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Reduction of Myeloid Suppressor Cell Derived Nitric Oxide Provides a Mechanistic Basis of Lead Enhancement of Alloreactive CD4⁺ T Cell Proliferation

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Abstract

The persistent environmental toxicant and immunomodulator, lead (Pb), has been proposed to directly target CD4⁺ T cells. However, our studies suggest that CD4⁺ T cells are an important functional, yet indirect target. In order to identify the direct target of Pb in the immune system and the potential mechanism of Pb-induced immunotoxicity, myeloid suppressor cells (MSCs) were evaluated for their ability to modulate CD4⁺ T cell proliferation after Pb exposure. Myeloid suppressor cells regulate the adaptive immune response, in part, by inhibiting the proliferation of CD4⁺ T cells. It is thought that the mechanism of MSC-dependent regulation involves the release of the bioactive gas, nitric oxide (NO), blocking cell signaling cascades downstream of the IL-2 receptor and thus preventing T cells from entering cell-cycle. In mixed lymphocyte culture (MLC), increasing numbers of MSCs suppressed T cell proliferation in a dose-dependent manner, and this suppression is strikingly abrogated with 5 μ M lead (Pb) treatment. The Pb-sensitive MSC population is CD11b⁺, GR1⁺ and CD11c[−] and thus phenotypically consistent with MSCs described in other literature. Inhibition of NO-synthase (NOS), the enzyme responsible for the production of NO, enhanced alloreactive T cell proliferation in MLC. Moreover, Pb attenuated NO production in MLC, and exogenous replacement of NO restored suppression in the presence of Pb. Significantly, MSC from iNOS^{−/−} mice were unable to suppress T cell proliferation. An MSC-derived cell line (MSC-1) also suppressed T cell proliferation in MLC, and Pb disrupted this suppression by attenuating NO production. Additionally, Pb disrupted NO production in MSC-1 cells in response to treatment with interferon- γ (IFN- γ) and LPS or in response to concanavalin A-stimulated splenocytes. However, neither the abundance of protein nor levels of mRNA for the inducible isoform of NOS (iNOS) were altered with Pb treatment. Taken together these data suggest that Pb abrogates an MSC-dependent suppression of alloreactive T cell proliferation by inhibiting the function, but not the expression of iNOS.

Keywords

metals; allogenicity; reactive nitrogen intermediates; immune modulators; immunotoxicity

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Introduction

The heavy metal, lead (Pb), is a widely dispersed environmental contaminant that is toxic to many organ systems including the immune system (Dietert and Piepenbrink, 2006). In mice, Pb attenuates the delayed-type hypersensitivity response (McCabe, Jr. *et al.*, 1999), skews the pattern of serum cytokine levels from type-1 to type-2 (Heo *et al.*, 1997), decreases host resistance to intracellular pathogens (Lawrence, 1981), and increases serum IgE levels (Lutz, 1999). However, underlying mechanisms for Pb-induced immune modulation have remained elusive. Although Pb exposure is thought to be associated with immune-based pathologies in humans (reviewed in Dietert and Piepenbrink, 2006), links to specific disorders have been difficult to establish given this lack of mechanistic understanding. A better understanding of the immune cell types targeted by Pb may lead to an improved understanding of its mechanism of action.

Many studies have implicated CD4⁺ T cells as functional targets of Pb-induced immunotoxicity. We have used the mixed lymphocyte culture (MLC) as an *in vitro* assay system to study Pb-induced changes in T cell function. Using this model system, we have demonstrated that levels of Pb as low as 0.1 μ M enhance alloreactive T cell proliferation (Farrer *et al.*, 2005; McCabe, Jr. *et al.*, 2001). These studies indicated that T cells are important functional, but not direct, targets for Pb. In MLCs, CD4⁺ T cells proliferate in response to antigen presented by allogeneic B cells in the presence or absence of syngeneic accessory cells. This culture system has proven useful for the identification of direct cellular targets for Pb and for the elucidation of possible mechanisms underlying Pb immunomodulation.

In previous studies we demonstrated that adherent splenocytes, most likely cells of myeloid lineage, were necessary for Pb immunomodulation to occur (Farrer *et al.*, 2005). Indeed, others have shown that Pb impairs several aspects of macrophage function including adherence (Villanueva *et al.*, 1997a), chemotaxis (Villanueva *et al.*, 1997b), and clearance of intracellular pathogens (Bishayi *et al.*, 2003; Lawrence, 1981). Interestingly, Pb has also been shown to inhibit nitric oxide (NO) production in myeloid cells such as macrophages (Tian *et al.*, 1996; Tian *et al.*, 1995), and macrophage-derived NO has been reported to suppress allo-T cell proliferation (Hoffman *et al.*, 1994).

Myeloid suppressor cells (MSC), a heterogeneous population of CD11b⁺GR-1⁺ macrophages, have emerged as key innate regulators of adaptive immunity (Bronte *et al.*, 2003). NO produced by MSC has a suppressive effect on CD4⁺ T cell proliferation *in vitro* by inhibiting critical signaling pathways downstream from the IL-2 receptor (Bronte *et al.*, 2003; Bingisser *et al.*, 1998; Mazzoni *et al.*, 2002). In response to IFN- γ and cell-cell interactions with activated CD4⁺ T cells, MSC produce large amounts of NO through the upregulation of the inducible form of NO-synthase (iNOS) (Aktan, 2004).

