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Lead effects on development and function of bone marrow-derived dendritic cells promotes Th2 immune responses

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Abstract

Although lead (Pb) has significant effects on the development and function of macrophages, B cells, and T cells and has been suggested to promote allergic asthma in mice and humans, Pb modulation of bone marrow (BM)-derived dendritic cells (DCs) and the resultant DC effects on Th1 and Th2 development have not been examined. Accordingly, we cultured BM cells with murine granulocyte macrophage-colony stimulating factor (mGM-CSF) $\pm$ PbCl₂. At day-10, culture supernatant (SN) and non-adherent cells were harvested for analysis. Additionally, day-10 non-adherent BM-DCs were harvested and re-cultured with mGM-CSF + LPS $\pm$ Pb for 2 days. The day-10 Pb exposure significantly inhibited BM-DC generation, based on CD11c expression. Although fewer DCs were generated with Pb, the existing Pb-exposed DCs had significantly greater MHC-II expression than did the non-Pb-exposed DCs. However, these differences diminished upon LPS stimulation. After LPS stimulation, CD80, CD86, CD40, CD54, and MHC-II were all up regulated on both Pb-DCs and DCs, but Pb-DCs expressed significantly less CD80 than did DCs. The CD86:CD80 ratio suggests a Pb-DC potential for Th2 cell development. After LPS stimulation, IL-6, IL-10, IL-12 (p70), and TNF- α levels significantly increased with both Pb-DCs and DCs, but Pb-DCs produced significantly less cytokines than did DCs, except for IL-10, which further supports Pb-DC preferential skewing toward type-2 immunity. *In vitro* studies confirm that Pb-DCs have the ability to polarize antigen-specific T cells to Th2 cells. Pb-DCs also enhanced allogeneic and autologous T cell proliferation *in vitro*, and *in vivo* studies suggested that Pb-DCs inhibited Th1 effects on humoral and cell-mediated immunity. The Pb effect was mainly on DCs, rather than on T cells, and Pb's modification of DC function appears to be the main cause of Pb's promotion of type-2-related immunity, which may relate to Pb's enhanced activation of the Erk/MAP kinase pathway.

Introduction

Environmental exposure to Pb has been reported to promote IgE production in children and to be an influence on asthma incidence (Lutz *et al.*, 1999b), which has been suggested to be related to Pb-enhancement of Th2 responses (Lawrence and McCabe, 2002). Pb preferentially enhances *in vivo* and *in vitro* Th2 development (Heo *et al.*, 1996; Heo *et al.*, 1997; Heo *et al.*, 1998) and affects development and phenotype of B cells (McCabe and Lawrence, 1990; McCabe and Lawrence, 1991) and macrophages (Kowolenko *et al.*, 1988; Kowolenko *et al.*, 1989; Song *et al.*, 2001; Sengupta and Bishayi, 2002). However, no study has reported Pb effects on the development of dendritic cells (DCs), the most important antigen-presenting cell

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(APC) for naïve T cells. DCs efficiently present antigens to T cells and, dependent on their phenotype, can polarize T cell differentiation. It has been reported that intracellular events, cytokine profiles, costimulation signals, and microenvironmental events affect the ability of DC to direct T cell activation and differentiation (MacDonald *et al.*, 2002; Steinman, 2003; Adams *et al.*, 2005; Jenkins and Mountford, 2005). DCs can modulate B cell humoral immunity (HI) indirectly by affecting T cell activation, which in turn affects B cell growth and immunoglobulin secretion, and by directly interacting with B cells so as to alter their differentiation and class switching (Banchereau and Steinman, 1998; Wykes *et al.*, 1998; Dubois *et al.*, 1999). Our hypothesis is that Pb preferentially induces the development of BM-DCs that promote Th2 immune responses.

In this study, we tested the effects of Pb on BM-DC development and examined the resultant BM-DC effects on Th1 and Th2 responses and on induction of allogeneic and autologous responses. We demonstrate that Pb-treated BM cells generate fewer DCs than does BM differentiated in the absence of Pb. However, DCs developed in the presence of Pb (Pb-DCs) express more MHC II (I-A^d), efficiently polarized antigen-specific T cells to Th2 cells, and enhanced allogeneic T cell proliferation. In addition, Pb-DCs induce Th2 skewing of HI and inhibit cell-mediated immunity (CMI) *in vivo*.

Material and methods

Mice

BALB/c, C57BL/6, and BALB/c DO11.10 (OVA-reactive TCR transgenic mice, OVA^{tg}) (2- to 4-month old) were obtained from the Wadsworth Center animal production unit. Mice were housed in our specified pathogen-free AAALAC-approved facility of the Wadsworth Center and were maintained on mouse chow and acidified water *ad libitum*. All of the studies were IACUC approved.

