



Decreased erythrocyte CD44 and CD58 expression link e-waste Pb toxicity to changes in erythrocyte immunity in preschool children

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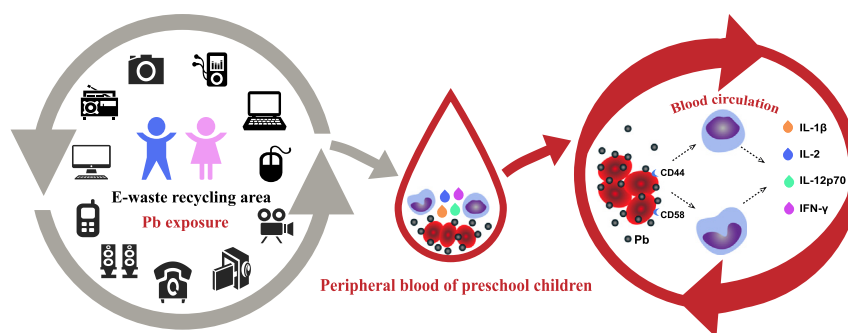
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HIGHLIGHTS

- Blood and erythrocyte Pb levels are higher in e-waste-exposed children.
- Erythrocyte CD44 and CD58 are significantly lower in e-waste-exposed children.
- Elevated erythrocyte Pb levels adversely affect CD44 and CD58 in all children.
- The association between Pb exposure and cytokines is mediated by CD44 and CD58.

GRAPHICAL ABSTRACT



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ABSTRACT

Lead (Pb) toxicity damages blood cells and disturbs the immune micro-environment. When Pb enters the circulatory system, >95% of Pb accumulates in erythrocytes. We therefore conducted this study to explore the long-term effect of Pb exposure on expression of erythrocyte adhesion molecules (CD44 and CD58) and related downstream cytokine concentrations. We enrolled a total of 267 preschool children, 2–7 years of age, from Guiyu (e-waste-exposed group, $n = 132$) and Haojiang (reference group, $n = 135$) in November and December 2015. We measured child blood Pb, biomarkers including erythrocyte CD44 and CD58, erythrocyte count, leukocyte count and inflammatory cytokines (IL-1 β , IL-12p70 and IFN- γ), and calculated erythrocyte Pb levels. Regression model demonstrated that higher erythrocyte Pb was associated with lower CD44 and CD58. Compared to low erythrocyte Pb levels (quartile 1), high erythrocyte Pb levels (quartile 4) were related to lower levels of erythrocyte CD44 and CD58. Elevated blood Pb correlated with higher IL-12p70 and IFN- γ , and lower IL-2. The mediation effect of erythrocyte CD44 on the relationship of erythrocyte Pb with IL-1 β and IL-12p70 was significant, and the effect of erythrocyte Pb on IFN- γ was mediated by erythrocyte CD58. The data provides novel translational insight into the relationship between elevated Pb exposure and the change of erythrocyte immunity and downstream cytokine secretion in preschool children.

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1. Introduction

The end-of-life electrical and electronic equipment, including computers, mobile phones, air conditioners, television sets and others, are regarded as electronic waste (e-waste). Recovery of precious metals, such as copper, aluminum and gold, make e-waste recycling profitable, thus promoting e-waste recycling development. It is like a Pandora's box and brings serious environmental and social problems on account of the potential environmental damage and health problems (Bakhiyi et al., 2018). Large amounts of heavy metals and persistent organic pollutants are discharged from e-waste by primitive dismantling or recycling processes, then released into the surrounding environment, threatening the health of unprotected employees and the local population (Grant et al., 2013; Wang et al., 2018). Surveying conducted in Guiyu, one of the most heavily lead (Pb)-polluted areas due to informal e-waste recycling activities, demonstrated high mean Pb concentrations in workshop dust (110,000 mg/kg) and adjacent roads (22,600 mg/kg), as well as in road side soil (540.9 mg/kg) and plants (18.7 mg/kg) (Alabi et al., 2012; Huo et al., 2007; Leung et al., 2008). Individuals can become exposed to hazardous materials of e-waste in dust, air, water, soil and food sources through routes, including dermal, ingestion and inhalation absorption (Xu et al., 2015). Since 2011, hazardous pollutants have decreased in Guiyu due to changes in e-waste disassembly methods in family workshops to centralized recycling. However, Pb exposure has remained high compared with other areas. Our research team has been conducting a long-term study in Guiyu and observed that 61.0%, 58.0%, and 57.5% of local children had blood Pb levels exceeding the limit (5 µg/dL) recommended by U.S. Center for Disease Control and Prevention (CDC) in 2012, 2013 and 2014, respectively (Betts, 2012; Zeng et al., 2017; Z. Zeng et al., 2018; Zhang et al., 2016).

Pb accumulates in the human body over time and can damage multiple systems, especially the hematologic, immune, respiratory and nervous systems, through affecting gene expression, membrane ion channels, signaling molecules, enzyme activity and neurogenesis (Grant et al., 2013; Zeng et al., 2016; X. Zeng et al., 2018; Z. Zeng et al., 2018). In preliminary work, we found that elevated Pb exposure was associated with adverse effects on blood morphology and hemoglobin synthesis (Dai et al., 2017; Liu et al., 2015). Another population study has suggested that blood Pb can play a role in the etiology of anemia (Henríquez-Hernández et al., 2017). In addition, our previous studies also observed that elevated Pb levels are correlated with lower erythrocyte CR1 expression, percentages of monocytes and natural killer (NK) cells, and counts of basophils and eosinophils, as well as altered concentrations of interleukin (IL)-1 β , IL-6 and IL-27, which might collectively comprise a tremendous threat to the adaptive and innate immune response in preschool children (Dai et al., 2017; Lu et al., 2018; Zhang et al., 2016; Zhang et al., 2017). Therefore, the negative effects of Pb exposure on erythrocytes and cytokines are well-established. However, the underlying mechanisms and their relationships are only partially understood.

