this area. Besides, our in vitro studies (**Chapters 6 and 7**) in this thesis used cell lines of immune cells (MonoMac 6 and Jurkat) to evaluate potential mechanisms by which heavy metals impact immune function.

In **Chapter 2**, the alterations in innate immune cells (including monocytes, eosinophils, neutrophils, basophils and NK cells) in preschool children from Guiyu and a non-e-waste-exposed area were evaluated. We found that Guiyu children had significantly higher absolute counts of monocytes, eosinophils, neutrophils and basophils but a lower NK cell percentage as compared with children in the non-e-waste-exposed area. Our further association analysis showed that lead exposure is positively associated with the counts of monocytes, eosinophils, and basophils, and cadmium is positively associated with the count and percentage of neutrophils. This suggests that exposure to e-waste-originated lead and cadmium might impact the development and proliferation of innate immune cells in children.

In **Chapter 3**, lead exposure and its effects on NK cells (total NK, CD56^{bright} and CD56^{dim} subpopulations) in Guiyu children were explored. We observed that children in Guiyu had a higher blood lead level and a lower NK cell percentage than children in the non-e-waste-

exposed group. Blood lead level was negatively associated with total and CD56^{bright} NK cell percentages, indicating that lead exposure may result in the reduction in percentages of total NK and CD56^{bright} cells. In addition, platelets, IL-1 β and IL-27, which are important for the development and differentiation of NK cells, were altered and associated with lead exposure. These data suggest that lead exposure might affect the proliferation, differentiation, and function of NK cells in children.

In **Chapter 4**, the effect of lead and cadmium on B cell apoptosis in Guiyu children were investigated. We observed a reduction in CD19⁺ B cell apoptosis, caspase-9 activation and IL-4 levels, and an elevation in NF- κ B transcription factor c-Rel mRNA level and IFN- γ /IL-4 ratio, as well as blood lead and cadmium levels in Guiyu children as compared with children in the non-e-waste-exposed group. Our findings after multiple correspondence analysis and linear regression analysis showed that both blood lead and cadmium levels were inversely associated with CD19⁺ B cell apoptosis. Thus, we concluded that exposure to lead and cadmium is likely to disturb the survival and development of B cells of children. This chapter also showed that NF- κ B signalling and the intrinsic apoptotic pathways are involved in heavy metal-induced immunotoxic action. The decrease in IL-4 production and the elevation in the IFN- γ /IL-4 ratio suggest that an imbalance of T helper cell (Th) 1/Th2 ratio might occur in Guiyu children. However, this needs further studies to validate.

In **Chapter 5**, the alterations in immune cells (neutrophils, monocytes, lymphocytes), pro-inflammatory cytokines (IL-1 β , IL-6, IL-8, TNF- α) and anti-inflammatory cytokines (IL-1RA, IL-4, IL-10, IL-13) in children of Guiyu and the non-e-waste-contaminated area were monitored. Guiyu children not only had high blood levels of lead, cadmium, mercury and arsenic, they also had increased IL-1 β and IL-6 but decreased lymphocyte count, IL-1RA and IL-13 levels as compared with the children in the non-e-waste-exposed group. Moreover, the neutrophil count was inversely associated with lead, cadmium and arsenic exposure. IL-1RA concentration negatively correlated with lead, cadmium, mercury and arsenic, while IL-1 β and IL-6 were positively correlated with blood lead level. These data suggest that exposure to heavy metals in children induced aberrant immune homeostasis and inflammatory activation. Although, we did not study the long-term health consequences of these heavy metals in the present studies, if these abnormalities in the immune system exist permanently, they may induce immune-related

disease in children in the future. Therefore, follow-up studies of Guiyu children's health are needed.

In **Chapter 6**, we firstly used the human monocytic leukemia reporter cell line (THP1-XBlue™-MD2-CD14) and seven human embryonic kidney (HEK) reporter cell lines (HEK-Blue™-hTLR2, 3, 4, 5, 7, 8 and 9) to determine whether lead and cadmium activate TLRs and NF-κB pathways. We observed that lead alone did not activate TLRs and NF-κB in THP1-MD2-CD14 or any of the TLR-expressing HEK reporter cells, while cadmium exposure (at 10 μM) had an inhibitory effect on TLR8 activation. Nevertheless, lead treatment could promote the activation of TLR4, 5, and 8 but attenuate TLR3 and 9 activation in the presence of their corresponding agonists; high exposure to cadmium reduced TLR2, 4, 5, 7 and 8 activation, while this may increase TLR3 activation, and the lower dose of cadmium increased TLR4, 5 and 8 activation induced by their agonists. These data suggest that lead and cadmium can affect TLR and NF-κB activation in the presence of TLR ligands.