As its name implies, iNOS is transcriptionally regulated. Its induction is mediated primarily through the NF- κ B pathway (Aktan, 2004) and/or the Jak/STAT pathway (Aktan, 2004; Rao, 2000). The iNOS promoter contains several binding elements for NF- κ B downstream from CD14 or IL-1 β -receptor engagement (Rao, 2000). Stimulation through the Jak/STAT pathway, downstream from the IFN- γ receptor, leads to the upregulation of IRF-1, a transcription factor that has a binding element on the iNOS promoter). Thus iNOS induction can be initiated by several inflammatory mediators including IFN- γ , TNF- α , IL-1 β , LPS, and even oxidative stress (Aktan, 2004). The overall effect of this MSC-iNOS-NO connection is that MSCs are able to regulate T cell proliferation via NO production in the presence of inflammatory mediators. We hypothesized that NO-dependent regulation of T cell proliferation was disrupted by Pb-induced inhibition of iNOS function in MSCs. Thus, Pb immunomodulation may be due to disruption of MSC-dependent suppression.

Materials and Methods

Mice

All mice were maintained in ALAAC accredited facilities located at the University of Rochester Medical Center, Rochester, NY. For most allo-MLC, 6–10 week-old female BALB/c mice (haplotype H-2^d) were used as the responder strain while 6–10 week old C57BL/6 females (haplotype H-2^b) were used as the stimulator strain. These mice were obtained from Jackson Laboratories (Bar Harbor, Maine). For use in studies, mice were euthanized by CO₂ inhalation followed by cervical dislocation. Spleens were harvested using aseptic technique.

Cell Lines

The MSC-1 cell line was generated by Vincenzo Bronte (Apolloni *et al.*, 2000) and generously donated to us by David M. Segal at the Experimental Immunology Branch, National Cancer Institute-National Institutes of Health, Bethesda, MD. Cells were maintained in 1640 RPMI medium (Gibco, Invitrogen Carlsbad, CA) supplemented with 10% FBS, 2 mM L-glutamine, 10 µM/mL of gentamicin, and 0.05 mM 2-mercaptoethanol at 37°C in 5% CO₂ and in a humidified atmosphere.

Splenocyte preparations

Spleens were homogenized between the frosted sides of two sterilized microscope slides into HBSS and cell suspensions were transferred to 15 mL centrifuge tubes. After 3 minutes on ice to allow debris to settle to the bottom of the tubes, supernatants were transferred to fresh centrifuge tubes and spun. All centrifuge spins were done at 300g for 10 minutes at 4° C. Red blood cells were eliminated by osmotic lysing using 4 mL RBC lysis buffer (Sigma-Aldrich Co., Inc., St. Louis, MO)/2 spleens. After 4 minutes in lysis buffer at room temperature, 6 mL HBSS was added and cells were spun and resuspended in HBSS such that additional debris bound to the inside of the pipette leaving a single-cell suspension free of RBCs. Cells were then used in subsequent applications.

Cell Separation

CD4⁺ T cell isolation—CD4⁺ T cells were negatively selected using a CD4⁺ T cell isolation kit from Miltenyi (Miltenyi Biotec Inc., Auburn, CA) that magnetically labeled all non-CD4⁺ T cells, following the manufacturer's instructions. Once cells were labeled, they were separated using an AutoMacs cell separator (Miltenyi). After cells were isolated, they were washed in HBSS and used in further applications. Purity was assessed flow cytometrically at ≥ 95%.

B cell isolation—B cells were isolated from splenocytes using a B cell isolation kit from Miltenyi according to the manufacturer's instructions in a negative selection process as described above. Purity was consistently assessed at ≥ 97%.

Adherent Cell isolation—Unfractionated splenocytes were plated at high concentrations (1.5–2 × 10⁷ cells/mL). These were incubated at 37° C for 24 hours after which plates were agitated on a Jitterbug platform (Boekel Industries Inc. Feasterville, PA) for 1 minute and media removed and replaced with sterile HBSS. Plates were again agitated on Jitterbug for 1 minute and HBSS removed. This was repeated twice more and media containing cells appropriate for MLC were added after last HBSS wash was removed.

CD11b⁺CD11c⁺ cell isolation—Unfractionated splenocytes were labeled with Miltenyi bead-conjugated monoclonal antibodies against CD11b. Splenocytes were then depleted of these labeled cells on the AutoMacs, and labeled cells were discarded. Unlabeled cells were

then labeled with Miltenyi bead-conjugated monoclonal antibodies against CD11c. Splenocytes were enriched for the labeled CD11c⁺ cells. Purity was assessed at 39% by flow cytometry.

CD11b⁺CD11c⁻ cell isolation—Unfractionated splenocytes were labeled with Miltenyi bead-conjugated monoclonal antibodies against CD11c, B220 and CD4. Splenocytes were then depleted of these labeled cells on the AutoMacs and labeled cells were discarded. Unlabeled splenocytes were then labeled with Miltenyi bead-conjugated monoclonal antibodies against CD11b. Splenocytes were then enriched for CD11b⁺ cells. Purity was assessed at ≥95% by flow cytometry.

Mixed Leukocyte Cultures (MLC)

BALB/c-derived CD4⁺ T cell responders were co-cultured with C57BL/6 APC stimulators at the cell densities indicated in the figure legends. As indicated in the figure legends, concentrations of Pb ranging from 1 to 5 μM were used. In iNOS^{-/-} MLC, C57BL/6- or B6.129P2-*Nos2^{tm1Lau}/J* (iNOS^{-/-})-derived adherent cells were isolated from unfractionated spleens as previously described (splenocyte preparations and adherent cell isolation). Negatively selected C57BL/6-derived CD4⁺ T cells and negatively selected BALB/c-derived B cells were co-cultured with C57BL/6 or iNOS^{-/-} adherent cells. Cultures were incubated at 37° C for 4 days in the presence of 0 or 1 μM Pb-glutamate. For proliferation analysis, triplicate or quadruplicate cultures were plated in 96-well, flat bottom plates. 1 μCi ³H-thymidine was added to each culture well for the final 6 hours of culture. Next cells were harvested using a 96-well plate cell harvester (Skatron; Molecular Devices Corporation, Sunnyvale, CA) such that DNA was trapped on glass filter paper. After drying, the amount of radioactivity incorporated into the DNA was assessed using liquid scintillation spectroscopy. For experiments in which culture supernatant and/or cell lysates were collected, MLC were set up in 24- or 12-well plates at the same cell densities as in 96-well plates. At the indicated time points, supernatants or cell lysates were harvested for further analysis.