Erythrocytes comprise approximately 70% of the total cell number of the human body and are the most common type of blood cells. In the past, erythrocytes were thought to only transport oxygen and carbon dioxide, and their role in immune function was neglected. Nishioka and Linscott (1963) discovered that cluster of differentiation (CD) 35 is responsible for erythrocyte immune adhesion. Subsequently, Siegel et al. proposed the concept of the erythrocyte immune system, after which an increasing number of erythrocyte adhesion molecules have been discovered, including CD44 and CD58 (Bianconi et al., 2013; Siegel et al., 1981). CD44 belongs to a cell-surface glycoprotein family that takes part in cell adhesion, migration, and cell-cell interactions, and has been suggested to be a lymphocyte homing receptor in humans (Hale et al., 1989). It participates in various cellular functions, including lymphocyte activation, recirculation and homing, hematopoiesis, and tumor metastasis (Bosco et al., 2011). Tumor cells transported in the blood circulation may be bound to CD44 on the surface of leukocytes

and erythrocytes, through hyaluronan expressed on tumor cells, and then taken to the endodermal reticular system for interception and clearance (Hosono et al., 2011). Therefore, erythrocyte CD44 and tumor cell surface hyaluronan may play a key role in erythrocyte adhesion to tumor cells and consequent development of inflammation and tumor metastasis. CD58, also known as lymphocyte function-associated antigen-3 (LFA-3), is the natural ligand of the lymphocyte receptor CD2, which together forms the main costimulatory pathway for CD28⁺CD8⁺ T cells in humans (Leitner et al., 2015; Yu et al., 2014). In particular, CD58 interacts with CD2 on the lymphocyte membrane to induce T lymphocytes to secrete cytokines, indirectly promoting T cell proliferation and differentiation (Antunes et al., 2011; Shivam et al., 2013). As a result, the expression of erythrocyte CD44 and CD58 is closely related to tumor metastasis and immune function.

In prior research, we found an association between elevated Pb exposure and low expression of erythrocyte CR1 in preschool children. We therefore explored the changes in erythrocyte surface expression of CD44 and CD58, as well as related cytokines, in response to Pb exposure, to offer a deeper knowledge of Pb toxicity on erythrocyte immunity in preschool children.

2. Materials and methods

2.1. Study participants

A cross-sectional study using stratified sampling was conducted from November to December 2015 in Guiyu and Haojiang, two towns located in Shantou, China. The reference area, Haojiang, is similar to Guiyu in population, socioeconomic status, residential lifestyle and cultural background, but lacks e-waste pollution. All participants were healthy preschool children with no history of hematological, autoimmune or infectious disease within one month before sample collection. We enrolled a total of 267 preschool children, 2–7 years of age, from Guiyu (e-waste-exposed group, $n = 132$) and Haojiang (reference group, $n = 135$). We obtained signed informed consent from the children parents or guardians, who also completed a questionnaire about the general characteristics of the parents and children, including their child's medical and health history, physical activity, nutrition intake, dieting habits, and residential environment, as well as parent education level and occupation, and family socioeconomic status. Our study protocol was approved by the Human Ethics Committee of Shantou University Medical College, China (SUMC2013XM-0076).

2.2. Blood sample preparation

Well-trained nurses collected 6 mL peripheral blood from each participant and stored the sample in three Pb-free tubes either containing sodium citrate or EDTA as an anticoagulant, or without anticoagulant. All blood samples were placed on ice and transported to the laboratory. The sodium citrate tube was centrifuged (200g for 5 min) to separate erythrocytes and then used to quantify erythrocyte CD44 and CD58. The blood sample in the EDTA tube was used for blood routine examination, and then stored at -20°C until measured for blood Pb. The blood sample without anticoagulant was centrifuged (855g for 10 min) to separate the serum, which was stored at -80°C until cytokine assay.

2.3. Determination of blood Pb level

For measurement of blood Pb levels, 900 µL 0.5% nitric acid (analytical reagent) and 100 µL blood sample were mixed by vortexing, followed by digestion for 10 min at room temperature. In line with our previous detection methods, we analyzed blood Pb using graphite furnace atomic absorption spectrophotometry (Jena Zeenit 650, Germany) (Liu et al., 2011; Yang et al., 2013). The accuracy of the method was confirmed by obtaining recoveries of between 95% and 107% from spiked blood samples, and the limit of detection (LOD) of

this method was determined to be 0.51 g/L (0.051 $\mu\text{g}/\text{dL}$). Using the hematocrit (HCT) as the fraction of whole blood, we then calculated erythrocyte Pb concentration using the following formula: erythrocyte Pb concentration ($\mu\text{g}/\text{dL}$) = blood Pb concentration ($\mu\text{g}/\text{dL}$)/HCT, as previously suggested by deSilva (1981).