In this chapter, we also used the human monocytic cell line (MonoMac 6) to identify how lead and cadmium affected monocytes via TLR4. We demonstrated that lead and cadmium induced cell death and cell apoptosis in the absence and presence of the TLR4 ligand LPS. Lead, in the absence of LPS, slightly increased ICAM-1 expression, while both lead and cadmium inhibited LPS-induced ICAM-1 expression on MonoMac 6 cells. Besides, an upregulation of p38 phosphorylation was found after lead and cadmium exposure in the presence of LPS, indicating that another downstream signalling pathway of TLRs, MAPK, is also activated. We concluded that p38 MAPK activation is associated with lead and cadmium exposure and this could explain the effect of lead and cadmium on MonoMac cell apoptosis and ICAM-1 expression. This chapter also showed that lead and cadmium increased TNF-α but inhibited IL-10 production in MonoMac 6 cells following LPS stimulation.

In **Chapter 7**, we used a T cell model, Jurkat cells, to evaluate the effect of lead and cadmium on cell survival, proliferation, NF-κB and mitochondria in the absence or presence of PMA/Ca²⁺ ionophore. Lead and cadmium were found to slightly decrease Jurkat cell viability, mainly in activated cells. In activated cells, i.e. in the presence of PMA/Ca²⁺ ionophore, lead and cadmium not only promoted cell apoptosis but also reduced IL-2 production. Further results showed that lead and cadmium increased NF-κB levels in non-activated cells. Cadmium also increased phosphorylation of NF-κB in non-activated cells, while lead inhibited p65

phosphorylation in activated cells. This suggests that the effect of lead and cadmium on NF- κ B activity may rely on Jurkat activation state. Additionally, we demonstrated that lead and cadmium enhanced mitochondrial mtDNA copy number in non-activated Jurkat cells, indicating that mitochondria in non-activated Jurkat cells are more susceptible to lead and cadmium stimulation than those in activated Jurkat cells. In line with this, our findings showed that lead and cadmium reduced *TFAM* and *NRF1* mRNA levels accompanied by a decreased *mtND2*, *mtCYB* and *mtATP8* transcription in non-activated Jurkat cells, which also suggests that the effect of lead and cadmium on mitochondrial biogenesis is predominant in non-activated Jurkat cells. Since these genes encode proteins to regulate the replication and transcription of mtDNA or constitute complexes I, III and V of the electron transport chain, we speculated that exposure to lead and cadmium might inhibit mitochondrial biogenesis and function of Jurkat cells, such as mitochondrial respiration and the ATP synthesizing capacity. Further studies are needed to substantiate this suggestion.

This thesis combines population and in vitro cell model studies to gain a better understanding of the toxic action of heavy metals on the immune system. Our current work provided evidence of the toxic effects of heavy metals on the immune system in a particularly susceptible population, i.e. children. On the other hand, in vitro studies with cell models helped us to evaluate several potential mechanisms of how heavy metals affect immune cells. These are TLRs, NF- κ B pathways, MAPK pathways and mitochondrial function. Future studies should also include animal models to study the effects of heavy metals on immune cells.