Antibodies

The following monoclonal-fluorescence-conjugated antibodies used in this study were obtained from BD-Biosciences Pharmingen (BD Biosciences San Jose, CA): anti-CD11b (M1/70) Alexa Fluor® 488-conjugated, anti-CD11c (HL3) Allophycocyanin-conjugated. The following microbead-conjugated antibodies were obtained from Miltenyi: biotin-conjugated CD4 T cell isolation kit, biotin-conjugated B cell isolation kit, anti-biotin microbead-conjugated antibodies, anti-CD11c beads (N418), anti-CD11b beads (M1/70.15.11.5).

Nitric Oxide Determination

In all experiments in which supernatants were analyzed for NO content, cultures were done in McCoy's Modified 5A culture media (Gibco, Invitrogen) with the same supplements as were used with RPMI 1640 as described above. This was to ensure that total nitrates measured were derived from cells and not from the media, as RPMI 1640 is high in nitrates (Assay Designs, 2005). For a reflection of NO production, supernatants from cell cultures were analyzed for total nitrates using a "Total Nitric Oxide" kit from Assay Designs (Ann Arbor, Michigan) according to manufacturer's instructions. A standard curve was generated by using defined standards included with the kit. Standards and samples were loaded into 96-well plates (Corning). Next, all nitrates in standards and samples were reduced to nitrite using the enzyme NADH-reductase with its cofactor, NADH. Then, 2% sulfanilamide (in 3 M HCl) and 0.04% *N*-(1-naphthyl)ethylenediamine dihydrochloride (Griess reagents) were added to samples after which absorbance was read at 562 nm using an absorbance plate-reader (Molecular Devices,

Sunnyvale, CA). Concentrations of total nitrates in the samples were calculated from the standard curve.

Quantitation of adherent cells

Adherent cells were quantified using a CyQUANT® (Molecular Probes, Inc., Eugene, OR) fluorometric assay according to manufacturer's instructions. After removing non-adherent cells and supernatant from culture wells, cells were frozen in plates and then treated with CyQUANT® reagents. Fluorescence was then measured using a fluorescence plate reader (Molecular Devices, Sunnyvale, CA) after which cell numbers were calculated using a standard curve generated by plating cells in known numbers and measuring their fluorescence after treatment with CyQUANT® reagents.

Immunoblotting

MSC-1 cells or primary adherent splenocytes were co-cultured with indicated numbers and types of primary mouse cells and treated with ConA for 1–3 days or with alloantigen in MLC for 3–5 days and 0 or 5 μ M Pb. After indicated time in culture, non-adherent cells and supernatants were washed away by vigorous trituration and discarded. This process was repeated 3X substituting fresh HBSS for media. Lysates of adherent cells were obtained by adding lysis buffer directly to plates and 2 freeze-thaw cycles at -80° C. Lysates were then collected and stored at -80° C. Cell lysates were subjected to SDS-PAGE (50 μ g protein per lane on a 7.5% polyacrylamide gel) followed by transfer to PVDF membranes. Membranes were probed with poly-clonal rabbit anti-mouse-iNOS antibodies (BD Biosciences cat. # 610332; San Jose, CA) at a 1:2500 dilution in 1% BSA in 1X TBS/0.1% Tween 20 (overnight at 4° C). The secondary antibody used was HRP-conjugated goat anti-rabbit IgG (Santa Cruz # sc-2004; Santa Cruz Biotechnology, Inc., Santa Cruz, California) at a 1:5000 dilution in 5% nonfat dry milk in 1X TBS/0.1% Tween 20 (1 h at RT). Blots were developed with ImmunoStar HRP Chemiluminescent substrate and imaged and analyzed using an Image Station 440CF (Kodak Digital Science, New Haven, CT). After imaging and analysis, some blots were probed with rabbit anti-actin antibody (Sigma # A 2066) at a 1:2400 dilution in 5% nonfat dry milk in 1X TBS/0.1% Tween 20 overnight at 4° C. Secondary antibody was HRP-conjugated goat anti-rabbit IgG as before. Blots were developed, imaged and analyzed as described above.

RT-PCR

MSC-1s were incubated in the presence of 0, 1, or 5 μ M Pb-glutamate and activated with 50 μ g/mL lipopolysaccharide and 100 U/mL IFN- γ for 6 hours. Cells were washed three times with PBS to prepare for RNA isolation. RNA was isolated using the RNEasy kit (Qiagen Inc., Valencia, CA) following the manufacturer's protocol. The iNOS forward primer 5'-TGTCTGCAGCACTTGGATCAGGAA-3', iNOS reverse primer 5'-GAAGCCACTGACACTTCGCACAAA-3', actin forward primer 5'-ACCCTGTGCTGCTCACCGA-3', and actin reverse primer 5'-CTGGATGGCTACGTACATGGCT-3' were used (Bio-Synthesis, Inc., Lewisville, TX) 100ng/ μ L of RNA was run by RT-PCR using the iScript™ One-Step RT-PCR Kit with SYBR® Green (Bio-Rad Laboratories, Hercules, CA). Samples were analyzed on a Roto-Gene 2000 (Corbet Research, San Francisco, CA) and normalized to actin.

