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Lead at 2.5 and 5.0 μM induced aberrant MH-II surface expression through increased MII exocytosis and increased autophagosome formation in Raw 267.4 cells

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ABSTRACT

Aberrant major histocompatibility complex class II (MHC-II) surface expression on antigen presenting cells (APCs) is associated with dysregulated immune homeostasis. Lead (Pb) is known to increase MHC-II surface expression on murine peritoneal macrophages *ex vivo* at concentrations exceeding 25 μM. Little data exist examining this effect at physiologically relevant concentrations. To address this deficit, we examined the effects of Pb on MHC-II surface expression, secondary T-cell activation markers (CD80, CD86, CD40), cell viability, cellular metabolic activity, and β-hexosaminidase activity in RAW 267.4 macrophage cell lines, with changes in cell ultrastructure evaluated by electron and confocal microscopy. Pb induced an increase in MHC-II, CD86, and lysosome-associated LAMP-1 and LAMP-2 surface mean expression during one doubling cycle (17 h), which was mirrored by increased β-hexosaminidase activity. Although cell viability was unaffected, cellular metabolism was inhibited. Electron microscopy revealed evidence of lipid vacuolization, macroautophagy and myelin figure formation in cells cultured with either Pb or LPS. Confocal microscopy with antibodies against LC3B showed a punctate pattern consistent with the presence of mature autophagosomes. Collectively, these data suggest that 2.5–5.0 μM Pb increased MHC-II surface expression by inhibiting metabolic activity, inducing autophagy, and increasing MHC-II trafficking in a macrophage cell line.

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1. Introduction

Pb is a widely dispersed heavy metal contaminant. In an Environmental Protection Agency (EPA) study from the late 1990s, it was estimated that over 3 million metric tons of inorganic Pb had been dispersed across shooting ranges and hunting areas throughout the United States in the form of spent pellets and bullet fragments. The EPA estimated that recreational and military shooting activity added an additional 80,000 tons each year (Craig et al., 1999). Pb pellet densities in the top 3 in. of soil of up to 1.5 billion per acre have also been described at skeet shooting ranges (Stansley et al., 1992). Over the course of 13 years, approximately 17% of metallic Pb transformed into organic compounds contaminating soil and water sources (Jørgensen and Willems, 1987; Kadi, 2009; Magrisso et al., 2009; Woodard et al., 2007). Prior to legislation restricting its use, Pb was also an important component of gasoline. Tetra-ethyl lead (TEL), an anti-knock agent, was the single biggest contributor to aerosolized Pb contamination until it was outlawed in 1978 (Davidson and Rabinowitz, 1992). Recent data show that despite this ban, TEL still exists as a major environmental contaminant, especially near roadways where byproducts of gasoline combustion were deposited. Further, over the course of 13 years, approximately 17% of metallic Pb transformed into organic compounds contaminating soil and water sources (Jørgensen and Willems, 1987; Kadi, 2009; Magrisso et al., 2009; Woodard et al., 2007).

While Pb exposure can occur via inhalation and topical application, the most common route is by oral ingestion. Organic Pb compounds may leach from soil into ground water where they are absorbed by plants and then consumed by animals, resulting in elevated blood Pb levels (Robinson et al., 2008; Stansley and Roscoe, 1996; Stansley et al., 1992; Trahan and Peterson, 2007; Wilde et al., 2005). Upon ingestion, metallic and organic Pb readily dissolves in the stomach of mammals, due to the low pH and is absorbed into the blood stream via the duodenum resulting in a rapid rise in Pb blood levels (Martinez-Haro et al., 2009; Quarterman and Morrison, 2007). Greater than 98% of the Pb absorbed by the blood is then sequestered in the cellular fraction (Cake et al., 1998).

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Pb affects several cells in the blood. In erythrocytes, Pb promotes phospholipid peroxidation and inhibits heme synthesis, and at high levels can result in hemolytic anemia (Lawton and Donaldson, 1991; McFarland, 2006). In the leukocytes, Pb and LPS treatment stimulated B cells to produce increased IgM, *in vitro*, (McCabe and Lawrence, 1990). Chronic Pb exposure in humans resulted in elevated serum IgA and IgE and depressed complement C3, IgG, and IgG response to antigen (Ewers et al., 1982; Rosenberg et al., 2007; Sun et al., 2003). In addition, peripheral blood T cell populations shifted toward a Th2 profile with an increased Th2:Th1 cell ratio, depressed IFN-γ, and elevated IL-4 following oral chronic Pb exposure in mice (Boscolo et al., 1999; Heo et al., 2007, 1996).

APCs mediate T-helper function primarily through MHC-II antigen presentation. T-cell activation is positively associated with increased surface expression of MHC-II on these APCs (Farrer et al., 2005). MHC-II surface expression can be modulated in APCs through two distinct trafficking pathways; multi vascular body rearrangement in mature APCs, and MHC-II positive compartment (MIIC) exocytosis in immature APCs (Farrer et al., 2005). Prior to MIIC exocytosis, MHC-II is expressed in the endoplasmic reticulum and then packaged into lysosome associated protein number 1 (LAMP-1) positive compartments for entry into the endolytic pathway (Turley et al., 2000). β-hexosaminidase, a glycolipolytic enzyme, is targeted to MIICs and thought to aid with antigen preparation (Dai et al., 2009; Pierre, 2001). MIICs fuse with endosomal compartments where lysosomal enzymes degrade their contents and load MHC-II with antigen prior to fusion of the compartment with the plasma membrane. MIIC exocytosis, modulated by intracellular calcium concentrations, leads to increased LAMP-1 and MHC-II surface expression and the release of β-hexosaminidase into the extracellular milieu (Bunbury et al., 2009).

