

Pb exposure attenuates hypersensitivity in vivo by increasing regulatory T cells

Liang Fang^{a,1}, Fang Zhao^{b,1}, Xuefeng Shen^b, Weiming Ouyang^c, Xinqin Liu^b, Yan Xu^b, Tao Yu^b, Boquan Jin^a, Jingyuan Chen^{b,*}, Wenjing Luo^{b,*}

^a Department of Immunology, Fourth Military Medical University, Xi'an 710032, China

^b Department of Occupational and Environmental Health and the Ministry of Education Key Lab of Hazard Assessment and Control in Special Operational Environment, School of Public Health, Fourth Military Medical University, Xi'an 710032, China

^c Laboratory of Cell Biology, Division of Monoclonal Antibodies, Office of Biotechnology Products, Center for Drug Evaluation and Research, United States Food and Drug Administration, Bethesda, MD, USA

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ABSTRACT

Pb is a common environmental pollutant affecting various organs. Exposure of the immune system to Pb leads to immunosuppression or immunodysregulation. Although previous studies showed that Pb exposure can modulate the function of helper T cells, Pb immunotoxicity remains incompletely understood. In this study, we investigated the effect of Pb exposure on T cell development, and the underlying mechanism of Pb-induced suppression of the delayed-type hypersensitivity (DTH) response in vivo. Sprague–Dawley rats were exposed to 300 ppm Pb-acetate solution via the drinking water for six weeks, and we found that Pb exposure significantly increased Pb concentrations in the blood by 4.2-fold ($p < 0.05$) as compared to those in the control rats. In Pb-exposed rats, the amount of thymic $CD4^+CD8^-$ and peripheral $CD4^+$ T cells was significantly reduced, whereas, $CD8^+$ population was not affected. In contrast to conventional $CD4^+$ T cells, Foxp3⁺ regulatory T cells (Tregs) were increased in both the thymus and peripheral lymphoid organs of Pb-exposed rats. In line with the increase of Tregs, the DTH response of Pb-exposed rats was markedly suppressed. Depletion of Tregs reversed the suppression of DTH response by Pb-exposed $CD4^+$ T cells in an adoptive transfer model, suggesting a critical role of the increased Tregs in suppressing the DTH response. Collectively, this study revealed that Pb-exposure may upregulate Tregs, thereby leading to immunosuppression.

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Introduction

Pb is a widespread environmental pollutant and its toxic effects have been recognized for centuries. Because Pb is so widely utilized in everyday life, contamination of the environment by Pb is one of the most common medical and political problems. Over the past decade, it has become increasingly evident that contact with Pb, even at very low levels, will produce serious adverse health effects, especially in children (Landrigan et al., 2000; Rosen, 1995). It has been documented that blood Pb levels as low as 10 µg per deciliter of whole blood (i.e., ~0.5 µM) are potentially neurotoxic; however, Pb virtually affects every system in the body. The subclinical effects of Pb intoxication on other organ systems have received increasing attention.

Animal and human studies indicate that the immune system may be a target organ for Pb toxicity. The interaction of Pb with the immune system leading to immunosuppression or immunodysregulation may

consequently cause allergy or autoimmunity. Previous studies showed that $CD4^+$ helper T cell function is particularly sensitive to the immunotoxic effects of Pb (Fischbein et al., 1993; Heo et al., 1996, 1997, 1998; McCabe and Lawrence, 1991; Neilan et al., 1980; Sata et al., 1998; Warner and Lawrence, 1986). Based on cytokine expression, $CD4^+$ helper T cells can be divided into distinct subsets, including Th1, Th2, Th17 and Tregs, that control different immune effector functions. Pb has been shown to enhance Th2 cell proliferation and/or regulatory activities and affect Th1 cell proliferation in vitro (Heo et al., 1996; McCabe and Lawrence, 1991). In vivo studies indicate that Pb preferentially enhances the development of Th2 cells (Heo et al., 1997, 1998) and that Pb affects Th1-mediated immune effector functions. T cell-mediated delayed type hypersensitivity (DTH or Type IV hypersensitivity) is modulated by Pb-exposure (Faith et al., 1979; McCabe et al., 1999; Miller et al., 1998; Muller et al., 1977). However, it remains largely unknown how Pb exposure affects these T-cell reactivities.

Regulatory T cells (Tregs) are one of the T cell subsets which have strong immune-suppressive activity. Tregs were originally identified as $CD4^+CD25^+$ T cells (Sakaguchi et al., 1995). Later on, the fork head box p3 (FOXP3) gene was identified as the master transcriptional factor of Tregs (Hori et al., 2003; Sakaguchi et al., 2008). Here, we showed that oral administration of Pb via the drinking water caused a significant reduction of $CD4^+$ T cells in the thymus and peripheral lymphoid organs.

* Corresponding authors. Fax: +86 29 84774862.

E-mail addresses: jy_chen@fmmu.edu.cn (J. Chen), luowenj@fmmu.edu.cn (W. Luo).

¹ Liang Fang and Fang Zhao contributed equally to this work.

In contrast, Foxp3⁺ Tregs were markedly increased in Pb-exposed rats. The increased Tregs played a critical role in mediating Pb-exposure-caused suppression of DTH reactivity.