NEDERLANDSE SAMENVATTING

Het produceren van elektronisch afval (e-waste) is een steeds groter wordend probleem, aangezien tijdens dit proces gevaarlijke chemicaliën vrijkomen die tot verontreinigingen kunnen lijden. Deze verontreinigingen vormen niet alleen een ernstige bedreiging voor het milieu, maar ook de gezondheid van de mens wordt in gevaar gebracht. Zware metalen die tijdens het ontmantelingsproces van e-waste worden gegenereerd, kunnen terecht komen in het milieu, het voedsel en ons drinkwater, waardoor mensen die binnen een bepaalde afstand van e-waste recyclingbedrijven wonen een verhoogd risico lopen op het ontwikkelen van ernstige gezondheidsproblemen. Het immuunsysteem van kinderen bevindt zich nog in de ontwikkelingsfase en is gevoeliger voor de effecten van giftige zware metalen in vergelijking met het immuunsysteem van volwassenen. Gegevens over hoe blootstelling aan zware metalen het immuunsysteem van kinderen beïnvloedt, vooral van degenen die in een gebied wonen dat aan e-waste is blootgesteld, zijn echter relatief schaars. Als een van de grootste recyclinggebieden voor e-waste in China en wereldwijd, heeft Guiyu veel aandacht gekregen. Onze populatiestudies (**Hoofdstukken 2, 3, 4 en 5**) hebben de blootstelling aan zware metalen (lood, cadmium, kwik en arseen) en hun effecten op het immuunsysteem van kinderen die in dit gebied wonen onderzocht. Bovendien hebben onze *in vitro* studies (**Hoofdstukken 6 en 7**), cellijnen van immuuncellen (MonoMac 6 en Jurkat) gebruikt om mogelijke mechanismen te evalueren waarmee zware metalen de immuunfunctie beïnvloeden.

In **Hoofdstuk 2**, hebben we de veranderingen in verschillende soorten aangeboren immuuncellen (inclusief monocyten, eosinofielen, neutrofielen, basofielen en NK-cellen) in kinderen uit Guiyu vergeleken met kinderen uit een niet aan e-waste blootgesteld nabij gelegen gebied. We ontdekten dat Guiyu-kinderen significant hogere absolute aantallen monocyten, eosinofielen, neutrofielen en basofielen hadden, maar een lager NK-celpercentage in vergelijking met kinderen uit het niet aan e-waste blootgestelde gebied. Onze verdere associatie-analyse toonde aan dat blootstelling aan lood positief geassocieerd is met het aantal monocyten, eosinofielen en basofielen, en dat cadmium positief geassocieerd is met het aantal en het percentage neutrofielen. Dit suggereert dat blootstelling aan lood en cadmium afkomstig

van e-waste, de ontwikkeling en proliferatie van aangeboren immuuncellen bij kinderen kan beïnvloeden.

In **Hoofdstuk 3**, werd de blootstelling aan lood en het effect ervan op NK-cellen (totale NK-, CD56^{bright}- en CD56^{dim}-subpopulaties) bij Guiyu-kinderen onderzocht. We zagen dat kinderen uit Guiyu een hoger loodgehalte in het bloed hadden en een lager NK-celpercentage dan kinderen in de niet aan e-waste blootgestelde groep. Het loodgehalte in het bloed was negatief geassocieerd met totale en CD56^{bright} NK-celpercentages, wat aangeeft dat lood blootstelling kan resulteren in een verlaging van de percentages van totale NK- en CD56^{bright} cellen. Bovendien bleken bloedplaatjes, IL-1 β en IL-27, die belangrijk zijn voor de ontwikkeling en differentiatie van NK-cellen, veranderd te zijn en geassocieerd met blootstelling aan lood. Deze gegevens suggereren dat blootstelling aan lood de proliferatie, differentiatie en functie van NK-cellen bij kinderen kan beïnvloeden.

In **Hoofdstuk 4**, werd het effect van lood en cadmium op B-cel apoptose bij Guiyu-kinderen onderzocht. We zagen een afname in apoptose van CD19⁺ B-cellen, afname in caspase-9-activering en IL-4-niveaus, en een verhoging in NF- κ B-transcriptiefactor c-Rel-mRNA-niveau en IFN- γ /IL-4-ratio; daarnaast was de cadmiumconcentratie in het bloed van de Guiyu-kinderen verhoogd, in vergelijking met kinderen in de niet aan e-waste blootgestelde groep. Na meervoudige correspondentie-analyse en lineaire regressie-analyse toonden onze bevindingen aan dat zowel lood- als cadmiumspiegels in het bloed omgekeerd geassocieerd waren met apoptose van CD19⁺ B-cellen. We concludeerden daarom dat blootstelling aan lood en cadmium de overleving en ontwikkeling van B-cellen van kinderen waarschijnlijk zal verstoren. Dit hoofdstuk toonde ook aan dat NF- κ B-signalerings- en de intrinsieke apoptotische routes betrokken zijn bij door zware metalen geïnduceerde immunotoxische werking. De afname van de productie van IL-4 en de verhoging van de IFN- γ /IL-4-ratio suggereren dat een onbalans van de T-helpercel (Th) 1/Th2-ratio kan optreden bij Guiyu-kinderen. Om deze resultaten te valideren, zullen verdere studies moeten worden uitgevoerd.