Chemicals

The following chemicals were all purchased from Sigma-Aldrich. Pb acetate and glutamic acid were obtained in powder and were dissolved separately in sterile, endotoxin-screened, distilled water at 40 mM. Equal volumes of these two solutions were mixed to form a 20 mM Pb glutamate stock. This stock was filter sterilized. Concanavalin A was dissolved in HBSS at 1 mg/mL and frozen until use upon which it was further diluted in HBSS to make a secondary

stock just prior to final dilution into cell culture media. N_G-monomethyl-L-Argine (NgMMA) was dissolved at 1 mg/mL in distilled water and refrigerated until use. Upon use it was further diluted to 10 mM in HBSS prior to final dilution into culture wells. S-nitroso-N-acetylpenicillamine (SNAP) was dissolved in HBSS at 1.4 mM just prior to final dilution and use. SNAP solutions were made fresh for each addition to culture.

Statistical Analysis

All statistical analysis was done using the JMP 6.0.0 statistical software package from the SAS Institute (Cary, NC).

Results

Pb abrogates an adherent cell-dependent suppression of alloreactive CD4⁺ T cell proliferation

We observed that Pb immunomodulation was dependent upon the presence of an adherent cell type (Farrer *et al.*, 2005). This dependence was further revealed upon co-culture of BALB/c-derived CD4⁺ T cells with increasing numbers of allogeneic C57BL/6-derived B cells (i.e., a source of alloantigen) in the presence or absence of BALB/c-derived adherent cells and 0 or 5 μ M Pb glutamate (Fig. 1A). After 3 days in culture, T cell proliferation was measured (Fig. 1A). There was a clear cell density-dependent increase in alloreactive T cell proliferation with increasing numbers of allo-B cells; the sharpest increase was between 1 and 6 $\times 10^5$ allo-B cells/well. However, in the presence of BALB/c-derived (syngeneic) adherent cells, T cell proliferation in the absence of Pb was suppressed by 2-fold at 3 $\times 10^5$ allo-B cells/well and 3-fold at 6 $\times 10^5$ allo-B cells/well relative to Pb-treated cultures. Pb had no effect on T cell proliferation in cultures lacking syngeneic adherent cells. Thus, Pb immunomodulation only occurred when adherent cells were present (Fig. 1A).

To verify that Pb disrupted an adherent cell-dependent suppression of alloreactive T cell proliferation, we established MLC in which BALB/c-derived CD4⁺ T cells were co-cultured with a fixed number (3 $\times 10^5$ cells/well) of C57BL/6-derived B cells and increasing numbers of BALB/c-derived adherent cells (Fig. 1B). With increasing adherent cell numbers, T cell proliferation decreased in control cultures, while T cell proliferation remained elevated regardless of adherent cell numbers in Pb-treated cultures (Fig. 1B). Taken together, these data suggest that an adherent cell is necessary for Pb immunomodulation to occur and that this enhancement may be more accurately described as relief of adherent cell dependent suppression.

The adherent cell type responsible for Pb-sensitive suppression of T cell proliferation in MLC is in the CD11b⁺ fraction and may represent an MSC

Adherent splenocytes are likely to be of myeloid lineage, and because the Pb-sensitive adherent cells in this experimental system suppressed T cell proliferation, we hypothesized that a myeloid suppressor cell (MSC) was essential for Pb immunomodulation. MSCs have previously been described as a heterogeneous population of CD11b⁺ cells (Bronte *et al.*, 2003; Mazzoni *et al.*, 2002). To verify that the adherent cell upon which Pb immunomodulation was dependent in our cultures was consistent with published characterizations, we used magnetic bead separation to first deplete CD11c⁺ cells and then enrich for CD11b⁺ splenocytes (Fig. 2). The relative proportion of CD11b⁺ and CD11c⁺ cells in unfractionated vs. CD11b-enriched splenocytes is shown in the left panels of Figure 2. Isolated cell populations were stained for CD11b and CD11c for immunophenotypic analysis by flow cytometry (Fig. 2). Additionally, we compared the ability of these enriched cell populations to suppress T cell proliferation in MLC with that of adherent splenocytes in the presence of 0 or 5 μ M Pb (Fig. 2 right panel). Isolation procedures yielded cells that were either 39% CD11c⁺ with few

detectable CD11b⁺ cells (data not shown) or 94% CD11b⁺ with few detectable CD11c⁺ cells (Fig. 2 left panel). Adherent splenocytes and splenocyte populations enriched for CD11b⁺ cells mediated a Pb-sensitive suppression of alloreactive T cell proliferation while splenocyte populations lacking CD11b⁺ cells did not significantly suppress T cell proliferation (Fig. 2 right panel). Consistent with the reported phenotype of MSCs, the CD11b-enriched cell population was heterogeneous with respect to GR1 and F4/80 expression (data not shown). These data suggest that cells with an MSC phenotype are necessary in MLC for Pb immunomodulation to occur and such cells may be a likely direct target for Pb.

The mechanism by which Pb disrupts adherent cell-dependent suppression involves inhibition of NO production

Release of NO is the primary way that murine MSCs inhibit T cell proliferation (Bronte *et al.*, 2003). To determine whether this pathway was important to Pb's mechanism of action in allo-MLC, we co-cultured CD4⁺ T cells with allo-B cells and syn-adherent cells in the presence of 0 or 5 μ M Pb glutamate and increasing concentrations of the non-specific NOS inhibitor N_gMMA. At 500 μ M, N_gMMA enhanced T cell proliferation in MLC to a level consistent with that in Pb-treated cultures (Fig. 3A). Moreover, Pb decreased the level of total nitrates (stable degradation products of NO) in the supernatants of these cultures 3-fold on day 3 of culture and 2.5-fold on day 4 (Fig. 3B). Furthermore, addition of 350 μ M of the NO-donor, SNAP, on day 2 of culture reduced T cell proliferation 5-fold in Pb-treated cultures (Fig. 3C) and resulted in total nitrate levels similar to those in non-Pb-treated control cultures (data not shown).