Materials and methods

Animal protocol and Pb²⁺ exposure. Male Sprague–Dawley rats (littermates, Fourth Military Medical University, Xi'an, China) aged 20–22 days weighing 30–50 g upon arrival, were assigned to two groups (n = 20) such that the group mean body weights were comparable. The animals were quarantined in our specified pathogen-free animal facility, and housed in stainless-steel cages in a temperature-controlled, 12/12 light/dark room, and given food ad libitum. On the third day (age of 22–24 days) after arrival, the animals started to receive Pb in drinking water. A stock solution of 50,000 ppm (250 mM) Pb-acetate (ACS Reagent Grade, Sigma, St. Louis, MO) was dissolved in deionized-distilled H₂O. Groups of rats were exposed to 0 or 300 ppm (1.5 mM) Pb-acetate solution via the drinking water for six weeks. Pb concentration (300 ppm Pb) in drinking water was chosen because intoxication with Pb could impair the generation of a robust DTH response at high doses (McCabe et al., 1999). The solutions were changed twice a week during the exposure period, and rats in each treatment group were monitored daily for food and water consumption. All procedures involving animals were carried out in strict accordance with the international standards of animal care guidelines (guide for the care and use of laboratory animals) and were approved by the institutional animal care and use committee of Fourth Military Medical University (Permit Number:12004).

Collection of blood. At the end of the study, eight animals from each group were decapitated. Blood samples were collected into heparinized syringes and aliquots were taken for the determination of blood Pb concentration by graphite furnace atomic absorption spectroscopy.

Flow cytometric analysis (FACS). Thymic, splenic, and lymph node cells were depleted of erythrocytes by hypotonic lysis, and were stained using predetermined optimal concentrations of different fluorochrome-labeled antibodies for 30 min at 4 °C. Cell phenotypes were determined using the following antibodies: anti-rat CD4-PE, anti-rat CD8α-FITC, and anti-rat CD25-APC. All antibodies were purchased from eBioscience (San Diego, CA). Intracellular Foxp3 staining was carried-out with kit from eBiosciences.

All samples were acquired and analyzed with a FACSaria flow cytometer (Becton Dickinson, San Jose, CA), and data were analyzed with the FCS Express 4 software (De Novo software).

Induction of DTH response. Delayed type hypersensitivity assays were induced using 2,4-dinitrofluorobenzene (DNFB) as described previously (Garrigues and Nicaud, 1994). Briefly, 300 μL of 0.5% DNFB diluted with acetone/olive oil was painted onto the shaved abdomen of the rats and another 30 μL was applied to each footpad on days 0 and 1. On day 5, the rats were repainted with 100 μL of 0.3% DNFB on both sides of the right ear. As a control, the left ear was painted with 100 μL of acetone/olive oil only. Ear thickness is recorded prior to and 24 h after antigen challenge. The control right ear was less than 0.4 mm for each treatment group.

Cytokine ELISA. IL-10 and IFN-γ concentrations in rat serum were determined using a sandwich ELISA kit from R&D systems following the manufacturer's instructions.

T cell purification. For adoptive transfer and in vitro suppression assays, CD4⁺CD25⁺ and CD4⁺CD25[−] T cells were isolated from rats by using the MagCelect Rat CD4⁺CD25⁺ Regulatory T Cell Isolation Kit (R&D systems, Minneapolis, MN) according to the manufacturer's instructions. Cells were checked for purity by flow cytometry.

Approximately 82% of CD4⁺CD25⁺ T cells stained positive for Foxp3, whereas purified CD4⁺CD25[−] cells contained <1% Foxp3⁺ cells.

Treg cell suppression assay. CD4⁺CD25⁺ regulatory T cells were isolated from spleens as described above. CD4⁺CD25[−] cells isolated from untreated rats were labeled by incubation with 5 μM CFSE (Molecular Probes, Eugene, OR) for 20 min at 37 °C, and used as responder T (Tresp) cells. Tresp cells were co-cultured with Tregs at ratios as indicated on a 96-well plate in the presence of 1 μg/mL anti-CD3 and 5 × 10⁵ irradiated splenocytes from untreated rats. Proliferation of CD4⁺CD25[−] cells was assessed after 72 h by CFSE dilution.

Adoptive T cell transfers. Purified populations of CD4⁺ and CD4⁺CD25[−] T cells were prepared as described using magnetic sorting. For the analysis of the effects of Tregs in DTH experiments, 2 × 10⁷ splenic CD4⁺ and CD4⁺CD25[−] T cells from the control and Pb-exposed rats were adoptively transferred into recipient rats via the tail vein 6 days before induction of DTH.

Histological analysis. Ears were fixed with 10% formalin buffered with 0.01 M phosphate buffer (pH 7.2) and embedded in paraffin. The fixed ears were sliced into 3 μm sections and stained with hematoxylin and eosin for histological examination.

Statistical analysis. All values are expressed as means ± S.D. The data were analyzed by ANOVA followed by Dunnett's *t*-test with statistical significance set at *p* < 0.05.

Results

Blood Pb level was increased following Pb exposure

Blood Pb concentrations were increased in proportion to the exposure level of Pb in the drinking water, and the variance in the blood Pb concentration within an exposure group was found to be remarkably low. Chronic exposure of rats to Pb in drinking water under the current dose regimen resulted in a 4.2-fold increase in blood Pb (BPb) as compared to those in the control rats (*p* < 0.05) (Fig. 1A). Intoxication with these concentrations of Pb did not produce overt signs of toxicity, in that, Pb-exposed rats did not show different body weight gain as compared to the controls (Fig. 1B).

Pb exposure impaired CD4⁺ thymic T cell development

Pb was reported to affect various populations of immune cells. To further study the potential effect of Pb exposure on T cell development, we examined the thymic cellularity post Pb-exposure. The rats developed normally and thymus sizes were normal following Pb exposure (data not shown). Total thymocyte numbers were normal, and there was no difference in the number of total thymic cells between control and Pb-exposed groups (Fig. 2B). Cell numbers of CD4[−]CD8[−] (DN) and CD4⁺CD8⁺ (DP) thymocytes remained unchanged as well. But Pb exposure resulted in 25% reduction of thymic CD4⁺CD8[−] cells in comparison to those in the controls. In contrast, thymic CD4[−]CD8⁺ cells were slightly increased in Pb-exposed rats (Figs. 2A and B).