Reagents

A stock solution of 10 mM PbCl₂ (Fisher Scientific, Pittsburgh, PA) was prepared in physiological saline (Baxter, Deerfield, IL), a stock solution of ovalbumin (OVA) was purchased from Calbiochem (San Diego, CA), and a stock solution of OVA peptide (OVA_p; ISQAVHAAHAEINEAGR-339) was provided by the Peptide Synthesis core of Wadsworth Center. All stock solutions were diluted with sterile saline and were confirmed to be endotoxin-free or low endotoxin by the use of Limulus Amebocyte Lysate QCL-1000 (Biowhittaker, Walkersville, MD) prior to addition to culture or injection into mice. Culture medium was RPMI 1640 supplemented with 1 mM nonessential amino acids, 1 mM sodium pyruvate, 1 % sodium bicarbonate from Biowhittaker, 2 mM glutamine (Sigma, St. Louis, MO), 50 µM β-mercaptoethanol (Fluka, Ronkonkoma, NY), 25 µg/ml gentamicin (Sigma), 1 % penicillin-streptomycin-neomycin mixture (Gibco, Grand Island, NY), and 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT). Sterile 1× DPBS used for cell preparation was purchased from Sigma. Red blood cell lysing buffer was made of 0.017 M Tris and 0.14 M NH₄Cl, pH 7.4.

Generation of BM-DCs

The protocol for generation of BM-DC was adapted from Lutz *et al.* (Lutz *et al.*, 1999a). Briefly, BM cells were obtained from femurs and tibias of BALB/c female mice and cultured in 100 mm (Falcon; Becton Dickinson, Franklin Lakes, NJ) bacteriological Petri dishes at 2×10⁶ BM leukocytes in 10 ml completed RPMI medium containing 200 U/ml mGM-CSF (Peprotech, Rocky Hill, NJ) with or without 25 µM PbCl₂ at 37°C in a low O₂ (5%) incubator. After 3 days incubation, additional 10 ml of complete RPMI containing the same amount of mGM-CSF with or without PbCl₂ were added to the Petri dishes. At day 6 and day 8, half of

the culture supernatant was collected and centrifuged, and the cell pellet was put back into the original dish with 10 ml fresh medium, containing mGM-CSF, in the present or absent of Pb. At day-10, non-adherent cells and supernatants (SNs) were collected for analysis. Additionally, day-10 (d10) non-adherent BM-DC were harvested and re-cultured with 100 U/ml mGM-CSF + lipopolysacchride (LPS, 1 µg/ml; Sigma) ± Pb for 2 days. Then the non-adherent cells and SN were collected for analysis. In a Pb dose-dependent study, 1, 5, and 25 µM PbCl₂ were used. For generating OVA-pulsed d10 BM-DC, at day-9, OVA (100 µg/ml) was added to culture. One day later, non-adherent cells were collected and washed two times with 1 × DPBS.

Immunization

BALB/c mice were immunized with 10⁶ OVA pulsed (100 µg/ml) d10 BM-DC on footpad, and 7 days later, the mice were bled and received OVA (200 µg/100 µl) subcutaneously (subQ). After 1 wk, these mice were bled, and the serum was stored for IgG1, IgG2a and IgE analyses. After bleeding, mice were challenged with OVA (100 µg in 25 µl saline) into the footpad for analysis of the DTH response. BALB/c mice immunized subQ twice with OVA served as positive controls.

Serum preparation

Peripheral blood was obtained by retro-orbital phlebotomy into 1.7-ml Eppendorf tubes. After clotting overnight at 4°C, serum was collected after centrifugation.

Splenocyte preparation

Splenocytes (SPLs) were prepared as described previously (Gao *et al.*, 2006).

CD4⁺ T cell isolation

CD4⁺ T cells were isolated from spleens with negative-selection columns (StemCell Technologies Inc), as described by the manufacturer. Briefly, SPLs were incubated with SpinSep antibody cocktails. After washing, the antibody-treated cells were mixed with SpinSep Dense Particles. Then, CD4⁺ T cells were separated from other cells by the density difference. The purity of CD4⁺ cells was 90 ± 3%.

Cytokine detection

Cytokine production was detected using ELISA kits from R & D System. The manufacturer's protocol was employed. Briefly, the plate was coated with anti-cytokine monoclonal antibody (mAb). After washing, serial diluted standards and samples were loaded on the plate. After incubation, biotin-labeled anti-cytokine mAb was applied, followed by avidin-peroxidase and finally the substrate. The plates were read using an ELISA reader (EL310; Bio-Tek, Burlington, VT) at 450 nm.

Flow-Cytometric Analysis

Single-cell suspensions were prepared and analyzed by multi-color flow cytometry (Becton Dickinson & Co., Mountain View, CA). Cells (10⁶) were suspended in 100 µl PBS, containing 0.1% sodium azide, Fc block (BD Pharmingen), and 1 µg of various directly conjugated mAbs. PerCp anti-mouse CD11b, PerCP or FITC anti-mouse Gr1, PE or APC anti-mouse CD11c, PE anti-mouse FcεR1, PE anti-mouse CD3, PE-anti-mouse CD19, PE anti-mouse CD45R/B220, FITC or PE anti-mouse I-A^d, FITC-anti mouse CD80, FITC-anti mouse CD86, FITC-anti mouse CD40, FITC anti-mouse ICAM-1 (BD Pharmingen), and FITC-anti mouse F4/80 (Caltag) were utilized. After 30-min incubation on ice, cells were washed and analyzed on the flow cytometer by gating out the majority of non-viable cells based on low forward angle light scatter.