2.4. Measurement of hematological indices

Hematological indices were measured with a Sysmex XT-1800i automated hematology analyzer (Sysmex Corporation, Kobe, Japan). The counts of erythrocytes and leukocytes, HCT, and ratio of monocytes and lymphocytes were assessed. Sample analysis was performed within 8 h of blood collection, and calibration standards and controls were obtained from the manufacturer.

2.5. Erythrocyte CD44 and CD58 measurements

On the basis of a previously published method, erythrocyte surface expression of CD44 and CD58 was determined (Senbagavalli et al., 2008). Briefly, whole blood (500 μL) was centrifuged (200g for 5 min), then 50 μL of erythrocytes in the sublayer was added to a tube containing 400 μL phosphate-buffered saline (PBS) (Shanghai BioScience Co., Ltd. China), followed by mixing and centrifugation (150g for 5 min). The supernatant fraction was decanted, and erythrocytes were washed twice again as above. After washing, 10 μL of packed erythrocytes was resuspended in 500 μL 2% BSA in PBS, after which 50 μL of resuspended erythrocytes was added to 1 mL 2% BSA in PBS followed by gentle vortexing, giving a diluted erythrocyte concentration of $10^6/\text{mL}$. The appropriate volume (20 μL) of CD44-PE and CD58-FITC monoclonal antibodies (BD Bioscience, USA) was added to 100 μL erythrocyte suspension, followed by incubation in the dark at room temperature for 30 min. Uncombined antibody was removed by washing once in 2% BSA in PBS (1 mL) and centrifuging (150g for 5 min), then cells were resuspended in 2% BSA in PBS (500 μL) for quantitation by flow cytometry using a BD Accuri™ C6 flow cytometer (BD Bioscience, USA). Data were analyzed using BD Accuri™ C6 software (Version 1.0.264.21, BD Bioscience, USA). The flow cytometry figures analyzed are displayed in Fig. S1.

2.6. Serum cytokine measurements

Concentrations of IL-1 β , IL-2, IL-12p70 and interferon (IFN)- γ , were measured using the ProcartaPlex Human Cytokine Chemokine Panel 1A (eBioscience, USA). Beads coated with anti-human IL-1 β , IL-2, IL-12p70 and IFN- γ were incubated with the serum samples and analyzed based on the manufacturer's instructions. We performed data acquisition by using a Luminex MagPix analyzer (Luminex, USA).

2.7. Statistical analysis

The Stata 13.0 statistical software (Stata, College station, TX, USA), Statistical Package for the Social Sciences (SPSS) 24.0 (SPSS Inc., Chicago, IL, USA), PROCESS software packages (Hayes) and GraphPad Prism 5.0 (GraphPad, San Diego, CA) were used to statistically analyze the data. Normal or skewed normally-distributed data are expressed as mean \pm standard deviation (SD) and were analyzed by an independent sample *t*-test. Non-normally distributed data are presented as interquartile ranges (IQRs) and were analyzed by the Mann-Whitney *U* test. Child blood Pb concentrations were stratified by dichotomization at 5 $\mu\text{g}/\text{dL}$ into low blood Pb and high blood Pb groups, in reference to the U.S. CDC threshold value revised in 2012 (Betts, 2012). Pearson's chi-square test was conducted to analyze the categorical data. Spearman correlations were performed to investigate the potential risk factors in Pb exposure and the associations between Pb exposure and immune indices, as well as presented as correlation coefficients (r_s) with *P*-values.

Simultaneously, blood and erythrocyte Pb was log-transformed using the natural logarithm. Considering the other confounding factors, such as catching a cold within a year, washing hands before eating, child contact with e-waste, yearly milk product consumption, smoking by a family member, residence as a workplace, e-waste contamination within 50 m of residence, monthly household income, and paternal and maternal education level, we conducted separate regression models adjusted with these factors by stepwise screening to better explore the relationships between erythrocyte Pb and CD44 or CD58. Erythrocyte Pb levels were categorized into quartiles, we calculated differences in mean values for the 2nd, 3rd and 4th quartiles compared with the 1st quartile. A parallel multiple mediators model was used to estimate the effects of erythrocyte membrane molecules on inflammatory cytokine levels of children exposed to Pb. Antecedent variables (erythrocyte Pb) were modeled as influencing consequent variables (IL-1 β , IL-12p70 and IFN- γ) directly and indirectly through multiple mediators (CD44 and CD58) (Hayes, 2013; Preacher and Hayes, 2008). The covariates including age and gender in the models and the significance of levels of the total, direct and indirect effects was set at 0.05, using a two-tailed test.

3. Results

3.1. Description of participant characteristics

The average age of children in the e-waste-exposed group was older than those from the reference group (Table 1). E-waste-exposed children manifested higher median blood and erythrocyte Pb concentrations compared with the reference group (all $P < 0.001$). >72.0% of the children in the e-waste-exposed group had blood Pb concentrations above 5 $\mu\text{g}/\text{dL}$, compared with 37.0% in the reference group ($P < 0.001$). Furthermore, 12.1% of the children in the e-waste-exposed group had blood Pb concentrations above 10 $\mu\text{g}/\text{dL}$, while no child in the reference group exceeded 10 $\mu\text{g}/\text{dL}$.