Peripheral T lymphocytes were reduced following Pb exposure

Next, we determined the effect of Pb exposure on peripheral T lymphocytes. The cellular composition of secondary lymphoid organs (spleen and lymph nodes) was quantified, and we found significant shifts in T cell numbers between the control group and Pb-exposed group. The absolute number of CD3⁺ cells was reduced by 23% in spleen and by 29% in lymph nodes (Fig. 3A). Then we analyzed the change of different subsets of T lymphocytes in Pb-exposed rats. As shown in Figs. 3B and C, the absolute number and percentage of CD4⁺ T cells

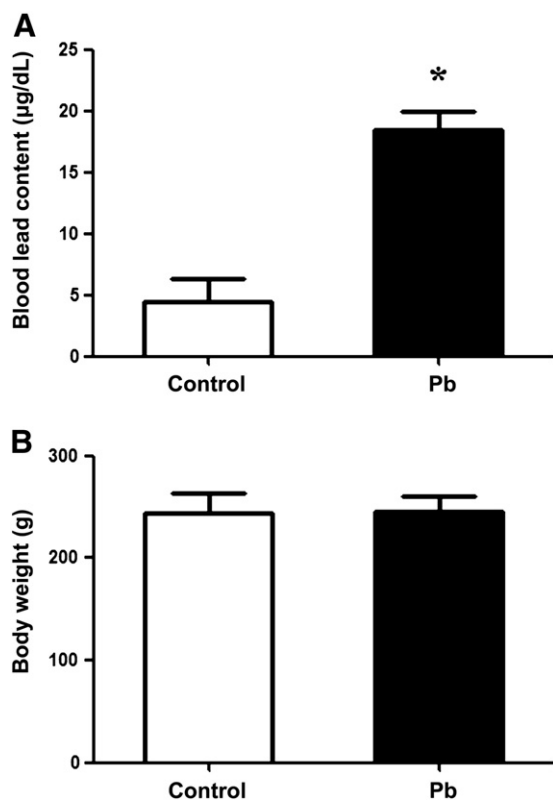


Fig. 1. Pb concentrations in the blood (A) and body weight (B) following Pb exposure. Weanling male Sprague–Dawley rats were exposed to Pb as Pb acetate in drinking water (300 ppm) daily for 6 weeks. Data represent mean $\pm$ SD ($n=8$) from three experiments performed, *: $p<0.05$ compared to controls.

were significantly reduced in the Pb-exposed rats as compared to the controls, while the number and percentage of CD8⁺ cells in Pb-exposed rats were unchanged.

Pb exposure increases thymic and peripheral Treg cells in rats

Treg is a T cell subset essential for keeping T cell in tolerance. To examine the potential effect of Pb-exposure on Tregs, we determined Tregs in Pb-exposed rats. As shown in Fig. 4A, the frequency and number of thymic and peripheral Tregs were increased. The number of thymic Tregs in Pb-exposed rats was approximately 1.59-fold of those in the controls. Splenic and lymph node Tregs were increased by 1.42 and 1.47-fold, respectively (Fig. 4B).

Increased Tregs played a critical role in Pb-exposure-induced immune suppression

Pb-exposure leads to immune suppression in vivo. Given the strong immune-suppressive activity of Tregs, we reasoned that the increased Tregs may cause the immune suppression in Pb-exposed rats. To test this hypothesis, we used the DTH, a T cell-mediated response model. DTH responsiveness was determined by measuring the thickness of swollen ears before and 24 h after DNFB challenge. Consistent to previous studies, Pb exposure caused a significant reduction of DTH response. The ear thickness of rats intoxicated with 300 ppm Pb was increased 0.22 mm (55% increase), while the ear thickness of the controls was increased 0.62 mm (157% increase) (Fig. 5B). The attenuation of the immune response against DNFB was confirmed by histological analysis. Dermal edema, infiltration of mononuclear cells, and vascular enlargement were observed in samples from control rats, while these characteristic features, including infiltration of mononuclear cells into the lesion, were attenuated in samples of Pb-exposed rats (Fig. 5A).