Cell signaling assay

Phosphorylated proteins were detected from d10 BM-DC lysates by the Upstate cell signaling kit (Upstate Biologicals, Lake Placid, NY). The assay protocol was provided by manufacturer. Briefly, cell lysates were incubated with beads coated with capture antibody to target protein for overnight. After washing, the beads were incubated with biotinylated reporter, which bound to only phosphorylated target protein. Streptavidin-PE was used as the fluorescent signal. The assay was run on a Luminex 100 instrument. The results were analyzed by using the software provided by the company. Since the assay kit lacked an internal control, all samples were loaded on one plate.

Protein assay

The protein concentration of cell lysates was tested by using BCA™ Protein Assay Kit (Pierce, Rockford, IL). The manufacturer protocol was utilized to perform the assay. In general, the samples were mixed with BCA reagent A (containing mainly bicinchoninic acid) and BCA reagent B (containing cupric sulfate). After 30 min incubation, the sample was read at 570 nm. The sample protein concentration was calculated based on the BSA standard.

Cell Culture

For evaluation of d10 BM-DC APC function *in vitro*, 10^6 Single-cell suspensions of OVA_{Ag} CD4⁺ T cells were cultured with 10^5 Pb treated or none treated d10 BM-DCs in present of 0.02 µg/ml OVA_{Ag}; in order to exam the allogeneic stimulatory ability of d10 BM-DC, 10^6 Single-cell suspensions of C57BL/6 spleen cells were cultured with 10^5 Pb-DCs or DCs; or for testing the syngeneic stimulatory ability of d10 BM-DC, 10^6 BALB/c CD4⁺ T cells were cultured with 10^5 Pb-DCs or DCs. All cultures (1 ml) were incubated at 37°C in a low O₂ (5%) incubator; 4 days later, all culture SNs were collected and stored at -20°C for cytokine analysis.

Cell Proliferation

The culture conditions were similar to those listed in the Cell Culture section, except that 2×10^5 CD4⁺ T cells or SPLs and 2×10^4 of Pb-DCs or DCs were used in 0.2 ml culture medium. After 4 days, each well was pulsed with 0.5 µCi/well [³H]-thymidine (Dupont-NEN, Wilmington, DE) for the last 6 hr. The phosphostimulated luminescence image was read by a BAS 2000 Fujix reader and analyzed by the TINA 2.0 program.

ELISA for IgG isotype and IgE

Total IgE, and OVA-specific IgG1, IgG2a, and IgE were measured by ELISA, as described (Gao *et al.*, 2006). For antigen-specific IgG1 and IgG2a, the wells were coated with OVA instead of KLH. For measurement of OVA-specific IgE, the wells were coated with rat anti-mouse IgE (BD Pharmingen). After blocking and washing, the sera were added, followed by washing and addition of OVA. After a second 2-hr incubation, the wells were washed, and biotin-anti-OVA (6-((biotinoyl)amino) hexanoic acid (Molecular Probes, Eugene, OR) was used to label anti-OVA antibody from Sigma) was added, followed by avidin-peroxidase and finally the substrate.

Statistical analysis

Statistical analysis was performed by SigmaStat (Jandel Scientific, San Rafael, CA) one-way ANOVA; $p \leq 0.05$ was considered significant.

Results

***In vitro* Pb exposure impairs development of BM-DC**

At day 10 (d10), non-adherent and adherent cells from mGM-CSF-promoted BM cultures were harvested and counted. Pb significantly ($p < 0.01$) impaired the yield of non-adherent cells, but not adherent cells (Fig. 1A). Cell viability was not significantly different between Pb-treated and control BM-DC cultures. Recovered d10 non-adherent cells were transferred to new cultures containing mGM-CSF and LPS, without any additional Pb. Two days later (d12), non-adherent and adherent cells were quantified. After LPS stimulation in the absence of Pb, the numbers of non-adherent cells from the d10 Pb-DC and control DC cultures were no longer different (Fig. 1B); however, the numbers of adherent cells were significantly lower ($p < 0.001$) for the cells previously exposed to Pb. The adherent cells after the indicated treatments have been suggested to be mature macrophages (Lutz *et al.*, 1999a).