In **Hoofdstuk 5**, hebben we gekeken naar de veranderingen in immuuncellen (neutrofielen, monocyt, lymfocyt), pro-inflammatoire cytokines (IL-1 β , IL-6, IL-8, TNF- α) en anti-inflammatoire cytokines (IL-1RA, IL-4, IL-10, IL-13) bij kinderen uit Guiyu en kinderen uit het niet-e-waste-besmette gebied. Guiyu-kinderen hadden niet alleen hoge bloedspiegels van lood, cadmium, kwik en arseen, ze hadden ook een verhoogd IL-1 β en IL-6, en een verlaagd

aantal lymfocyten, IL-1RA en IL-13 in vergelijking met de kinderen in de niet aan e-waste blootgestelde groep. Bovendien was het aantal neutrofielen omgekeerd evenredig met blootstelling aan lood, cadmium en arseen. De IL-1RA-concentratie was negatief gecorreleerd met lood, cadmium, kwik en arseen, terwijl IL-1 β en IL-6 positief gecorreleerd waren met het loodgehalte in het bloed. Deze gegevens suggereren dat blootstelling aan zware metalen bij kinderen een afwijkende immuunhomeostase en inflammatoire activering veroorzaakt. Hoewel we de gezondheidsgevolgen van deze zware metalen op de lange termijn niet hebben bestudeerd in de huidige studies, kunnen deze afwijkingen in het immuunsysteem in de toekomst tot immuungerelateerde ziekten bij kinderen leiden. Daarom zijn vervolgonderzoeken naar de gezondheid van Guiyu-kinderen nodig.

In **Hoofdstuk 6**, hebben we gebruik gemaakt van de menselijke monocytische leukemie reporter cellijn (THP1-XBlue™-MD2-CD14) en zeven menselijke embryonale nier (HEK) reporter cellijnen (HEK-Blue™-hTLR2, 3, 4, 5, 7, 8 en 9) om te bepalen of lood en cadmium de TLR's en NF- κ B-routes activeren. Lood alleen activeerde TLR's en NF- κ B niet in THP1-MD2-CD14 of een van de TLR-tot expressie brengende HEK-reportercellen, terwijl blootstelling aan cadmium (bij 10 μ M) een remmend effect had op TLR8-activering. Echter, zou het behandelen met lood de activering van TLR4, 5 en 8 kunnen bevorderen, maar de activering van TLR3 en 9 kunnen verzwakken in de aanwezigheid van hun overeenkomstige agonisten; hoge blootstelling aan cadmium verminderde TLR2, 4, 5, 7 en 8 activering, terwijl dit TLR3 activering kan verhogen, en de lagere dosis cadmium verhoogde TLR4, 5 en 8 activering geïnduceerd door hun agonisten. Deze gegevens suggereren dat lood en cadmium de TLR- en NF- κ B-activering kunnen beïnvloeden in aanwezigheid van TLR-liganden.

In dit hoofdstuk hebben we ook de menselijke monocytische cellijn (MonoMac 6) gebruikt om te identificeren hoe lood en cadmium monocytten beïnvloeden via TLR4. We hebben aangetoond dat lood en cadmium celdood en celapoptose kunnen induceren in de afwezigheid en aanwezigheid van de TLR4-ligand LPS. Lood, in de afwezigheid van LPS, verhoogde de expressie van ICAM-1 enigszins, terwijl zowel lood als cadmium de door LPS geïnduceerde ICAM-1-expressie op MonoMac 6-cellen remde. Bovendien werd een toename van p38-fosforylering gevonden na blootstelling aan lood en cadmium in de aanwezigheid van LPS, wat aangeeft dat een andere signaalroute van TLR's, MAPK, ook geactiveerd is. We concludeerden dat p38 MAPK-activering geassocieerd is met blootstelling aan lood en cadmium en dit zou een reden kunnen zijn waarom lood en cadmium de celapoptose en ICAM-1-expressie van

MonoMac 6-cellen veranderen. Verder toonde dit hoofdstuk aan dat lood en cadmium TNF- α verhoogden, maar de IL-10-productie in MonoMac 6-cellen remde na LPS-stimulatie.