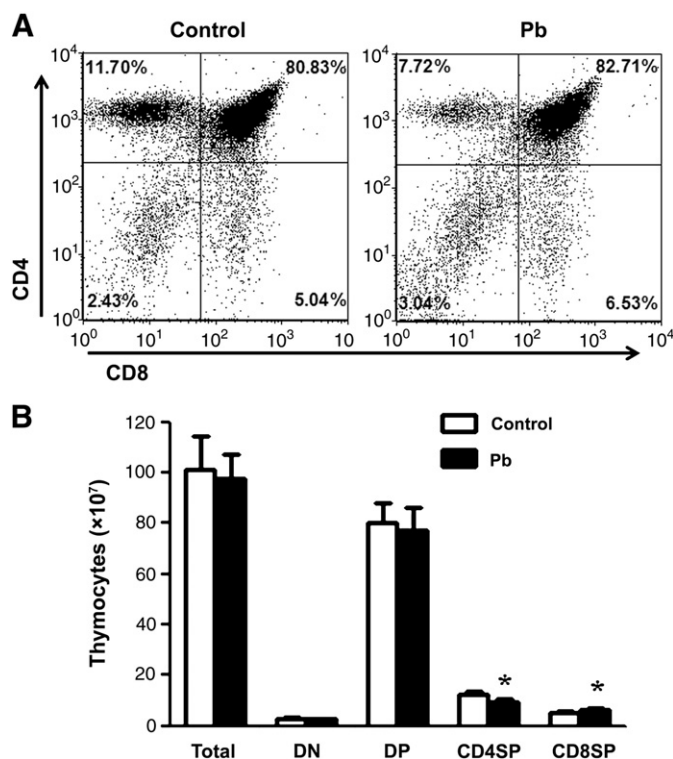


Fig. 2. The effect of Pb exposure on thymocyte development. (A). CD4 versus CD8 flow cytometric analysis of Pb-exposed rat thymocytes and the controls. Numbers indicate the percentages of the corresponding T cell populations. (B). The absolute cell numbers of DN, DP, SP (CD4⁺CD8⁻ and CD4⁻CD8⁺) thymocytes. Data represent mean $\pm$ SD ($n=8$) from three experiments performed, *: $p<0.05$ compared to controls.

To examine the potential role of the increased Tregs in Pb-exposure-induced immune suppression of DTH response, we used a T cell transfer model. Total CD4⁺ T cells or CD25⁺ Tregs-depleted CD4⁺ T cells were isolated from Pb-treated and control rats by using the MagSelect Rat CD4⁺CD25⁺ Regulatory T Cell Isolation Kit, and then adoptively transferred into unexposed recipient rats. The recipient rats were challenged with DNFB to induce DTH response. Consistently, rats receiving total CD4⁺ T cells isolated from Pb-exposed rats showed a lower DTH response as compared to those receiving total CD4⁺ T cells from control rats (Figs. 6A and B). The transferable suppression of Pb-exposed CD4⁺ T cells was diminished after depletion of CD25⁺ Tregs, suggesting that the increased Tregs played a critical role in Pb exposure-induced immune suppression.

In addition to the amount of Tregs, Pb-exposure may also affect suppressive function of Tregs. We examined this possibility by using an in vitro Treg suppression assay. CD4⁺CD25⁺ T cells were isolated from the control and Pb-exposed rats, and then co-cultured with CFSE-labeled CD4⁺CD25⁻ responder T cells isolated from untreated rats. As shown in Fig. 6C, Pb-exposure did not affect the suppressive function of Tregs. These data suggest that Pb-exposure increased the quantity but not the quality of Tregs that leads to the immune suppression.

Discussion

Pb is a common environmental pollutant which has been reported to affect many organ systems, including the central nervous system, hematological system, and reproductive system. The immune system is also recognized as a target of Pb (Mishra, 2009). Many studies have addressed that Pb can affect various populations of immune cells (Heo et al., 1998; Lawrence, 1981; McCabe and Lawrence, 1990; Queiroz et al., 1993; Yucesoy et al., 1997).

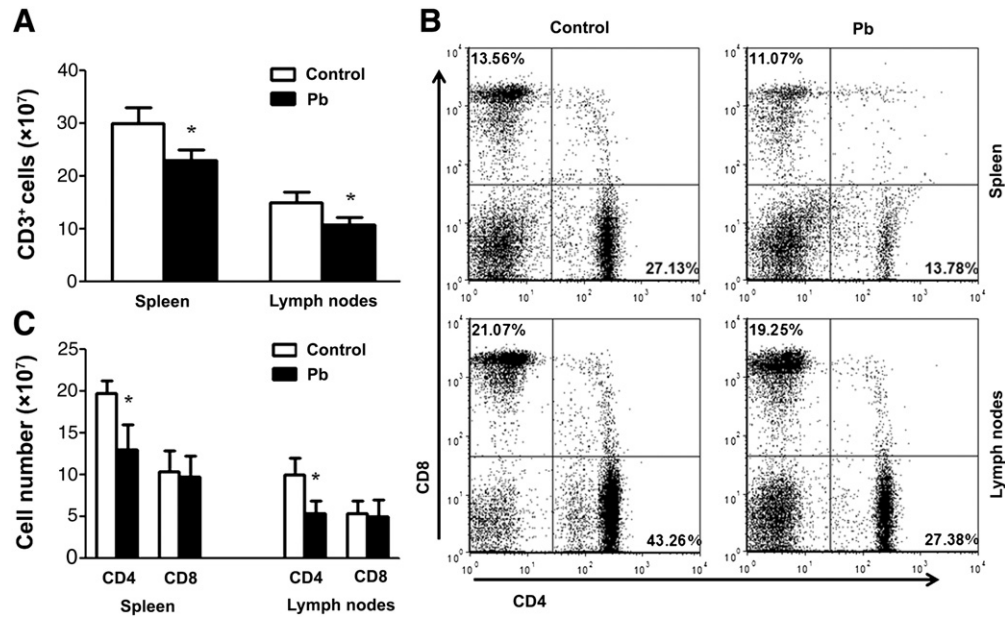


Fig. 3. Pb exposure reduces peripheral CD4⁺ T cells in rats. (A). The absolute cell numbers of peripheral T cells in the Pb-exposed rats and the controls. (B). CD4 versus CD8 flow cytometric analysis of Pb-exposed splenic, lymph node cells and the controls. (C). The absolute cell numbers of peripheral CD4⁺ and CD8⁺ T cells. Data represent mean ± SD (*n* = 8) from three experiments performed, *: *p* < 0.05 compared to controls.