***In vitro* Pb-exposure impairs generation of CD11c⁺ BM-DCs, but the Pb-DCs have elevated MHC-II expression**

To investigate Pb effects on phenotypic changes of BM-derived cells, we assayed the developing BM-DC cultures without Pb (DCs) and with Pb (Pb-DCs) for expression of various markers by flow cytometry (Fig. 2). By d8, in the absence of Pb, there appear to be three distinct CD11c⁺ populations with low, intermediate and high levels of MHC-II (I-A^d) expression. CD11c is the main biomarker for DCs, and MHC-II is usually used to distinguish immature from mature DCs. A recent study also described a third subset of DC with low MHC-II expression (Goth *et al.*, 2006). In any case, Pb-DCs mainly expressed the intermediate level of MHC-II. Based on all of the cells present at d10, the expressions of CD11b, Gr-1, CD14, CD19, B220, F4/80, FcεR-1, CD123, CD83, CD34, CD31, CD3, CD4, CD8, CD80, CD86, CD40, and ICAM were not significantly altered by Pb (data not shown). However, the numbers of Pb-DCs expressing CD11c ($39.6 \pm 4.7\%$), as well as the amount of CD11c per cell (GeoMean fluorescence intensity (GMFI) of 429 ± 33.9) was significantly ($p < 0.001$) impaired, relative to the control DC population ($69.9 \pm 3.6\%$ with a GMFI of 777 ± 72.7) (Fig. 3). As shown in dot-plots (Fig. 2), the majority of CD11c⁺ cells expressing low levels of MHC-II were missing with Pb, but there was a group of CD11c^{low}MHC-II⁺ cells. The d10 MHC-II⁺/CD11c⁺ Pb-DCs expressed significantly more CD31/Gr1 (Table 1). Although fewer CD11c⁺ cells developed in the presence of Pb, expression of MHC-II on the CD11c⁺ cells was significantly enhanced by Pb in a dose-dependent manner (Fig. 4).

Pb inhibits LPS-induced expression of CD80, but not CD86

At d10, non-adherent cells were collected and recultured with LPS $\pm$ Pb. Two days later (d12), cells were harvested and analyzed. LPS stimulation significantly up-regulated expression of CD80, CD86, CD40, CD54, and MHC-II on CD11c⁺ cells (data not shown) and abolished the phenotypic difference previously observed between the d10 DCs and Pb-DCs in expression of CD11c and MHC-II. Interestingly, when d10 Pb-DCs were stimulated with LPS, they expressed equivalent levels of CD80 and CD86 as DCs; however, if they were cultured for the last two days with LPS + Pb, expression of CD80, but not CD86, on CD11c⁺ cells was significantly inhibited (Fig. 5).

***In vitro* LPS-stimulated Pb-DCs and DCs have different cytokine profiles**

The amounts of various cytokines in d10 BM-DC culture SN was very low to non-detectable (data not shown). After LPS stimulation $\pm$ Pb for 2 days, IL-18, IL-4, and IFN- γ production was still not detectable. However, IL-6, IL-10, IL-12p70 (IL-12), and TNF- α were significantly enhanced by LPS-stimulated DCs and Pb-DCs. Although IL-6, IL-12, and TNF- α were significantly increased by LPS-stimulated Pb-DCs in comparison to no LPS stimulation, they

produced significantly ($p < 0.01$) less IL-6, IL-12 and TNF- α than did the LPS-stimulated DCs (Fig. 6). There was no significant difference of IL-10 production between the LPS-stimulated Pb-DCs and DCs (Fig. 6). The IL-10:IL-12 ratio was significantly ($p < 0.01$) increased in the LPS-stimulated Pb-DC cultures (ratio was 9.9 ± 1.8 ; $n=5$), compared to the LPS-stimulated DC cultures (ratio was 1.6 ± 0.6 ; $n=5$). An IL-10:IL-12 imbalance was also observed in female rat offspring exposed to Pb during early or late embryonic development (Bunn *et al.*, 2001). The presence of Pb for the last 2 days of culture did not further alter the cytokine production profile (Fig. 6A), indicating that Pb effects on cytokine production mainly occurred during development but not LPS-induced maturation and stimulation of DCs.

***In vitro* Pb exposure altered phosphorylation of signal transduction pathway of BM-DC**

The d10 Pb-DCs or DCs were lysed and assayed for phosphorylation of cell signaling pathways with an Upstate Cell Signaling kit. Pb-DCs demonstrated more phosphorylated Erk/Map kinase than did the control DCs (Fig. 7).

***In vitro* Pb-DCs preferentially induce skewing toward Th2 responses**

In order to evaluate the APC function of the Pb-DC and DC, we cultured CD4⁺ T cells from naïve OVA_{Ag} BALB/c mice with Pb-DCs or DCs and OVA_p for 4 days, then assessed T cell proliferation and cytokine production. The specific CD4⁺ T cell to DC ratio (10:1) and the concentration of OVA_p were selected because these conditions have been demonstrated to evoke a mixed Th1 and Th2 response (Whelan *et al.*, 2000; Boonstra *et al.*, 2003). CD4⁺ T cells cultured with DCs or Pb-DCs only yielded low to undetectable cytokines (data not shown). Due to the variation among the four independent experiments, the mean IFN- γ production was not significantly different for Pb-DC and DC cultures. However, Pb-DCs consistently inhibited IFN- γ production by about 50 % (Fig. 8a), a degree of suppression similar to previous results with splenic cultures or Th1 clones (Heo *et al.*, 1996; Heo *et al.*, 1998). Addition of Pb to the CD4⁺ T cell/Pb-DC cultures did not further affect IFN- γ production (Fig. 8a), suggesting that Pb affects APCs to a greater extent than it does T cells. In contrast to inhibition of IFN- γ production, Pb-DCs enhanced IL-4, IL-5, and IL-6 production (Fig. 8b), but had no effect on IL-10 production (data not shown). Taken together, the cytokine production profiles suggest that the responding T cells are mainly Th2 cells. Nevertheless, Pb-DCs slightly enhanced antigen-specific T cell proliferation (data not shown).