All routine blood indicators were within the normal range. Erythrocyte and leukocyte counts, and lymphocyte ratio displayed no significant differences between the two groups ($P > 0.05$). However, compared to the reference group, the e-waste-exposed group had a higher HCT ($P < 0.05$). We found that the child monocyte ratio was elevated in the e-waste-exposed group relative to the reference group ($P < 0.05$). In contrast, the median value of the lymphocyte-to-monocyte ratio (LMR) was 7.707% in the e-waste-exposed group, lower than that in the reference group (8.725%) ($P < 0.05$). Moreover, comparison of the differences of these cell parameters in the lower ($\leq 5 \mu\text{g}/\text{dL}$) and high ($> 5 \mu\text{g}/\text{dL}$) blood Pb preschool children in the reference group showed that LMR was significantly decreased only in the low blood Pb children (57.49%), compared with the high blood Pb children (72.81%) ($Z = -2.394, P = 0.017$) (data not shown).

3.2. Potential factors in relation to Pb exposure

We observed Pb exposure positively correlated with child age, catching cold within a year, contact with e-waste, having a smoking family member, using residence as a workplace and living within 50 m away from an e-waste contamination site. Negative correlations were obtained for child hand washing before eating, yearly milk product consumption, monthly household income, and paternal and maternal education level (Table 2). To sum up, child dietary and hygiene habits, residential environment, and family status were the important factors affecting blood Pb levels.

3.3. Erythrocyte CD44 and CD58 and serum cytokines

To better understand the factors in the erythrocyte immune micro-environment that are associated with immune adhesion molecules and related cytokines, we measured the expression of adhesion

Table 1
Demographic characteristics of the participants.

Demographic variables	Reference group (n = 135)	Exposed group (n = 132)	Statistics	P-value
Age (years) (mean ± SD)	4.215 ± 1.091	4.533 ± 0.928	t = 2.573	0.011
Gender [n (%)]				
Male	83 (61.481%)	78 (59.091%)	$\chi^2 = 0.159$	0.690
Female	52 (38.519%)	54 (40.909%)		
Blood Pb level (µg/dL) [median (IQRs)]	4.411 (3.293, 5.569)	6.513 (4.616, 8.214)	Z = -7.430	<0.001
>5 µg/dL [n (%)]	50 (37.037%)	96 (72.727%)	$\chi^2 = 34.307$	<0.001
>10 µg/dL [n (%)]	0 (0.000%)	16 (12.121%)	$\chi^2 = 17.407$	<0.001
Erythrocyte Pb level (µg/dL) [median (IQRs)]	11.771 (9.082, 15.105)	16.605 (12.638, 21.484)	Z = -7.044	<0.001
Erythrocyte count ($\times 10^{12}/L$) [median (IQRs)]	4.650 (4.490, 4.930)	4.740 (4.600, 4.938)	Z = -1.640	0.101
HCT [median (IQRs)]	0.370 (0.360, 0.390)	0.380 (0.370, 0.390)	Z = -2.078	0.038
Leukocyte count ($\times 10^9/L$) [median (IQRs)]	8.090 (7.240, 9.900)	8.150 (7.123, 9.510)	Z = -0.495	0.620
Lymphocyte ratio (%) [median (IQRs)]	49.440 (42.840, 55.140)	48.490 (39.515, 54.915)	Z = -1.133	0.257
Monocyte ratio (%) [median (IQRs)]	5.540 (4.740, 6.440)	5.890 (5.140, 7.040)	Z = -2.639	0.008
LMR (%) [median (IQRs)]	8.725 (7.042, 10.949)	7.707 (6.182, 9.704)	Z = -2.852	0.004

Values of $P < 0.05$ were considered statistically significant. HCT: hematocrit; LMR: lymphocyte-to-monocyte ratio; IQRs: interquartile ranges.

molecules and cytokines in child peripheral blood. Surface expression of CD44 and CD58 on erythrocyte membranes was decreased in the e-waste-exposed group (mean ± SD: 68.083 ± 10.726% and 40.768 ± 7.067%, respectively) compared to the reference group (mean ± SD: 76.154 ± 9.453% and 46.322 ± 7.262%, respectively) (all $P < 0.01$) (Fig. 1a). Erythrocyte CD44 and CD58 expression were lower in children with high blood Pb group (mean ± SD: 70.854 ± 10.885% and 42.302 ± 7.755%, respectively) compared with children with low blood Pb in both groups (mean ± SD: 73.744 ± 10.669% and 45.114 ± 7.317%, respectively) (all $P < 0.05$) (Fig. 1b). As shown in Fig. 2a and Table S1, compared with reference children, e-waste-exposed preschool children had higher median levels, for IL-1 β (0.490 pg/mL vs. 0.250 pg/mL), IL-12p70 (2.310 pg/mL vs. 0.150 pg/mL) and IFN- γ (4.590 pg/mL vs. 3.200 pg/mL) (all $P < 0.05$). Additionally, we found that median concentrations of IL-2 were substantially lower in the e-waste-exposed group (4.820 pg/mL) compared to the reference group (8.340 pg/mL) ($P < 0.05$). In the high blood Pb group, both IL-12p70 (0.430 pg/mL vs.

0.290 pg/mL, $P < 0.05$) and IFN- γ (4.590 pg/mL vs. 3.350 pg/mL, $P < 0.01$) levels were increased in preschool children relative to those in the low blood Pb group (Fig. 2b and Table S1). Taken collectively, e-waste-exposed preschool children and high blood Pb groups had lower erythrocyte adhesion molecule expression and higher cytokine levels, except for IL-2, compared with those in the reference and low blood Pb groups.