T cells are believed to be critical functional targets of Pb immunotoxicity (Fischbein et al., 1993; McCabe et al., 2001; McCabe and Lawrence, 1991; Mishra et al., 1998; Neilan et al., 1980; Sata et al., 1998), and it is of importance to ascertain the biological effect of Pb on the T cells. A functional diverse T cell repertoire is generated in the thymus and maintained in the periphery. In this study, we found that exposure to Pb in drinking water impaired thymocyte development. The percentage of CD4⁺ single positive (SP) thymocytes was significantly lower in the Pb-exposed rats as compared to that in the controls, while there was no notable difference in the cell numbers of total thymocytes. In periphery, CD4⁺ but not CD8⁺ T cells were significantly reduced in Pb-exposed rats. These data suggest that CD4⁺ SP thymocytes and CD4⁺ T cells in the periphery may be targets of Pb toxicity. Interestingly, Pb exposure was previously reported to enhance T cell proliferation in mixed lymphocyte culture experiments. But nonetheless, Pb exposure did not affect T cell proliferation induced by mitogens or superantigen (Fischbein et al., 1993; McCabe et al., 2001; McCabe and Lawrence, 1991; Mishra et al., 1998).

It is unclear whether the reduction of CD4⁺ T cells in Pb-exposed rats is due to a decrease of cell proliferation.

The concentration of Pb in drinking water used for this study is 300 ppm. Similar high dose of Pb has been shown to induce immunotoxicity in previous studies (McCabe et al., 1999; Miller et al., 1998). Notably, control rats in this study had blood Pb (BPb) just under 5 µg/dL, and this would be considered as a level of concern for neurodevelopmental endpoints for children in the United States (Jusko et al., 2008). The low-level blood Pb (BPb) in control animals may be due to the contamination of rat chow or metal cages. For future experiments, purified diet and high quality stainless-steel cages should be used to reduce the unintended source of Pb exposure to control animals.

The DTH reaction, a T cell mediated immune response, has been shown to negatively correlate with Pb exposure. Müller et al. reported a dose-dependent, negative correlation between blood Pb levels and DTH reactivity to SRBC challenge in BALB/c mice (Müller et al., 1977).

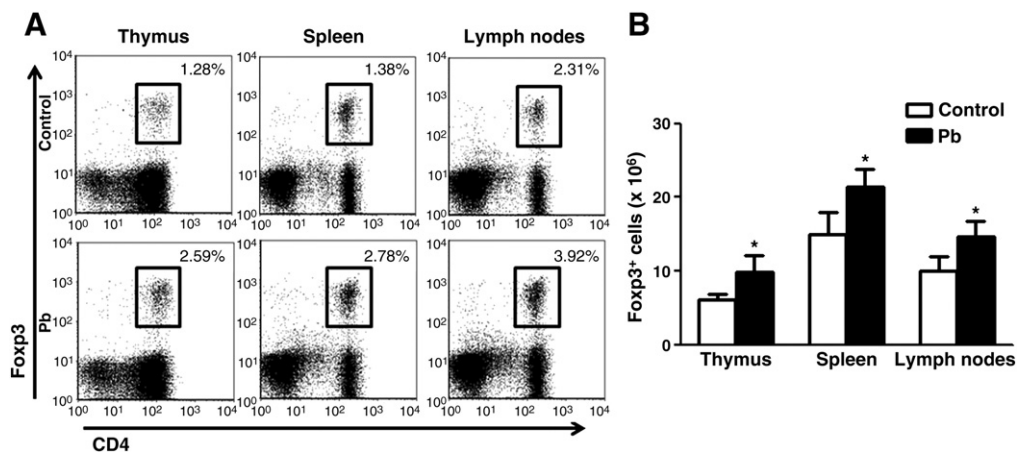


Fig. 4. The effect of Pb exposure on Tregs in rats. (A). Foxp3 expression in CD4⁺ T cells of the thymus, spleen and lymph nodes was determined by FACS. (B). The number of Foxp3⁺ Tregs was shown. Data represent mean ± SD (*n* = 8) from three experiments performed, *: *p* < 0.05 compared to controls.