Pb-DCs are better *in vitro* stimulators of allogeneic T cells than are DCs

Pb-DCs and DCs (d10) were compared for their ability to stimulate allogeneic T cells from C57BL/6 (B6) mice. Day 4 cultures were assessed for responder T cell proliferation and cytokine production. Pb-DCs induced significantly ($p = 0.01$) greater allogeneic responses than DC (Fig. 9a). The Pb-DC-induced stimulation index (SI) was 195 ± 31 % of the DC-induced SI. Due to the variations among the 9 independent experiments, the mean IFN- γ production was not significantly altered by Pb-DCs. However, Pb-DC cultures produced overall 15% less IFN- γ than did the DC cultures. Additionally, Pb-DC cultures had greater IL-6, IL-2, and IL-10 levels to those of the DC cultures (Fig. 9b).

Pb-DCs generate a slightly greater autologous responses than DCs

Pb-DCs and DCs (d10) were compared for their ability to induce proliferation and cytokine production of syngeneic CD4⁺ T cells. Pb-DC (3122 ± 560.8 PSL) induced slightly greater proliferation of syngeneic CD4⁺ T cells than did DCs (2202 ± 766.4 PSL). The Pb-DC-induced SI was 143 ± 19 % of the DC-induced SI. However, cytokine production was very low for both.

Pb-DCs induced Th2 skewing of HI *in vivo*

At day 9, DC and Pb-DC cultures were pulsed with OVA. At day 10, these cells were washed and injected into footpads of naïve BALB/c mice. After 7 days, the “OVA primed” mice received a subQ OVA boost. After another 7 days, mice were bled, and their sera were analyzed for IgG1 and IgG2a anti-OVA and total IgE production. Pb-DCs and DCs induced equivalent priming of the IgG1 anti-OVA response; however, Pb-DC priming for the IgG2a anti-OVA response was significantly impaired ($p < 0.01$). Total IgE production was not affected by the OVA-pulsed Pb-DC (Fig. 10). OVA-specific IgE was also not affected by the OVA-pulsed Pb-DC (data not shown).

Pb-DCs inhibited DTH responses *in vivo*

After assessment of IgG1, IgG2a, and IgE anti-OVA responses, mice were challenge with 100 µg OVA in the footpad. The footpad swelling was measured 24 hours later. The mice that received “OVA primed” Pb-DCs developed significantly ($p < 0.005$) weaker DTH response (0.1 ± 0.02 mm; $N = 6$ from 3 independent experiments) compared to controls (0.17 ± 0.008 mm; $N = 6$ from 3 independent experiments). DTH response is usually considered to be a type-1 immune response (Cher and Mosmann, 1987; Fong and Mosmann, 1989). Therefore, Pb-DCs inhibited Th1 responses.

Discussion

It has been shown that immunosuppressive and anti-inflammatory drugs, dietary products, and even tick saliva have modulatory effects on the differentiation and function of murine BM-DCs (Mehling *et al.*, 2000; Hackstein *et al.*, 2001; Kim *et al.*, 2004; Bronnum *et al.*, 2005; Cavassani *et al.*, 2005; Kim *et al.*, 2005). Here, we report that the environmental toxicant Pb also can modulate the development and functions of BM-DCs.

We report that Pb inhibits GM-CSF driven development of DC precursors. Fewer total cells, including BM-DC, were generated in the presence of Pb. The Pb-DC population expressed less CD11c, the classical marker of DCs, but they expressed greater levels of MHC-II per cell, as though they were already mature DCs. The Pb-DCs had some similarities with plasmacytoid DCs (pDCs) (LeibundGut-Landmann *et al.*, 2004); however, the Pb-DCs that developed after 10 days lacked other required markers, such as CD123 and B220 (Asselin-Paturel *et al.*, 2001; Adams *et al.*, 2005). Therefore, it is unlikely that Pb skewed the development of BM precursors to pDCs. The Pb-DCs also were not CD8⁺ DCs (Vremec *et al.*, 1992). In this study, we found that the expression of CD31/Gr1 was higher on Pb-DCs. Since CD31/Gr1/CD11b positive cells have been suggested to be an intermediate immature population (Bronte *et al.*, 2000), Pb may simply slow down the development of BM-DCs, while promoting heightened MHC-II expression. Recently, a novel DC population has been identified as CD11c⁺NK1.1/DX5⁺ (NKDC) in humans and rodents (Josien *et al.*, 1997; Fanger *et al.*, 1999; Homann *et al.*, 2002; Pillarisetty *et al.*, 2005; Chen *et al.*, 2007). These described NKDCs were about 15 to 20 % of the CD11c⁺ cells in the BM. They did not express MHC Class II and showed a strong cytotoxic rather than antigen-presenting ability (Chen *et al.*, 2007). Compare to DCs, Pb-DCs were missing a CD11c⁺MHC II⁺ population (Fig. 2), suggesting that Pb may interfere with development of the newly identified NKDC cells. Pb-DCs had an ability to mature upon LPS stimulation similar to that of control DCs. Only when Pb was present in the cultures with LPS was the expression of CD80 inhibited. This result is consistent with the previous finding that Pb exposure inhibits CD80 expression on APC (macrophages or B cells), but did not affect CD86 expression (Selgrade *et al.*, 1997). Several studies have suggested that the differential expression of the B7 family on APCs influences the generation of Th1 or Th2 cells (Kuchroo *et al.*, 1995; Lenschow *et al.*, 1995; Selgrade *et al.*, 1997; De Becker *et al.*, 1998). In fact, expression of CD80 preferentially enhances the generation of Th1 cells, and expression of

CD86 preferentially enhances the generation of Th2 cells (Kuchroo *et al.*, 1995; Selgrade *et al.*, 1997). Our flow cytometric study suggests that Pb-DCs have Th2 polarization potential. However, with regard to CD80 expression, this potential required Pb to be continuously present.