3.4. Immune parameters in relation to Pb exposure

We investigated the association of peripheral blood cells and cytokines with blood Pb levels (Table 3). Blood Pb was negatively correlated with leukocyte and lymphocyte counts, LMR, and IL-2. In contrast, blood Pb was positively correlated with IL-12p70 and IFN- γ . However, significant correlations among blood Pb and erythrocytes, HCT, monocytes, and IL-1 β were lost.

3.5. Erythrocyte CD44 and CD58 in relation to Pb exposure

To further illustrate the relationship between erythrocyte Pb and adhesion molecules, we used separate regression models (model 1 and 2) to evaluate the relationship between erythrocyte Pb and CD44 or CD58 expression in all preschool children after adjusting for gender and age (Fig. 3a and Table S2). Expression of CD44 and CD58 decreased with elevated erythrocyte Pb when categorized in quartiles ($P < 0.05$ for the trends of both). High erythrocyte Pb levels (quartile 4) were associated with lower levels of erythrocyte CD44 [B_{Q4} (95% CI) = -5.444 (-9.106, -1.729)] and CD58 [B_{Q4} (95% CI) = -4.278 (-6.899, -1.676)], compared with erythrocyte Pb in quartile 1 (both $P < 0.01$). As shown in Fig. 3b and Table S2, the results of model 3 (adjusted by age, gender, and residence as workplace) and model 4 (adjusted by age, gender, residence as workplace and family member smoking) showed that compared with erythrocyte Pb in quartile 1, high erythrocyte Pb concentrations (quartile 4) were significantly related to lower erythrocyte CD44 [B_{Q4} (95% CI) = -5.626 (-9.659, -1.592)] and CD58 expression [B_{Q4} (95% CI) = -3.719 (-6.562, -0.876)] (both $P < 0.01$). In summary, higher erythrocyte Pb levels are linked to reduced expression of adhesion molecules.

3.6. Mediation analysis of the effects

To further examine the effects of mediators, parallel multiple mediator models were utilized with mediator variables including CD44 and CD58, and the direct effect factors erythrocyte Pb, and IL-1 β , IL-12p70 and IFN- γ as outcome variables (Table 4). Bias-corrected 95% CI no crossed zero represent for statistically significant. For IL-1 β , the total indirect effect and indirect effect through CD44, and the proportion of CD44-mediated effects in the total effect was 13.00%. For IL-12p70,

Table 2
Potential influencing factors related to child body burden of Pb exposure.

Relevant factors	Blood Pb level	
	r_s	P-value
Personal information		
Age	0.144	0.019
Catching cold within a year	0.238	<0.001
Playing and hygiene habits		
Time spent outdoors	-0.068	0.271
Washing hands before eating	-0.201	0.001
Sucking/biting nails	0.079	0.198
Chewing pencils	0.098	0.110
Sucking/biting toys	0.069	0.265
Child contact with e-waste	0.191	0.002
Dietary habits		
Yearly preserved egg consumption	0.086	0.162
Yearly milk product consumption	-0.194	0.002
Yearly soy product consumption	0.109	0.077
Yearly marine product consumption	-0.093	0.131
Yearly canned food consumption	0.093	0.132
Yearly Ca-Fe-Zn supplement consumption	0.078	0.206
Yearly iron-rich food consumption	-0.106	0.086
Yearly vitamin supplement consumption	0.077	0.211
Residential environment		
Family member smoking	0.171	0.005
Residence as workplace	0.149	0.016
Ventilation of house	0.007	0.905
Distance of residence from road	-0.067	0.278
E-waste contamination within 50 m of residence	0.174	0.005
Family status		
Monthly household income	-0.295	<0.001
Paternal education level	-0.370	<0.001
Maternal education level	-0.356	<0.001

Values of $P < 0.05$ were considered statistically significant.

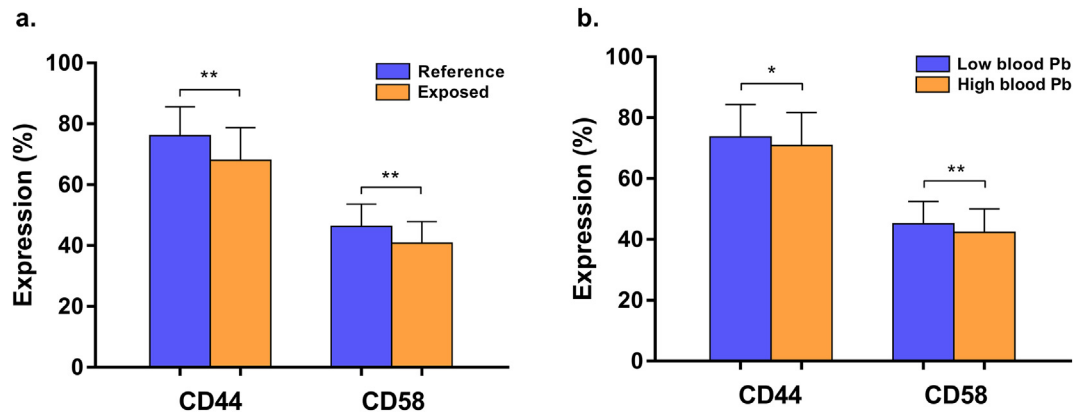


Fig. 1. Comparison of surface membrane CD44 and CD58 levels of erythrocytes in preschool children. Reference group, n = 135; exposed group, n = 132. Low blood Pb group, n = 121; high blood Pb group, n = 146. Results are represented as mean \pm SD; data analysis by the independent sample *t*-test. **Significant at $P < 0.01$. *Significant at $P < 0.05$.

results showed a significant direct effect, total indirect effect and indirect effect through CD44, and the proportion of CD44-mediated effects in the total effect was 7.26%. For IFN- γ , results demonstrated a significant direct effect, total indirect effect and indirect effect through CD58. The proportion of CD58-mediated effects in the total effect was 9.02%. However, no mediation effects of CD44 and CD58 on the relationship between erythrocyte Pb with IL-2 were found (data not shown). Collectively, we found that erythrocyte CD44 and CD58 are different mediators of the association between erythrocyte Pb and various cytokines.