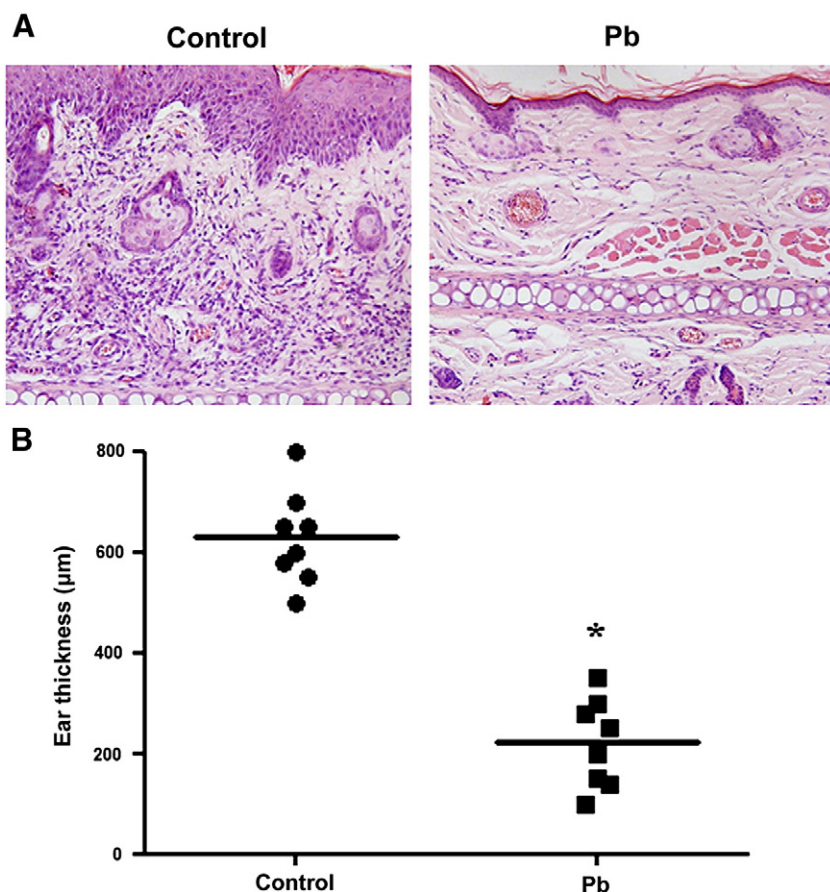


Fig. 5. Intoxication with Pb impairs the generation of a robust DTH response. (A). Histopathological characteristics of the swollen ears. At 24 h after painting with DNFB. Sections of the swollen ears were stained with hematoxylin and eosin (magnification: $\times 200$). The characteristic features of inflammation, such as infiltration of mononuclear cells, dermal edema and vascular enlargement, were observed in the sections of control rats, whereas these features were markedly attenuated in samples from Pb-exposed rats. (B). Ear swelling in Pb-exposed and control rats. The rats were sensitized by DNFB on day 0, 1 and challenged on day 5. Data are displayed as scatter plots of the ear thickness difference of 8 individual control and 8 individual Pb-exposed rats prior to and 24 h after challenged with DNFB from three experiments performed. *: $p < 0.05$ compared to controls.

In another study, Faith et al. reported that exposure to Pb resulted in a significant reduction in DTH reactivity to purified protein derivative (PPD) of tuberculin in the offspring of Pb-exposed dams (Faith et al., 1979). A recent study has been reported that a persistent decrease in the DTH reactivity of the offspring from Pb-exposed dams was observed (Miller et al., 1998). McCabe et al. have reported that Pb intoxication suppresses DTH reactivity when antigen sensitization occurs via an intravenous route of antigen administration (McCabe et al., 1999). The cellular mechanism of Pb exposure-impaired DTH response has not been well established, and there are few advanced studies in this field in recent years. Potential mechanisms include: (1) direct inhibition of antigen-specific T cell activation through the Pb-induced elevation of cAMP-dependent signaling pathway (Heo et al., 1998), (2) inability of activated T cells to activate macrophages (Lawrence, 1995), and (3) indirect inhibition of helper T cells mediated by an impairment of antigen presenting cells (McCabe et al., 1999).

Tregs are one of the T cell subsets that have strong immune-suppressive activity. Recently, it has been reported that the Treg-mediated immunosuppression plays very important roles in DTH (Gorbachev and Fairchild, 2001; Honda et al.,). It has long been established that Pb enhances rather than inhibits suppressor cell activity (Cohen et al., 1989). McCabe et al. also found no effect of Pb exposure on the generation of suppressor activity (McCabe et al., 1999). In this study, we observed that thymic and peripheral Tregs were increased in Pb-exposed rats, although conventional $CD4^+$ T cells were decreased. Tregs can be naturally generated in the thymus as a functionally mature subpopulation of T cells (nTreg) and be induced from naive T cells in the

periphery (iTreg). Currently, it remains unclear how Pb exposure increases Treg number. It will be an interesting topic for future study.

Tregs could modify DTH at multiple levels. There is a model of Treg-mediated suppression: (1) upon antigenic stimulation, antigen-specific Tregs are swiftly recruited via chemokines to dendritic cells presenting the antigen and out-compete antigen-specific naive T cells in aggregating around the dendritic cells; (2) antigen-activated Tregs contacting dendritic cells then downmodulate dendritic cell function, thereby hindering the activation of other T cells that are recruited to the dendritic cells; and (3) Tregs may then further differentiate to secrete some immunosuppressive cytokines (such as IL-10) (Sakaguchi et al., 2008). In this study, we found that Pb-exposure increased Treg cell number and frequency in the thymus and peripheral lymphoid organs. However, we did not find any changes of Treg-derived immunosuppressive cytokines (such as IL-10) in the Pb-exposed rat blood (data not shown). The in vitro suppressive function assay indicated that Pb exposure did not affect the function of regulatory T cells. In our view, the most rational possibility would be the increased Tregs suppress effector T cells in the lymph nodes (LN) and inhibit leukocyte influx into the periphery. In addition, Pb has been reported to impair DTH through its effect on Th1- and Th2-derived cytokines (Mihara et al., 1991). DTH is characterized as a local inflammatory reaction as a result of a large influx of non-specific inflammatory cells in response to T-cell-derived cytokines. It is also possible that Pb suppresses the local inflammatory response and monocytic infiltration characteristics of the afferent phase of the DTH response through inhibiting IFN- γ production. In our study, we observed similar reduction of interferon- γ in