Many publications have suggested that IL-12 produced by DC mediates Th1 development (Macatonia *et al.*, 1995; De Becker *et al.*, 1998; Moser and Murphy, 2000; Maldonado-Lopez *et al.*, 2001; Adams *et al.*, 2005), whereas enhanced production of IL-10, which suppresses IL-12 production, promotes production of Th2 cells and type-1 regulatory T cells (Tr1) (McGuirk *et al.*, 2002; Yi *et al.*, 2002; Lavelle *et al.*, 2003). Nevertheless, McDonald *et al.* argue that production of IL-12 from the host, but not from DCs, polarizes toward Th1 (MacDonald and Pearce, 2002), and Feili-Hariri *et al.* argue that IL-12 may induce Th1 differentiation, but not the Th1 response (Feili-Hariri *et al.*, 2005). At d10, both DCs and Pb-DCs produced low levels of cytokines. After LPS stimulation, both LPS-matured DCs and Pb-DCs secreted significant amounts of IL-6, IL-10, IL-12, and TNF- α . Control DCs were able to make both Th1 and Th2 cytokines, whereas Pb-DCs generally made lower quantities of cytokines; only IL-10 production was equivalent. Based on most current opinion, the significantly lower IL-12 production in the presence of Pb suggests that Pb-DCs are less able to drive Th1 differentiation. Unlike the need for continuous Pb exposure for inhibition of CD80, Pb did not have to be present during the LPS stimulation to inhibit IL-6, IL-12, or TNF α production. IL-6 is suggested to be involved in DC generation and development (Santiago-Schwarz *et al.*, 1996; Brasel *et al.*, 2000). Impaired production of IL-6 by the Pb-treated BM cultures correlates well with our suggestion that the Pb-DCs are intermediately matured DC with lower expression of CD11c⁺ cells and higher expression of CD31/Gr1.

Besides altering the development and cytokine production of BM-DCs, Pb also affected normal BM-DC function. Pb-DCs slightly enhanced antigen-specific CD4⁺ T cell proliferation, but preferentially promoted Th2 cell development. In the mixed lymphocyte culture (MLC), Pb-DCs significantly enhanced allogeneic T cell proliferation. Although IL-2 is not considered to be a typical Th2 cytokine, IL-2 has been suggested to play a central role in Th2 differentiation (Cote-Sierra *et al.*, 2004; Yamane *et al.*, 2005). Additionally, the Pb-DCs enhanced generation of Th2 cytokines IL-6 and IL-10 and lowered the amount of the Th1 cytokine IFN- γ . Therefore, the cytokine profiles from the MLC responses support the suggestion that Pb-DCs promoted Th2 cell development and proliferation.

In addition, Pb-DCs induced an *in vivo* HI response different from that induced by DCs. It has been reported that antigen-pulsed DCs and peritoneal macrophages, but not resting B cells, are able to induce the synthesis of specific antibody isotypes *in vivo* (De Becker *et al.*, 1994), suggesting the B cell response can be regulated by the nature of the APC. Since the Pb-DCs induced IgG1 antibodies and IgE to an extent comparable to that of the DCs, but a significantly lower IgG2a antibody response, Pb-DCs seem to be responsible for preferential promotion of type-2 immune responses, as previously suggested (Heo *et al.*, 1996; Heo *et al.*, 1998). It has been reported that the secretion of IgG1 and IgE is Th2 cytokine-mediated (Vitetta *et al.*, 1984; Bossie *et al.*, 1987), whereas the secretion of IgG2a is Th1 cytokine-mediated (Snapper and Paul, 1987; Stevens *et al.*, 1988). Therefore, our data imply that Pb-DCs suppress Th1 development. Our DTH results also support the suggestion that Pb-DCs are more Th2 than Th1 promoting. Our finding that Pb-DCs suppress DTH responses is in agreement with previous publications in mice and rats (Muller *et al.*, 1977; Faith *et al.*, 1979; Miller *et al.*, 1998; McCabe *et al.*, 1999; Bunn *et al.*, 2001; Chen *et al.*, 2004). It had been suggested that Pb-induced suppression of DTH responses is due to the alteration of macrophage functions by Pb (Muller *et al.*, 1977; Miller *et al.*, 1998; Bunn *et al.*, 2001). In addition, it had been previously reported that Pb inhibited development of macrophage in Colony Stimulating Factor-1 (CSF1)-induced BM cultures (Kowolenko *et al.*, 1989). Here, our data suggest that alteration of DC

functions by Pb plays a role in suppression of DTH responses. Thus, developmental exposure to Pb may skew DC development toward the Pb-DC phenotype reported here, further supporting the contention that Pb exposures are involved in the development of allergies (van Rijt and Lambrecht, 2005).