4. Discussion

In this cross-sectional study, we found marked changes in erythrocyte immune adhesion molecules (CD44 and CD58) and serum cytokines, including IL-1 β , IL-2, IL-12p70 and IFN- γ , in e-waste-exposed children and in association with higher Pb exposure. Results from this study suggest that Pb-exposed populations, especially for susceptible preschool children, may have higher risk for decreased erythrocyte immunity due to long-term environmental Pb pollution.

Informal e-waste recycling activities have been dramatically increasing hazardous metal pollution, which has aggravated the environmental burden for the Guiyu ecosystem (Huo et al., 2007; Tansel, 2017). Blood Pb levels can represent short-term exposure, because of its short half-life (9.96 ± 3.92 days) in children (Specht et al., 2016). The value of erythrocyte Pb or HCT has been used for the adjustment of blood Pb for hematotoxicity studies of Pb toxicity (Kim and Lee, 2013; Liu et al., 2015). We also noticed that erythrocyte Pb levels could better evaluate Pb toxicity to erythrocytes in a previous study (Dai et al., 2017). In our study, e-waste-exposed children had higher median blood Pb concentration at $6.513 \mu\text{g/dL}$, and 72.7% of them had a concentration over the

safety threshold ($5 \mu\text{g/dL}$), which was nearly 2 times higher than children from reference area. By contrast, the overall median blood Pb level of 0- to 6-year-old children from Shanghai in China was $1.95 \mu\text{g/dL}$, with only 2.7% having concentrations over $5 \mu\text{g/dL}$ (Cao et al., 2014). Children breathe more air and consume more food than adults per surface area of the respiratory tract and pound body weight, and at the same time, they are still growing and developing, and at certain stages of development, exposure to environmental Pb can lead to irreversible damage (Heacock et al., 2018). Water, diet, house dust and industrial activities are the primary sources of internal exposure of Pb (Bi et al., 2018; Cheng and Hu, 2010; Yoshinaga et al., 2014). Therefore, we investigated multifarious interference factors by questionnaire, including personal information, child play and hygiene habits, dietary habits, residential environment and family status. Our results indicate that long-term exposure along with age, e-waste and residential environment may increase the burden of Pb in the body. However, washing hands before eating, consumption of milk products, and monthly household income, as well as paternal and maternal education level are protective factors decreasing the opportunity of daily exposure. As a result, developing good hygiene, increasing dairy consumption and improving the family environment will minimize the effects of Pb exposure on preschool children. Kordas et al. (2018) also noticed that the consumption of a calcium-rich diet is related to lower blood Pb levels of school-age children.

Acute Pb exposure induces erythrocyte hemolysis arising from osmosis and erythrocyte membrane brittleness and destruction in the spleen, as well as decreases hemoglobin synthesis (Mrugesh et al., 2011; Palacios et al., 2012). Adult short-term occupational exposure to Pb is related to increased counts of leukocytes and decreased hemoglobin levels (Dobrakowski et al., 2016). Our previous report demonstrates

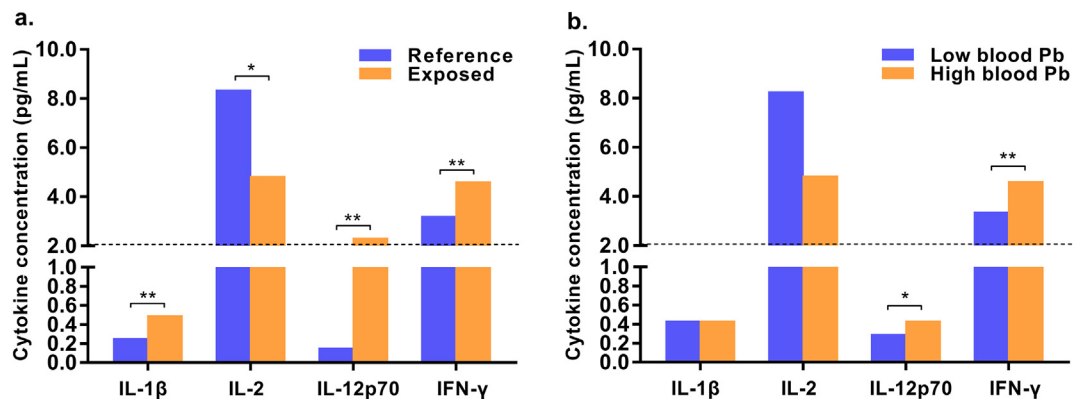


Fig. 2. Comparison of cytokine concentrations in preschool children serum. Reference group, n = 101; exposed group, n = 108. Low blood Pb group, n = 91; high blood Pb group, n = 118. Data are represented by medians. *P*-values are the result of a Mann-Whitney *U* test. IL: interleukin; IFN: interferon. **Significant at $P < 0.01$. *Significant at $P < 0.05$.