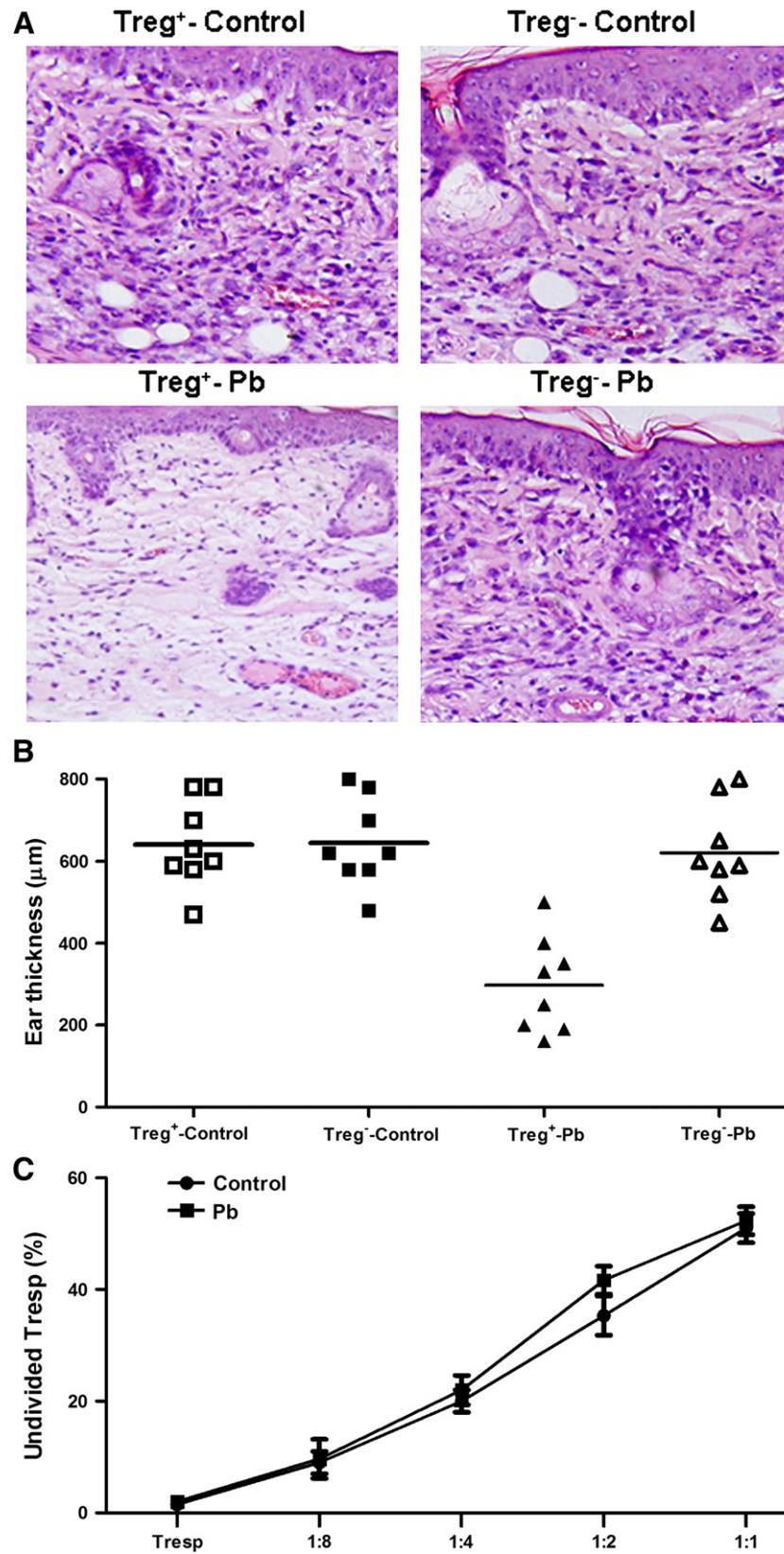


Fig. 6. The effect of increased Treg cell number on suppression of DTH in Pb-exposed rats. (A). Histopathological characteristics of the swollen ears. (B). Ear swelling in unexposed recipient rats adoptively transferred with total CD4⁺ T cells (Treg⁺) or with CD4⁺CD25⁺ Tregs-depleted CD4⁺ T cells (Treg⁻) from the control and Pb-exposed rats. Data are displayed as scatter plots calculated by comparing the thickness of the challenged ear at 24 and 0 h. (C). CD4⁺CD25⁺ Tregs from spleens were isolated from the control and Pb-exposed rats. CD4⁺CD25⁻ T cells from untreated rats were labeled with CFSE, and used as responding T cells (Tresp). Cell division was assessed by CFSE dilution. The percentages of undivided Tresp cells (Y axis) and the ratios of cell numbers between Treg and Tresp cells (X axis) were plotted. These are representative of three independent experiments. *: $p < 0.05$ compared to controls.

Pb-exposed rats (data not shown). However, our transfer experiment showed that Pb-induced DTH suppression can be transferred by adoptive transfer of T cells isolated from Pb-exposed rats into suitable recipients, and the transferred suppression was eliminated after removal of Tregs. These results strongly suggest that increased Tregs play an essential role in mediating Pb exposure-induced DTH suppression.

In conclusion, we show that Pb exposure could increase Tregs, which preceded suppression of the DTH response. These findings support the hypothesis that Treg may play a role in the immunomodulatory role of toxic heavy metal Pb. It should be noted that this study examined only the relationship between Tregs and suppression of the DTH response. Further studies are required to elucidate the underlying molecular mechanisms associated with Pb immunotoxicity.

Conflict of interest

There is no financial or any other interest with regard to this paper which might be construed as a conflict of interest.

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Abbreviations

DN	double negative
DP	double positive
DTH	delayed type hypersensitivity
LN	lymph node
PPD	purified protein derivative
SP	single positive
Treg	regulatory T cell

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References

- Cohen, N., Modai, D., Golik, A., Weissgarten, J., Peller, S., Katz, A., Averbukh, Z., Shaked, U., 1989. Increased concanavalin A-induced suppressor cell activity in humans with occupational lead exposure. *Environ. Res.* 48, 1–6.
- Faith, R.E., Luster, M.I., Kimmel, C.A., 1979. Effect of chronic developmental lead exposure on cell-mediated immune functions. *Clin. Exp. Immunol.* 35, 413–420.
- Fischbein, A., Tsang, P., Luo, J.C., Roboz, J.P., Jiang, J.D., Bekesi, J.G., 1993. Phenotypic aberrations of CD3+ and CD4+ cells and functional impairments of lymphocytes at