There are 3 isoforms of nitric oxide synthase (NOS). Endothelial-NOS (eNOS) and neuronal-NOS (nNOS) are both constitutively expressed in resting cells and are activated to produce NO by increases in cytosolic Ca²⁺ levels and calmodoulin binding. In contrast to eNOS and nNOS, the inducible isoform (iNOS) is transcriptionally regulated (Alderton *et al.*, 2001). Furthermore, eNOS and nNOS generate much less NO/unit enzyme than iNOS (Aktan, 2004). The 2-day lag between initiation of MLC and measurable NO in the media and the quantities of NO observed (Fig. 3B) suggested kinetics more consistent with iNOS. When iNOS^{-/-} mice were used as the source of adherent cells in MLC, T cells proliferated as well as in Pb-treated cultures where adherent cells were iNOS-competent, and Pb had no effect on proliferation in cultures where adherent cells were iNOS^{-/-} (Fig. 3D). Taken together, these data suggest that in untreated cultures, adherent cells regulate alloreactive T cell proliferation by the iNOS-dependent release of NO and that Pb attenuates this release.

iNOS protein expression is not affected by Pb in primary MSC

Data to this point indicated that Pb-alloenhancement was based on Pb's disruption of MSC-dependent NO signaling, and that this NO was primarily produced by the activity of iNOS. Therefore, we hypothesized that Pb inhibited the function and/or expression of iNOS. To test this hypothesis, we co-cultured CD4⁺ T cells with allo-B cells and syn-adherent splenocytes in the presence of 0 or 5 μ M Pb glutamate. After 4 days, protein from adherent cells was isolated and probed for iNOS (Fig. 4, *top*). Pb had no effect on iNOS protein abundance in primary adherent cells. Total nitrate analysis of the supernatants from the same cultures indicated a marked decrease in NO production with Pb treatment (Fig. 4, *bottom*). Cumulatively, these data suggest that Pb inhibits iNOS function without decreasing its abundance.

MSC-1 cell line is functionally similar to primary adherent splenocyte MSC

To confirm that Pb inhibited iNOS function without modulating its abundance, we employed an MSC-derived cell line that shares phenotypic characteristics with the suppressor adherent cells identified in figure 2, namely high expression of CD11b and functional suppression of T cell proliferation via NO release. The MSC-1 cell line is an H-2^d cell line derived from BALB/

c mice that expresses iNOS upon stimulation by IFN- γ and an undefined cell-cell interaction with activated T cells (Mazzoni, *et al.*, 2002).

To verify that the MSC-1 line behaved similarly to our primary cultured MSCs in MLC, we co-cultured BALB/c-derived CD4⁺ T cells and C57BL/6-derived B cells with irradiated (6000 cGy) MSC-1 cells in the presence or absence of 1 mM NgMMA, 5 μ M Pb, or both. After 3 days in culture, T cell proliferation was assessed by ³H-thymidine incorporation (Fig. 5A). MSC-1 cells suppressed T cell proliferation in a cell density-dependent manner. MSC-1-dependent suppression was disrupted with Pb and/or NgMMA treatment. Pb-treated cultures and cultures treated with both Pb and NgMMA demonstrated significantly greater T cell proliferation in response to alloantigen than control cultures or those treated with NgMMA alone (Fig. 5A).

To demonstrate that Pb could inhibit MSC-1-dependent NO production, we co-cultured BALB/c-derived CD4⁺ T cells with irradiated MSC-1 cells and C57BL/6-derived B cells in the presence of 0 or 5 μ M Pb or 1 mM NgMMA and harvested supernatants for total nitrate analysis on days 1–4 of culture (Fig. 5B). Total nitrates from all groups remained below 10 μ M until day 3 when levels rose sharply (3-fold) in control culture-derived supernatants in contrast to both Pb and NgMMA-treated cultures, which remained near 10 μ M. This difference in nitrate levels persisted through day 4. However, total nitrates did increase 0.6-fold in Pb-treated cultures between days 3 and 4. These data indicate that Pb inhibited MSC-1-dependent NO production (Fig. 5B).

Pb decreases NO production but not iNOS protein abundance in indirectly stimulated MSC-1 cells

Pb could attenuate NO production either by impairing the expression or the function of iNOS. To determine whether Pb could decrease the abundance of iNOS, we cultured MSC-1 cells with ConA-stimulated BALB/c splenocytes, collecting supernatant for NO analysis (Fig. 6A) and adherent cell lysates (Fig. 6B) to probe for the levels of iNOS protein from the same cultures on days 1–3 of culture. Pb significantly suppressed NO production (by 24.5%) by day 3 of culture (Fig. 6A), but did not change the abundance of iNOS protein (Fig. 6B).

Pb decreases NO production but not iNOS expression or abundance in MSC-1 cells directly stimulated with IFN- γ and LPS

To separate any extraneous T cell-mediated changes in the MSC-1 cell NO response, we directly stimulated MSC-1 cells, in the absence of any other cell type, with 100 U/mL IFN- γ and 5 μ g/mL LPS. IFN- γ + LPS treatment induced MSC-1 cells to produce large amounts of NO, and this production was decreased 30% with 5 μ M Pb treatment (Fig. 7C). This decrease in NO production did not correlate with a decrease in iNOS protein abundance, as measured by western blot (Fig. 7A), or message, as measured by RT-PCR (Fig. 7B). Taken together, these data suggest that Pb inhibits iNOS function without altering iNOS expression.

Discussion

Pb is a widespread environmental contaminant that adversely affects many organ systems. Its broad dispersion and persistence in the environment make it a relevant and significant threat to anyone living in an industrialized or developing nation. Additionally, Pb is primarily stored within the mineralized structure of bone where it can remain for decades. During periods of higher bone turnover the toxicokinetic properties of Pb change increasing the potential for the release of Pb back into the blood and re-exposure of soft tissues (Hu, *et al.* 2007).