In **Hoofdstuk 7**, we gebruikten een T-celmodel, Jurkat-cellen, om het effect van lood en cadmium op celoverleving, proliferatie NF- κ B en mitochondria in de afwezigheid of aanwezigheid van PMA/Ca²⁺ ionofoor. Lood en cadmium bleken de levensvatbaarheid van Jurkat-cellen te onderdrukken, voornamelijk in geactiveerde cellen. In geactiveerde cellen, d.w.z. in Jurkat-cellen met PMA/Ca²⁺ ionofoorstimulatie, bevorderden ze niet alleen celapoptose, maar verminderden ook de productie van IL-2. Verdere resultaten toonden aan dat lood en cadmium de NF- κ B-niveaus in niet-geactiveerde cellen verhoogden. Cadmium verhoogde ook de NF κ B-fosforylering in niet-geactiveerde cellen, terwijl lood de p65-fosforylering in geactiveerde cellen remde. Dit suggereert dat het effect van lood en cadmium op NF- κ B-activiteit kan afhangen van de Jurkat-activeringstoestand. Bovendien hebben we aangetoond dat lood en cadmium het aantal mitochondriale mtDNA-kopieën in niet-geactiveerde Jurkat-cellen deed toenemen, wat aangeeft dat mitochondriën in niet-geactiveerde Jurkat-cellen gevoeliger zijn voor lood- en cadmiumstimulatie dan die in geactiveerde Jurkat-cellen. Verder toonden onze bevindingen aan dat lood en cadmium een verlaagd TFAM- en NRF1-mRNA-niveau konden induceren, samen een verminderde mtND2-, mtCYB- en mtATP8-transcriptie in niet-geactiveerde Jurkat-cellen, wat suggereert dat lood en cadmium in niet-geactiveerde Jurkat-cellen voornamelijk effect hebben op mitochondriale biogenese. Omdat deze genen coderen voor eiwitten die de replicatie en transcriptie van mtDNA reguleren of coderen voor eiwitten van de complexen I, III en V van de elektronentransportketen, is een hypothese dat de blootstelling aan lood en cadmium de mitochondriale biogenese en functie van Jurkat-cellen zou kunnen remmen, zoals mitochondriale ademhaling en het ATP-synthetiserend vermogen. Verdere studies zijn nodig om deze suggestie te onderbouwen.

Dit proefschrift combineert populatie- en *in vitro* celmodelstudies om een beter begrip te krijgen van de toxische werking van zware metalen op het immuunsysteem. Ons huidige werk leverde bewijs van de toxische effecten van zware metalen op het immuunsysteem bij een bijzonder gevoelige populatie, namelijk kinderen. Aan de andere kant hielpen *in vitro* studies met celmodellen ons om verschillende mogelijke mechanismen te evalueren van hoe zware metalen immuuncellen beïnvloeden. Mogelijke mechanismes zijn TLRs, NF- κ B -routes, MAPK-routes en mitochondriale functie. Toekomstige studies zouden ook diermodellen moeten gebruiken om de effecten van zware metalen op immuuncellen te bestuderen.

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Here is the last section of my thesis, however, I'm struggling with structuring my words for it! Writing these pages of this section means that my PhD journey is coming to an end. I'm reluctant to say goodbye to the past years because it was an excellent time to get along with many active and enthusiastic people. You offered all the help and support for my study, life, and the completion of this thesis. I would like to express my sincere gratitude to all of you by this chance.