- low-level occupational exposure to lead. *Clin. Immunol. Immunopathol.* 66, 163–168.
- Garrigues, J.M., Nicaud, P., 1994. Solitary ulcer of the rectum. *Ann. Chir.* 48, 140–149 (discussion 149–150).
- Gorbachev, A.V., Fairchild, R.L., 2001. Regulatory role of CD4+ T cells during the development of contact hypersensitivity responses. *Immunol. Res.* 24, 69–77.
- Heo, Y., Parsons, P.J., Lawrence, D.A., 1996. Lead differentially modifies cytokine production in vitro and in vivo. *Toxicol. Appl. Pharmacol.* 138, 149–157.
- Heo, Y., Lee, W.T., Lawrence, D.A., 1997. In vivo the environmental pollutants lead and mercury induce oligoclonal T cell responses skewed toward type-2 reactivities. *Cell. Immunol.* 179, 185–195.
- Heo, Y., Lee, W.T., Lawrence, D.A., 1998. Differential effects of lead and cAMP on development and activities of Th1- and Th2-lymphocytes. *Toxicol. Sci.* 43, 172–185.
- Honda, T., Miyachi, Y., Kabashima, K., 2010. The role of regulatory T cells in contact hypersensitivity. Recent patents on inflammation & allergy drug discovery 4, 85–89.
- Hori, S., Nomura, T., Sakaguchi, S., 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299, 1057–1061 (New York, N.Y.).
- Jusko, T.A., Henderson, C.R., Lanphear, B.P., Cory-Slechta, D.A., Parsons, P.J., Canfield, R.L., 2008. Blood lead concentrations <10 microg/dL and child intelligence at 6 years of age. *Environ. Heal. Perspect.* 116, 243–248.
- Landrigan, P.J., Boffetta, P., Apostoli, P., 2000. The reproductive toxicity and carcinogenicity of lead: a critical review. *Am. J. Ind. Med.* 38, 231–243.
- Lawrence, D.A., 1981. In vivo and in vitro effects of lead on humoral and cell-mediated immunity. *Infect. Immun.* 31, 136–143.
- Lawrence, D.A., 1995. Posited mechanisms of metal immunotoxicity. *Hum. Exp. Toxicol.* 14, 114–116.
- McCabe Jr., M.J., Lawrence, D.A., 1990. The heavy metal lead exhibits B cell-stimulatory factor activity by enhancing B cell Ia expression and differentiation. *J. Immunol.* 145, 671–677.
- McCabe Jr., M.J., Lawrence, D.A., 1991. Lead, a major environmental pollutant, is immunomodulatory by its differential effects on CD4+ T cells subsets. *Toxicol. Appl. Pharmacol.* 111, 13–23.
- McCabe Jr., M.J., Singh, K.P., Reiners Jr., J.J., 1999. Lead intoxication impairs the generation of a delayed type hypersensitivity response. *Toxicology* 139, 255–264.
- McCabe Jr., M.J., Singh, K.P., Reiners Jr., J.J., 2001. Low level lead exposure in vitro stimulates the proliferation and expansion of alloantigen-reactive CD4(high) T cells. *Toxicol. Appl. Pharmacol.* 177, 219–231.
- Mihara, M., Ikuta, M., Koishihara, Y., Ohsugi, Y., 1991. Interleukin 6 inhibits delayed-type hypersensitivity and the development of adjuvant arthritis. *Eur. J. Immunol.* 21, 2327–2331.
- Miller, T.E., Golemboski, K.A., Ha, R.S., Bunn, T., Sanders, F.S., Dietert, R.R., 1998. Developmental exposure to lead causes persistent immunotoxicity in Fischer 344 rats. *Toxicol. Sci.* 42, 129–135.
- Mishra, K.P., 2009. Lead exposure and its impact on immune system: a review. *Toxicol. In Vitro* 23, 969–972.
- Mishra, K.P., Rani, R., Yadav, V.S., Naik, S., 2010. Effect of lead exposure on lymphocyte subsets and activation markers. *Immunopharmacology and immunotoxicology* 32, 446–449.
- Muller, S., Gillert, K.E., Krause, C., Gross, U., Age-Stehr, J.L., Diamantstein, T., 1977. Suppression of delayed type hypersensitivity of mice by lead. *Experientia* 33, 667–668.
- Neilan, B.A., Taddeini, L., McJilton, C.E., Handwerker, B.S., 1980. Decreased T cell function in mice exposed to chronic, low levels of lead. *Clin. Exp. Immunol.* 39, 746–749.
- Queiroz, M.L., Almeida, M., Gallao, M.I., Hoehr, N.F., 1993. Defective neutrophil function in workers occupationally exposed to lead. *Pharmacol. Toxicol.* 72, 73–77.
- Rosen, J.F., 1995. Adverse health effects of lead at low exposure levels: trends in the management of childhood lead poisoning. *Toxicology* 97, 11–17.
- Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., Toda, M., 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 155, 1151–1164.
- Sakaguchi, S., Yamaguchi, T., Nomura, T., Ono, M., 2008. Regulatory T cells and immune tolerance. *Cell* 133, 775–787.
- Sata, F., Araki, S., Tanigawa, T., Morita, Y., Sakurai, S., Nakata, A., Katsuno, N., 1998. Changes in T cell subpopulations in lead workers. *Environ. Res.* 76, 61–64.
- Warner, G.L., Lawrence, D.A., 1986. Cell surface and cell cycle analysis of metal-induced murine T cell proliferation. *Eur. J. Immunol.* 16, 1337–1342.
- Yucesoy, B., Turhan, A., Ure, M., Imir, T., Karakaya, A., 1997. Effects of occupational lead and cadmium exposure on some immunoregulatory cytokine levels in man. *Toxicology* 123, 143–147.