In this study, the observed data support our hypothesis that Pb disrupts an MSC-dependent suppression of alloreactive CD4⁺ T cell proliferation by inhibiting the function, but not altering the expression, of iNOS. The function of MSCs has traditionally been measured by their ability to suppress T cell responses *in vitro* (Bronte *et al.*, 2003; 1999; Mazzoni *et al.*, 2002), and our data identify Pb as a potential modulator of MSC function in the *in vitro* assay, MLC.

The mechanisms underlying the immunotoxicity of Pb are not been well understood, however, data presented here identify cells of myeloid lineage as potential target cell types. Pb has previously been shown to target macrophage function (Sengupta *et al.*, 2002; Song *et al.*, 2001; Villanueva *et al.*, 1997a; Villanueva *et al.*, 1997b) and even NO production by macrophages (Tian *et al.*, 1996; Tian *et al.*, 1995), but never in the context of the immunomodulatory properties of NO.

Inhibition of NO production could explain some of the major observations of Pb-induced immunomodulation including inability of macrophages to clear intracellular pathogens (Bishayi *et al.*, 2003; Lawrence, 1981), enhanced T cell proliferation in MLC (Fig. 3) (Farrer *et al.*, 2005; McCabe, Jr. *et al.*, 2001), and modulation of antigen processing (Farrer *et al.*, 2005). In the presence of reactive oxygen species in the phagolysosome, NO is rapidly converted to peroxy-nitrite which contributes to the general oxidative environment designed to kill phagocytosed pathogens as well as the nitrosylation of tyrosine residues. A decrease in NO production would lead to a depressed ability to oxidatively destroy pathogenic organisms. This has been born out in a study in which Pb-treated and vehicle-treated mice were infected with *Staphylococcus aureus*. After sufficient incubation time for mice to recover from the infection, macrophages from Pb-treated mice still contained viable bacteria, whereas none were recovered from the macrophages of control mice (Bishayi *et al.*, 2003). As demonstrated in the present study, decreases in NO due to Pb exposure also dysregulate NO-dependent suppression of T cell proliferation. Finally, our previously published data indicate that Pb may alter antigen processing (Farrer *et al.*, 2005). Antigenic peptides and catalytic sites on some cathepsins and other proteases involved in the processing of antigenic peptides are normally nitrosylated. Specifically, peroxy-nitrite nitrosylates tyrosine residues, and NO nitrosylates cysteine residues. Although speculative, a Pb-induced reduction of NO, could affect both pathways of antigen processing potentially leading to an altered array of antigenic peptides presented in the context of MHC. This could reduce the amount of immunogenic peptide fragments of a given pathogen presented to responding T cells leading to a weakened host response to infection. Moreover, such a scenario could lead to the release of cryptic epitopes or altered self. Altered self due to decreases in S-nitrosylation of critical peptides and proteases could be the mechanism by which Pb exacerbates autoimmune diseases in mice prone to such disorders (Hudson *et al.*, 2003).

Another area of possible significance for Pb-induced decreases in NO production could be allograft survival. MLC is based on allogeneic immunity, the same type of immunity underlying organ transplant rejection. *In vitro*, the allogeneic response appears to be enhanced with Pb treatment based solely on alloreactive CD4⁺ T cell proliferation. *In vivo*, however, decreases in NO have been shown to prolong allograft survival and increased NO levels are associated with increased rates of rejection (Pieper *et al.*, 2002; Pieper *et al.*, 2000; Roza *et al.*, 2000; Shiraishi *et al.*, 1997; Winlaw *et al.*, 1995). This discrepancy has yet to be resolved. Observation of the effects of Pb on allograft survival has not been directly assessed, however the results of such studies could be instructive in determining whether dysregulation of T cell proliferation or overall decreases in NO levels would have the greater immunological impact *in vivo*.

Of course, NO serves many functions outside of the immune system such as neurotransmission, regulation of vasodilation, and even in triggering the acrosome reaction in the head of

spermatozoa. NO production for many of its non-immunological functions is catalyzed by constitutively expressed isoforms of NOS such as endothelial (eNOS) or neuronal (nNOS) (Alderton *et al.*, 2001). While iNOS is transcriptionally regulated, the constitutively expressed isoforms are more rapidly regulated by the binding of calcium via calmodoulin. We have shown here that Pb inhibits the function of iNOS, but not its expression. This is of broad significance because it suggests that Pb may impair the function of the constitutive isoforms of NOS as well. Such findings could serve as the key to unlock the mystery of Pb's mechanism of action in many tissues, simultaneously providing a basis from which to better evaluate and monitor the overall impact of Pb exposure on human health rather than only the immunological or neurological effects.

Heme is a prime candidate for study as part of the mechanism by which Pb suppresses iNOS function. In order to form an active enzyme, iNOS must form a tight homodimer. This dimerization is dependent upon the cofactor heme (as reviewed in (Aktan, 2004)). It has long been known that Pb impairs heme synthesis by blocking the action of δ -aminolevulinic acid dehydratase (ALAD) (Goldstein *et al.*, 1975; Kajimoto *et al.*, 1983; Sharma *et al.*, 2000). Furthermore, induction of heme-oxygenase-I (HO-1) may be a contributing factor to lowered heme levels in certain cell types (Cabell *et al.*, 2004; Opanashuk *et al.*, 1995; Vargas *et al.*, 2003). Blocking synthesis and increasing degradation may produce an overall reduction of heme, which may result in reduced activity of heme-dependent enzymes such as iNOS. Specifically, depletion of heme may prevent iNOS (and other NOS isozymes) from dimerizing to form active enzyme.