Table 3
Spearman rank correlations (r_s) of blood Pb level and immune parameters in preschool children.

Variables	Blood Pb level	
	r_s	P-value
Erythrocyte count	0.110	0.072
HCT	0.040	0.513
Leukocyte count	-0.125	0.041
Lymphocyte ratio	-0.164	0.007
Monocyte ratio	0.117	0.056
LMR	-0.180	0.003
IL-1 β	0.045	0.519
IL-2	-0.154	0.026
IL-12p70	0.196	0.004
IFN- γ	0.223	0.001

Values of $P < 0.05$ were considered statistically significant.
HCT: hematocrit; LMR: lymphocyte-to-monocyte ratio; IL: interleukin; IFN: interferon.

that long-term Pb exposure can affect heme synthesis, as well as damage the antimicrobial activity of hemoglobin and cause anisocytosis in preschool children (Dai et al., 2017). As mentioned earlier, erythrocytes play a dual role in peripheral blood, and erythrocyte-mediated immunity is the first line of defense in innate immunity. Our previous results show that erythrocyte complement regulatory protein CD35 declines in e-waste-exposed children (Dai et al., 2017). In this study, CD44 and CD58 expression at the erythrocyte membrane of Guiyu children are significantly reduced, and are negatively associated with erythrocyte Pb, particularly in 4th quartile of erythrocyte Pb levels (>18.88 $\mu\text{g}/\text{dL}$). Interestingly, we did not find any correlations within the 2nd and 3rd quartiles for erythrocyte Pb, which indicates that only when the Pb exposure is high does Pb-mediated reduction of erythrocyte CD44 and CD58 become manifest. A recent study reported that exposure to Pb is associated with decreased host immune defenses against numerous microorganisms and cancer (Kasten Jolly and Lawrence, 2014). Moreover, it has been pointed out that the expression of erythrocyte CD44 and CD58 in cancer patients is significantly lower than those of healthy individuals, which suggests that the quantities of CD44 and CD58 are related to the degree of tumor metastasis and deterioration (Hoffmann et al., 1996; Zhao et al., 2007). Cai et al. (2015) showed that CD44 in children with hepatoblastoma is significantly higher. This is opposite to our findings, with the possible reason being Guiyu children are chronically exposed to a relatively low level of Pb, and they are still healthy without any obvious disease characteristics. We propose that determination of erythrocyte adhesion molecules, such as CD35, CD44 and CD58, is capable of predicting the health of child erythrocyte immunity, especially for children under long-term Pb exposure.

Certainly, erythrocyte immunity is related to multiple factors, such as monocytes, lymphocytes, NK cells and multiple cytokines, all of which can combine with erythrocyte CD44 and CD58 to promote immunity (Dooms and Abbas, 2010; Ebert et al., 2009; Galandrini et al., 1994; Sharma et al., 2000). In this study, we also measured lymphocytes and monocytes, and show the percentage of monocytes is higher in the e-

Table 4
Summary of the mediating effect of CD44 and CD58 on the relationship between ln-transformed erythrocyte Pb level and cytokines.

Model ^a	Product of coefficients		Bootstrapping bias-corrected 95% CI		Proportion of mediated effect (%)
	Coeffi.	SE	Lower	Upper	
IL-1β					
Direct effect	0.081	0.045	-0.008	0.169	-
Indirect effects					
Total	0.018	0.011	0.001	0.043	18.00
CD44	0.013	0.010	0.000	0.041	13.00
CD58	0.005	0.008	-0.007	0.029	5.00
Contrasts					
CD44 vs. CD58	0.008	0.015	-0.019	0.043	-
IL-12p70					
Direct effect	0.988	0.231	0.533	1.443	-
Indirect effects					
Total	0.133	0.064	0.030	0.290	11.77
CD44	0.082	0.052	0.006	0.224	7.26
CD58	0.051	0.047	-0.006	0.196	4.51
Contrasts					
CD44 vs. CD58	0.031	0.075	-0.108	0.191	-
IFN-γ					
Direct effect	1.431	0.439	0.566	2.296	-
Indirect effects					
Total	0.165	0.110	0.000	0.440	10.34
CD44	0.021	0.062	-0.085	0.180	1.32
CD58	0.144	0.104	0.005	0.428	9.02
Contrasts					
CD44 vs. CD58	-0.124	0.131	-0.447	0.074	-

IL: interleukin; IFN: interferon; SE: standard error; CI: confidence interval.
^a All regression analyses are controlled for age and gender; 5000 bootstrap samples; n = 209.

waste-exposed group. Also, the lymphocyte ratio is positively correlated with blood Pb. This is consistent with research on lead-exposed workers (Niu et al., 2015). LMR, as a systemic inflammatory indicator, has been explored to predict the prognosis for a wide variety of tumors, with a low LMR being an indicator of poor prognosis in several malignancies (Deng et al., 2015; Han et al., 2015; Ying et al., 2014). Our results show that LMR is lower in e-waste-exposed children, and it negatively correlates with Pb exposure in all preschool children, which suggests that children living in high Pb-exposed areas are at higher risk of inflammation.