It has been reported that parasite-related proteins are also able to induce Th2-driven DCs (DC2) (Whelan *et al.*, 2000; MacDonald *et al.*, 2002; Balic *et al.*, 2004; Jenkins and Mountford, 2005). Unlike Pb-induced DC2s, the majority of the parasite-induced DC2s had minimal effects on MHC II expression. Some of the parasite-induced DC2s had upregulated CD86 expression (Balic *et al.*, 2004; Jenkins and Mountford, 2005), but no change in CD80 expression. The cytokine profile also was different between Pb-induced DC2 and these DC2s. Taken together, the phenotype and cytokine information of Pb-DCs indicates that they are not the same as the parasite-induced DC2s.

It has been shown that Pb alters CD4⁺ T-cell-mediated immunity by inducing a Th2 skewing (Heo *et al.*, 1996; Heo *et al.*, 1997; Heo *et al.*, 1998). However, the mechanisms involved have not been clearly delineated. Lawrence and McCabe had proposed possible mechanisms, in which Pb could directly affect T cell function or indirectly modulate T cell activity via altering APC functions (Lawrence and McCabe, 2002). More recently, Dietert *et al.* summarized the theorized effects of Pb on immunity that have been proposed to date (Dietert and Piepenbrink, 2006). The data in the present report indicate that Pb is capable of modulating BM-DC development and consequent APC functions, which in turn, inhibits Th1 development and preferentially promotes Th2 development. It seems that Pb has less effect directly on T cells. A recent study also suggested that Pb alters CD4⁺ T cell proliferation by targeting APC function (Farrer *et al.*, 2005). It has been proposed that p38 and JNK1/2 kinase pathway (Adams *et al.*, 2005; Kim *et al.*, 2005; Miranda *et al.*, 2005) and the STAT3-dependent pathway (Imada and Leonard, 2000; Nefedova *et al.*, 2005; Rathinam *et al.*, 2005) are also involved in DC development and function; however, these pathways were not affected by Pb. The proposed mechanism of Pb alteration of DC function is Pb stimulation of Erk/MAPK phosphorylation in DCs, which stabilizes the transcription factor c-Fos (Okazaki and Sagata, 1995; Vial and Marshall, 2003), a suppressor of IL-12. With promotion of Erk signaling, IL-12 production is suppressed (Roy *et al.*, 2000; Agrawal *et al.*, 2003; Dillon *et al.*, 2004). Alternatively, Pb-stimulated Erk signaling promotes the production of IL-10, which mediates negative feedback regulation of IL-12 production (Yi *et al.*, 2002; Xia *et al.*, 2005). Along with higher MHC class II expression, the Pb-DCs had a significantly greater IL-10:IL-12 ratio than the DCs supporting these alternative influences on Th2 development.

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Figure 1.

Effect of Pb on the numbers of BM cells induced by mGM-CSF. BM cells were cultured with mGM-CSF $\pm$ 25 μ M PbCl₂ and assayed for non-adherent (NAd) and adherent (Ad) cells on day-10 (A). Then, day-10 NAd cells were recultured in the presence of mGM-CSF + LPS (1 μ g/ml) for two days and assayed again for NAd and Ad cells (B). Data are presented as mean $\pm$ SEM for 18 separate experiments; * indicates statistically significant ($p < 0.05$) differences from cultures without Pb.

Figure 2.

Dot plots of BM-DC development. BM cells were cultured with mGM-CSF $\pm$ 25 μ M PbCl₂ and collected and immunophenotyped on the days indicated. Cells were labeled with the designed monoclonal antibodies (mAbs) and analyzed by flow cytometry. The quadrants shown were set based on isotype control staining. Results are representative of three independent experiments for day 3, 6, and 8; and nine independent experiments for day-10.

Figure 3.

Pb effect on CD11c expression. BM cells were cultured with mGM-CSF $\pm$ PbCl_2 (1-25 μM) and collected on day 10. Cells were labeled with mAbs and analyzed by flow cytometry. Results are shown as the mean percentage $\pm$ SEM (A) and mean GMFI $\pm$ SEM (B) for three separate experiments with Pb concentrations 1 and 5 μM and nine independent experiments for no Pb and 25 μM Pb; * designates $p < 0.05$ between no Pb and 25 μM Pb groups.

Figure 4.

Pb alters MHC-II expression on CD11c $^+$ cells. BM cells were cultured with mGM-CSF $\pm$ PbCl_2 (1-25 μM) and collected on day-10. Cells were labeled with the designed mAbs and analyzed by flow cytometry. Results are shown as the mean percentage $\pm$ SEM (A) and mean GMFI $\pm$ SEM (B) for three separate experiments with Pb concentrations 1 and 5 μM and nine independent experiments for no Pb and 25 μM Pb; * designates $p < 0.05$ between no Pb and 25 μM Pb cultures.