There has been some controversy regarding the role of iNOS expression and NO production by human in comparison to murine macrophages (Macmicking *et al.* 1997). With respect to the antimicrobial activities of NO, it's clear that while human macrophages can express functional iNOS in response to microbial pathogens and endotoxin, they are much less sensitive than murine macrophages and NO production may not be the preferred antimicrobial pathway available to human macrophages. It is not surprising, then, that human MSCs in comparison to murine MSCs may have alternative pathways with respect to the immunoregulatory activities of NO. However, it is clear that human macrophages express higher levels of iNOS and NO in response to more specialized stimuli (Pham *et al.* 2003; Pechkovsky *et al.*, 2002). It remains to be seen if the immunoregulatory activities of human MSCs are equally sensitive to Pb.

In summary, Pb enhances alloreactive CD4⁺ T cell proliferation by disrupting MSC-dependent suppression. Specifically, Pb inhibits the function of iNOS without decreasing its expression. The overall significance of data reported here is that NO production via modulation of iNOS function is a key mediator in Pb-induced immunomodulation and possibly other NO-dependent bio-regulatory processes.

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Figure 1. Pb immunomodulation depends on the presence of a suppressor adherent cell in MLC
A, BALB/c-derived CD4⁺ T cells (3×10^5 /well) were co-cultured with or without syngeneic adherent splenocytes (5×10^4 /well) and increasing numbers of C57BL/6-derived B cells in the presence of 0 or 5 μ M Pb. On the third day of culture, T cell proliferation was assessed using ³H-thymidine incorporation. Data represent mean $\pm$ SEM (n= 3 independent experiments). Multiple linear regression analysis was performed on the four plots and comparisons in the presence *versus* absence of Pb and presence *versus* absence of adherent cells were made using a least squares means Student's t-test. In the presence of syngeneic adherent cells a significant difference in ³H-thymidine incorporation was found in Pb-treated (closed squares) vs. control cultures (open squares) (*p<0.001). Also, Pb-free cultures

containing adherent cells (open squares) yielded significantly lower ^3H -thymidine incorporation in comparison to cultures lacking adherent cells (open and closed circles) ($p < 0.001$). **B**, BALB/c-derived CD4^+ T cell ($3 \times 10^5/\text{well}$) were co-cultured with C57BL/6-derived B cells ($6 \times 10^5/\text{well}$) and increasing numbers of syngeneic, BALB/c-derived adherent cells in the presence of 0 or 5 μM Pb. On the third day of culture, T cell proliferation was assessed using ^3H -thymidine incorporation. Data represent mean $\pm$ SEM ($n = 3$ independent experiments). Linear regression analysis was performed on both plots and comparisons between responses in the presence and absence of Pb were made using a least squares means Student's t-test. Comparing Pb (+) with Pb (–) responses $p \leq 0.001$.

Figure 2. The Pb-sensitive myeloid suppressor cell is found within the CD11b⁺ cell fraction
BALB/c-derived CD4⁺ T cells (3×10^5 /well) and C57BL/6- derived allogeneic B cells (3×10^5 /well) were cultured in the presence (*closed bars*) or absence (*open bars*) of 5 $\mu\text{M Pb}$. As indicated, BALB/c-derived adherent cell populations (5×10^4 /well) were added to the allo-MLC. Cultures devoid of additional adherent cells are indicated with a “-”; cultures containing splenic adherent cells are indicated as “unsep”; cultures containing CD11b⁺ adherent cells (i.e., CD11c depletion followed by CD11b enrichment) or CD11c⁺ adherent cells (i.e., CD11b depletion followed by CD11c enrichment) are indicated as CD11b⁺ and CD11b⁻, respectively. T cell proliferation was assessed on day 4 by ^3H -thymidine incorporation. Data are mean $\pm$ SD of quadruplicate cultures and are representative of 2 independent experiments. Student’s t-test revealed significant differences between Pb-treated and control cultures are indicated by an * ($p < 0.01$). Representative bivariate plots for CD11c and CD11b markers for the unseparated and CD11b⁺ enriched cell populations are shown.

Figure 3. Pb reverses NO-dependent inhibition of T cell proliferation by attenuating NO production

A, A NOS inhibitor has the same effect on T cell proliferation as Pb treatment. BALB/c-derived CD4⁺ T cells (3×10^5 /well) and adherent splenocytes (4×10^4 /well) were co-cultured with C57BL/6-derived B cells (3×10^5 /well) in the presence of 0 or 5 μM Pb and increasing concentrations of NgMMA. On the fourth day of culture, T cell proliferation was assessed using ³H-thymidine incorporation. Data represent mean $\pm$ SEM (n = 3 independent experiments). Linear regression analysis was performed on both plots and comparisons between responses in the presence and absence of Pb were made using a least squares means Student's t-test. Comparing Pb (+) with Pb (–) responses $p \leq 0.001$. **B**, Pb suppresses NO production. Cells were plated at the same ratios as in panel A in nitrate-free culture media in 24-well plates and treated with 0 or 5 μM Pb and 0 or 500 μM NgMMA. On days 1–4 of culture, media was harvested, filtered, and analyzed for total nitrate content. Data are mean $\pm$ SEM (n = 3 independent experiments). Linear regression analysis was performed on the three plots and comparisons in the presence *versus* absence of Pb and presence *versus* absence of NGMMA were made using a least squares means Student's t-test. Pb-treated and NGMMA-treated cultures produced significantly less NO in comparison to control ($p < 0.001$). **C**, Addition of

SNAP (a NO donor) to Pb-treated cultures restored suppression of alloreactive T cell proliferation. BALB/c-derived CD4⁺ T cells (3×10^5 /well) and adherent splenocytes (4×10^4 /well) were co-cultured with C57BL/6-derived B cells (3×10^5 /well) in the presence of 0 or 5 μ M Pb. Zero or 350 μ M SNAP was added to cultures on day 2. On the fourth day of culture, T cell proliferation was assessed using ³H-thymidine incorporation. Data represent mean $\pm$ SEM (n= 3 independent experiments). (*) Indicates statistically significant difference between Pb-treated and control cultures (p<0.01) as assessed by Student's t-test. **D**, Adherent cells derived from iNOS^{-/-} mice do not suppress T cell proliferation and are not sensitive to Pb. C57BL/6-derived CD4⁺ T cells (3×10^5 /well) and BALB/c-derived allogeneic B cells (3×10^5 /well) were cultured in the presence (*closed bars*) or absence (*open bars*) of 1 μ M Pb and in the absence or presence of 4×10^4 /well adherent cells from iNOS wild type or iNOS^{-/-} C57BL/6 mice. On the fourth day of culture, T cell proliferation was assessed using ³H-thymidine incorporation. Data represent mean $\pm$ SD from triplicate cultures (representative of 3 independent experiments). (*) Indicates statistically significant difference between Pb-treated and control cultures (p<0.01) as assessed by Student's t-test.