Although erythrocytes do not secrete cytokines directly, they can affect cytokine secretion by other lymphocytes and monocytes, and participate in the immune response (Danesh et al., 2014; Sharma et al., 2000). We observed that IL-12p70 and IFN- γ levels are increased in e-waste-exposed children and children with high blood Pb. In addition, IL-12p70 and IFN- γ concentrations positively correlate with blood Pb, which suggests that pro-inflammatory cytokines activate immune responses in response to environmental Pb exposure. Erythrocytes can regulate T cell growth and survival, and IL-12p70 released by dendritic cells can participate in Th1 cell proliferation together with their effector cytokine IFN- γ (Arosa et al., 2003; Schakel et al., 2006).

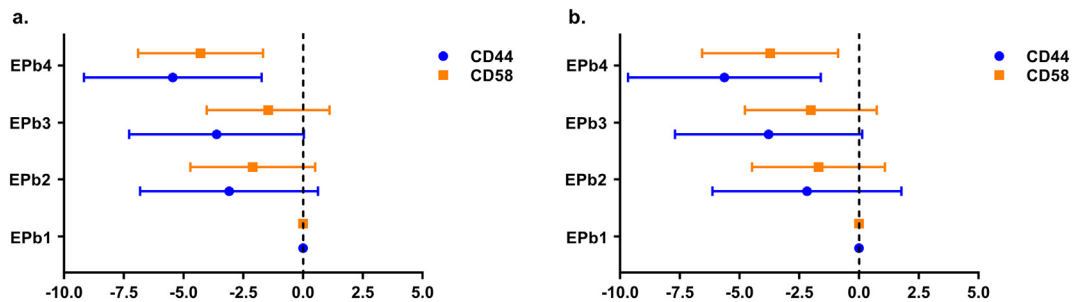


Fig. 3. Effect estimates and 95% confidence intervals for quartiles of erythrocyte Pb with CD44 and CD58 expression. (a) Model 1 (CD44) and model 2 (CD58) were adjusted by age and gender. (b) Model 3 (CD44) was adjusted by age, gender and residence as workplace; model 4 (CD58) was adjusted by age, gender, residence as work place and family member smoking. Dashed horizontal line represents the null association. EPb: erythrocyte Pb level.

We have previously investigated the effects of Pb on a variety of immune cells, such as NK cells, lymphocytes and granulocytes, which produce cytokines in preschool children (Cao et al., 2016; Zhang et al., 2016; Zhang et al., 2017). IL-1 β , as a pleiotropic cytokine, can facilitate angiogenesis, tumor invasiveness and carcinogenesis (Elkabets et al., 2010). In addition, it can stimulate T cell activation and induce endothelial cells to synthesize hyaluronan to promote lymphocyte and monocyte penetration into sites of inflammation through CD44 (Clark et al., 1996; DeGrendele et al., 1997; Estess et al., 1998; Mohamadzadeh et al., 1998). Our results show that IL- β is not increased in the high blood Pb group and is also not associated with blood Pb levels. Nevertheless, we found that erythrocyte CD44 plays a significant mediation effect on the association between erythrocyte Pb and IL-1 β or IL-12p70. In addition, erythrocyte CD58 also displays a significant mediation effect on the association between erythrocyte Pb and IFN- γ . Furthermore, IL-2 levels negatively correlate with blood Pb in all children and are lower in e-waste-exposed children. As a non-specific immune stimulating factor, CD58 can stimulate monocytes to secrete IL-2, which takes part in T cell growth, supports T cell proliferation and development, and widely takes part in regulation of the immune response (Dooms and Abbas, 2010). However, we did not find that erythrocyte CD58 plays a mediating role in the relationship between Pb exposure and IL-2, which may be mediated by other immune cells. To be sure, along with the erythrocyte Pb concentrations being elevated, the levels of IL-1 β , IL-12p70 and IFN- γ are also increased through erythrocyte CD44 and CD58. Collectively, our results demonstrate that erythrocyte adhesion molecules, CD44 and CD58, are involved in the release of immune-related cytokines due to Pb exposure, especially in an elevated Th1 immune response.

Again, several limitations in this study have to be considered. A possible reason is that this cross-sectional study proposes a relationship between Pb exposure and erythrocyte immunity, but the causality cannot be identified, which is essential for ascertaining specific links in the next prospective cohort studies. Second, other organic pollutants and heavy metals may also affect erythrocyte function, but we only focus on the effect of Pb exposure on erythrocyte immunity in present study due to limitations of blood volume. Nevertheless, we explore the impact of Pb toxicity on erythrocytes because when Pb reaches the systemic circulation, >95% accumulates in erythrocytes (Jang et al., 2011). Third, we only measured the expression of erythrocyte CD44 and CD58, but did not directly measure their immune adherence function, which would confirm a functional role. Future research should concentrate on the joint influences of multiple pollutants and erythrocyte immunity.

5. Conclusions

In summary, we propose that long-term exposure to Pb can impair erythrocyte adhesion molecule expression, such as CD44 and CD58 expression, and the increase of Pb exposure can slightly stimulate cytokine secretion, including IL-1 β , IL-12p70 and IFN- γ , which are mediated by erythrocyte CD44 and CD58. Our results provide novel translational insight into the relationship between Pb exposure and the change of erythrocyte immunity and downstream cytokine secretion in preschool children. It will be necessary to pay additional attention to the development of the immune system in children and decrease environmental Pb exposure, to reduce the body accumulation and prevent detrimental health outcomes.

Conflict of interest

All authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2019.02.040>.

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