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PUBLICATION LIST

- [1] **Zhang Y**, Huo X, Lu X, Zeng Z, Faas MM, Xu X. Exposure to multiple heavy metals associate with aberrant immune homeostasis and inflammatory activation in preschool children. *Chemosphere*. **2020**. 257:127257.
- [2] **Zhang Y**, Xu X, Sun D, Cao J, Zhang Y, Huo X. Alteration of the number and percentage of innate immune cells in preschool children from an e-waste recycling area. *Ecotoxicol Environ Saf*. **2017**. 145:615-622.
- [3] **Zhang Y**, Huo X, Cao J, Yang T, Xu L, Xu X. Elevated lead levels and adverse effects on natural killer cells in children from an electronic waste recycling area. *Environ Pollut*. **2016**. 213:143-150.
- [4] Zeng X, Xu C, Xu X, **Zhang Y**, Huang Y, Huo X. Elevated lead levels in relation to low serum neuropeptide Y and adverse behavioral effects in preschool children with e-waste exposure. *Chemosphere*. **2020**. 269:129380.
- [5] Zheng X, Huo X, **Zhang Y**, Wang Q, Zhang Y, Xu X. Cardiovascular endothelial inflammation by chronic coexposure to lead (Pb) and polycyclic aromatic hydrocarbons from preschool children in an e-waste recycling area. *Environ Pollut*. **2019**. 246:587-596.
- [6] Zeng Z, Huo X, **Zhang Y**, Hylkema MN, Wu Y, Xu X. Differential DNA methylation in newborns with maternal exposure to heavy metals from an e-waste recycling area. *Environ Res*. **2019**. 171:536-545.
- [7] Cai H, Xu X, **Zhang Y**, Cong X, Lu X, Huo X. Elevated lead levels from e-waste exposure are linked to sensory integration difficulties in preschool children. *Neurotoxicology*. **2019**. 71:150-158.
- [8] Zhang S, Huo X, **Zhang Y**, Huang Y, Zheng X, Xu X. Ambient fine particulate matter inhibits innate airway antimicrobial activity in preschool children in e-waste areas. *Environ Int*. **2019**. 123:535-542.
- [9] Huo X, Dai Y, Yang T, **Zhang Y**, Li M, Xu X. Decreased erythrocyte CD44 and CD58 expression link e-waste Pb toxicity to changes in erythrocyte immunity in preschool children. *Sci Total Environ*. **2019**. 664:690-697.

- [10] Lu X, Xu X, **Zhang Y**, Zhang Y, Wang C, Huo X. Elevated inflammatory Lp-PLA2 and IL-6 link e-waste Pb toxicity to cardiovascular risk factors in preschool children. *Environ Pollut.* **2018.** 234:601-609.
- [11] Zeng Z, Huo X, **Zhang Y**, Xiao Z, Zhang Y, Xu X. Lead exposure is associated with risk of impaired coagulation in preschool children from an e-waste recycling area. *Environ Sci Pollut Res Int.* **2018.** 25(21):20670-20679.
- [12] Cao J, Xu X, **Zhang Y**, Zeng Z, Hylkema MN, Huo X. Increased memory T cell populations in Pb-exposed children from an e-waste-recycling area. *Sci Total Environ.* **2018.** 616-617:988-995.
- [13] Lu X, Xu X, Lin Y, **Zhang Y**, Huo X. Phthalate exposure as a risk factor for hypertension. *Environ Sci Pollut Res Int.* **2018.** 25(21):20550-20561.
- [14] Dai Y, Huo X, **Zhang Y**, Yang T, Li M, Xu X. Elevated lead levels and changes in blood morphology and erythrocyte CR1 in preschool children from an e-waste area. *Sci Total Environ.* **2017.** 592:51-59.
- [15] Hou W, Xu X, Lei Y, Cao J, **Zhang Y**, Chen L, Huo X. The role of the PM2.5-associated metals in pathogenesis of child *Mycoplasma Pneumoniae* infections: a systematic review. *Environ Sci Pollut Res Int.* **2016.** 23(11):10604-10614.
- [16] Liu W, Huo X, Liu D, Zeng X, **Zhang Y**, Xu X. S100 β in heavy metal-related child attention-deficit hyperactivity disorder in an informal e-waste recycling area. *Neurotoxicology.* **2014.** 45:185-91.

MANUSCRIPT FINISHED

- [1] **Zhang Y**, Huo X, Zhou X, Lu X, Faas MM, Xu X. Exposure to lead and cadmium and their effects on CD19⁺ B cell apoptosis in children from an e-waste recycling area.
- [2] **Zhang Y**, Silva-Lagos L, de Haan B, Xu X, Huo X, de Vos P, Faas MM. Toll-like receptor, NF- κ B and p38 Mapk signaling activation in MonoMac 6 cells exposed to heavy metals lead and cadmium.
- [3] **Zhang Y**, Silva-Lagos L, de Haan B, Xu X, Huo X, de Vos P, Faas MM. Effects of lead and cadmium on NF- κ B activity/mitochondrial dynamics and the role for the survival and activation of Jurkat cells.