Figure 4. Pb inhibits the production of NO in MLC but does not reduce iNOS expression
BALB/c-derived CD4⁺ T cells (3×10^5 /well) and adherent splenocytes (4×10^4 /well) were co-cultured with C57BL/6-derived B cells (3×10^5 /well) in the presence of 0 or 5 μM Pb. After 4 days, supernatants (**B**) and adherent cells (**A**) were harvested. In panel **A**, protein was isolated from primary adherent cell lysates, separated using SDS-PAGE electrophoresis, transferred to a PVDF membrane, and probed for iNOS using a polyclonal rabbit-anti-mouse antibody. Data are a representative of 3 blots and 2 independent experiments. In panel **B**, supernatants were analyzed for total nitrates using the Griess reaction. Data are mean $\pm$ SD of triplicate cultures and are representative of 2 independent experiments. Linear regression analysis was performed

on both plots and comparisons between responses in the presence and absence of Pb were made using a least squares means Student's t-test. Comparing Pb (+) with Pb (-) responses $p \leq 0.001$.

Figure 5. Pb modulates the function of the MSC-1 cell line

In panel **A**, BALB/c-derived CD4^+ T cells ($3 \times 10^6/\text{mL}$) were co-cultured with C57BL/6-derived B cells ($3 \times 10^6/\text{mL}$) and increasing numbers of irradiated (6000 cGy) MSC-1 cells in the presence of 0 or 1 mM NgMMA and 0 or 5 μM Pb. On the third day of culture, T cell proliferation was measured using ^3H -thymidine incorporation. Data are mean $\pm$ SD of triplicate samples and are representative of 2 independent experiments. Statistical analysis was done by one-way ANOVA followed by Tukey-Kramer HSD comparison of means. (*) Indicates statistically significant differences between Pb-treated and non-Pb-treated cultures ($p < 0.01$). (#) Indicates statistically significant difference between control culture and all other cultures at the same MSC-1 cell dose ($p < 0.01$). In panel **B**, BALB/c-derived CD4^+ T cells ($3 \times 10^6/\text{mL}$)

were co-cultured with C57BL/6-derived B cells ($3 \times 10^6/\text{mL}$) and irradiated (6000 cGy) MSC-1 cells ($6.25 \times 10^4/\text{mL}$) in the presence of 0 or 1 mM NgMMA or 5 μM Pb. Supernatants were collected on days 1–4 and analyzed for total nitrates using the Griess reaction. Data are mean $\pm$ SD of triplicate cultures and are representative of 2 independent experiments. Linear regression analysis was performed on the three plots and comparisons in the presence *versus* absence of Pb and presence *versus* absence of NGMMA were made using a least squares means Student's t-test. Pb-treated and NGMMA-treated cultures produced significantly less NO in comparison to control ($p < 0.001$).

A

B

Figure 6. Pb attenuates NO levels but does not inhibit iNOS expression following indirect activation of the MSC-1 cell line

BALB/c-derived splenocytes ($2 \times 10^6/\text{mL}$) were co-cultured with MSC-1 cells ($5.6 \times 10^5/\text{mL}$) in the presence of 0 or 2 $\mu\text{g/mL}$ ConA and 0 or 5 μM Pb. Supernatants (A) and MSC-1 cell lysates (B) were harvested on days 1–3 of culture. In panel A, supernatants were analyzed for total nitrates using Griess reaction as above. Data are mean $\pm$ SD from triplicate cultures and representative of 2 independent experiments. Statistical analysis was done by one-way ANOVA followed by Tukey-Kramer HSD comparison of means. (#) Indicates a statistically significant difference between the ConA alone treatment versus Pb plus ConA treatment ($p < 0.01$). (*) Indicates a statistically significant difference between the control and ConA treated cultures ($p < 0.01$). In panel B, protein was isolated from MSC-1 cell lysates, separated using SDS-PAGE electrophoresis, transferred to a PVDF membrane, and probed for iNOS using a polyclonal rabbit-anti-mouse antibody. Data are a representative of 3 blots and 2 independent experiments.

Figure 7. Pb attenuates NO levels but does not inhibit iNOS expression following direct activation of the MSC-1 cell line

MSC-1 cells were plated at 5×10^5 cells/mL in bulk culture in the presence of 0, 1 or 5 μ M Pb and with or without stimulation consisting of 100 U/mL IFN- γ and 5 μ g/mL LPS. (A) After 2 days in culture, protein isolated from MSC-1 cell lysates was separated using SDS-PAGE electrophoresis, transferred to a PVDF membrane, and probed for iNOS using a polyclonal rabbit-anti-mouse antibody. The blot was stripped and re-probed for actin normalization. (B) After 6 hours in culture, RNA was isolated and 100 ng/ μ l was subjected to RT-PCR for iNOS and then normalized to actin mRNA levels. In panel (C), supernatants harvested on day 2 were analyzed for total nitrates using the Greiss reaction. Data values are the mean $\pm$ SD from triplicate cultures and the asterisk indicates statistically significant differences between control and Pb (* $p < 0.01$) cultures as assessed by Student's t-test. All data are representative of at least 3 independent experiments.