Multiple studies suggest that Pb mimics calcium by binding to a number of regulatory proteins and thereby modulates the calcium dependent pathways that regulate MIIC exocytosis (Bridges and Zalups, 2005). In a recent study, dendritic cells cultured with 100 μM Pb had a significant increase in surface MHC-II expression when compared to vehicle-exposed control cells (Gao et al., 2007). However, concentrations at this high level, equivalent to a blood Pb concentration of 2070 μg/dL, are rarely encountered *in vivo*, and if so, would likely be fatal. In mice, blood Pb levels up to 450 μg/dL (22 μM) have been observed, but this is three times the maximum lethal dose for humans of 150 μg/dl (7.2 μM) (Carmouche et al., 2005). Collectively, these studies underscore the gap in data regarding lower physiologic Pb concentrations (<20 μM) and their impact on innate immune mechanisms such as MIIC exocytosis. Thus, the focus of this study was to assess the effect of environmentally relevant levels of Pb on MHC-II surface expression, secondary T-cell activation markers (CD80, CD86, CD40), cell viability, cellular metabolic activity, and β-hexosaminidase activity in RAW 267.4 macrophage cell lines, a representative APC, and to further document these changes in cell ultrastructure using electron and confocal microscopy.

2. Materials and methods

2.1. Cells

Raw 267.4 cells were acquired from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in DMEM media supplemented with 10% Fetal Bovine Serum (FBS) (MediaTech, Herndon, VA) in T-75 cm^2 flasks (Corning, Corning, NY) at 37 °C, 5% CO_2, and 95% humidity. All cells assayed were from three sequential passages and data generated were representative of a minimum of three experiments/assay.

2.2. Pb treatment and reagents

All reagents were acquired from Sigma (St. Louis, MO) unless otherwise noted. Prior to treatment, cells were allowed to reach confluence usually by 48 h, harvested, enumerated with a T4 automated cell counter (Nexcelom, Lawrence, MA), and standardized at 2.0×10^6 cells/ml (Bunbury et al., 2009). Aliquots of cells were then added to 24 well plates in 1 ml complete media containing pre-prepared 1 ml serial dilutions of $PbNO_3$, ionomycin or LPS, and incubated for 17 h. Final concentrations of 0.0, 2.5, 5.0, and 10.0 μM $PbNO_3$, 30 μM ionomycin, and 100 μg/ml LPS were analyzed.

2.3. Cellular viability

Cellular viability was analyzed via staining with 7-amino-actinomycin D (7-AAD). Briefly, following treatment cells were collected, washed, enumerated and diluted to 2.0×10^5 cells in 200 μL phosphate buffered saline (PBS). Cells from each treatment were aliquoted in triplicate tubes and co-incubated on ice in the dark for 30 min with 0.2 μg/ml 7-AAD as per the manufacturers protocol. Samples were then analyzed on a C6 Cytometer (Accuri, Ann Arbor, MI). Data were expressed as mean% unstained ± SEM.

2.4. Alamar Blue™ metabolic proliferation assay

One hundred microliters of 2.0×10^5 cells/well were added to a 96 well flat bottom microtiter plate containing 100 μL of media, Pb, ionomycin or LPS and incubated for 17 h. Twenty microliters of Alamar Blue™ were added to each well and plates incubated for an additional 2 h. Absorbance (O.D) was then measured in a Synergy 4 spectrophotometer (BioTek, Winooski, VT) at 570 nm and 600 nm. Data were expressed as mean delta (Δ) 570–600 nm O.D ± SEM following blank subtraction.

2.5. Cell surface marker and apoptosis analysis

Aliquots of cells at 2.0×10^5 cells/well in duplicate were fixed in 4% paraformaldahyde for 30 min and then blocked with unlabeled Abs against CD16/32 (BD Biosciences, San Jose, CA) for 60 min. Cells were then washed 3× in cold PBS. Saturating optimized amounts (0.13 μg/well) of the following anti-mouse antibodies combined in triple color (Table 1); MHC-II, LAMP-1, LAMP-2, CD80, CD86, CD40 and intracellular apoptotic fluorescent probe 7-AAD (BD Biosciences) were then added to each well in FACS staining buffer (PBS, 0.1% BSA, 0.01% NaN_3) and incubated on ice in the dark for 30 min. For each triple color set, antibodies were

Table 1
Cell surface marker antibodies.

Reactivity	Color	Concentration	Cat. No.
Pre-stain			
CD32/16	N/A	0.500 ng/well	14-0161-85
Colorset 1			
CD80	FITC	0.250 ng/well	11-0801-85
CD86	PE-Cy5	0.100 ng/well	15-0862-82
CD40	PE	0.020 ng/well	12-0401-83
Colorset 2			
MHC-II	PE-Cy5	0.015 ng/well	15-5321-82
CD11b	FITC	0.125 ng/well	11-0112-85
CD11c	PE	0.500 ng/well	12-0114-83
Colorset 3			
MHC-II	PE-Cy5	0.015 ng/well	15-5321-82
CD107a	PE	0 500 ng/well	12-1071-83
CD107b	FITC	0.500 ng/well	11-1072-85

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