Figure 5.

Pb alters CD80 and CD86 expression on CD11c⁺ cells upon LPS stimulation. BM cells were cultured with mGM-CSF $\pm$ 25 μ M PbCl₂, and on d10, the expression of CD80 and CD86 on CD11c⁺ cells was assessed by flow cytometry (**A**). At day-10, cells were washed and recultured with mGM-CSF + LPS $\pm$ Pb for 2 days, and again assayed for CD80 and CD86 (**B**). Results are shown as the mean GMFI $\pm$ SEM (N=4, from 4 independent experiments); * indicates significant difference from d10 DC + LPS + Pb cultures.

Figure 6.

Pb effects LPS-induced cytokine production by d10 BM-DC cultures. (A) BM cells were cultured with mGM-CSF $\pm$ 25 μ M PbCl₂ for 10 days; then, 10⁶ cells were washed and recultured with mGM-CSF + LPS $\pm$ PbCl₂ for 2 days (A). Additionally, BM cells were cultured with mGM-CSF + 0-25 μ M PbCl₂ for 10 days; then, 10⁶ cells were washed and recultured with mGM-CSF + LPS for 2 days (B). SNs were collected for analysis of cytokine production. Results are presented as the mean $\pm$ SEM (N=5, from 5 independent experiments); *, **, and *** indicate significant difference from control (0 μ M Pb) cultures.

Figure 7.

Pb effect on phosphorylation of cell-signaling molecules of d10 DC. The d10 DC or Pb-DC was lysed and tested for phosphorylation of six cell-signaling molecules. Data are presented as mean $\pm$ SEM of MFI vs. total protein from 6 separate experiments; * indicates significant difference from the comparable non-Pb DC cultures.

Figure 8.

Pb-DC effect on cytokine production by antigen-specific CD4⁺ T cells. OVA_{Ag} CD4⁺ T cells were purified and cultured (10^6) with d10 DC or Pb-DC (10^5) and OVAp for 4 days. On day 4, SNs were harvested to quantify production of IFN- γ (A) or Th2 cytokines (B) by ELISA. For A, results are shown as the mean $\pm$ SEM of percentage of production of the control (CD4⁺ T cells + d10 DC + OVAp, 100%); four separate experiments. For B, data are shown as mean $\pm$ SEM of three separate experiments; * indicates significant difference from the comparable non-Pb group and ** indicates significant difference from the comparable non-Pb group by paired *t*-test only.

Figure 9.

(A) Pb effect on stimulatory ability of BM-DC with responder allogeneic T cells in vitro. C57BL/6 SPLs (5×10^5) were cultured with d10 DC or d10 Pb-DC (2×10^4) for 4 days and pulsed with [^3H]-thymidine for last 6 hr followed by quantification of incorporated radioactivity by phosphoimager (PSL units). Data are presented as the mean $\pm$ SEM (N=10 out of 7 independent experiments). (B) Pb effect on cytokine production by BM-DC stimulated allogeneic T cells. C57BL/6 SPLs (10^6) were cultured with d10 DC or d10 Pb-DC (10^5) for 4 days. On d4, the SN was used to quantify cytokine production by ELISA. Data are presented as the mean $\pm$ SEM (N=7, from 5 independent experiments); * designates significant difference

from the comparable non-Pb group and ** indicates significant difference from the comparable non-Pb group by paired *t*-test only.

Figure 10.

Induction of IgG1 (A) and IgG2a (B) anti-OVA and IgE (C) by priming naïve BALB/c mice with OVA-pulsed BM-DC. Naïve BALB/c mice received 10^6 of *in vitro* OVA-pulsed (24 hr) d10 DC or d10 Pb-DC into the footpad. Seven days later, the mice were given 200 µg OVA subQ and on d14, mice were bled. Mice that received two *in vivo* OVA immunizations served as the positive control. The results for d10 DC + OVA priming group (N = 7) and d10 Pb-DC + OVA group (N = 7) are presented as the mean $\pm$ SEM from three separate experiments; * indicates significant difference from the comparable non-Pb group.

Table 1

Pb effect on expression of surface markers on MHC-II⁺/CD11c⁺ BM-DCs.

Markers	DCs % (GMFI)	Pb-DCs % (GMFI)	P value
CD31/Gr1	5.4 ± 1.1 (78.0 ± 8.3)	9.5 ± 1.0 (144.0 ± 15.1)	0.03 (0.008)
B220/Gr1	2.1 ± 0.2 (66.5 ± 7.6)	3.0 ± 0.7 (70.7 ± 7.8)	> 0.05
CD16/32/CD11b	20.0 ± 1.1 (57.5 ± 0.1)	16.2 ± 0.1 (58.0 ± 2.3)	> 0.05
CD83	12.2 ± 0.9 (267.8 ± 22.5)	14.8 ± 0.6 (311.6 ± 47.2)	> 0.05

BM cells were cultured with mGM-CSF ± PbCl₂ (25 μM) and collected on day 10. Cells were labeled with mAbs and analyzed by flow cytometry. Results are shown as the mean ± SEM from two to four independent experiments. *p* < 0.05 indicates statistical difference from the comparable non-Pb group.