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Prenatal cadmium exposure alters postnatal immune cell development and function

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Abstract

Cadmium (Cd) is generally found in low concentrations in the environment due to its widespread and continual use, however, its concentration in some foods and cigarette smoke is high. Although evidence demonstrates that adult exposure to Cd causes changes in the immune system, there are limited reports of immunomodulatory effects of prenatal exposure to Cd. This study was designed to investigate the effects of prenatal exposure to Cd on the immune system of the offspring. Pregnant C57Bl/6 mice were exposed to an environmentally relevant dose of CdCl₂ (10 ppm) and the effects on the immune system of the offspring were assessed at two time points following birth (2 and 7 weeks of age). Thymocyte and splenocyte phenotypes were analyzed by flow cytometry. Prenatal Cd exposure did not affect thymocyte populations at 2 and 7 weeks of age. In the spleen, the only significant effect on phenotype was a decrease in the number of macrophages in male offspring at both time points. Analysis of cytokine production by stimulated splenocytes demonstrated that prenatal Cd exposure decreased IL-2 and IL-4 production by cells from female offspring at 2 weeks of age. At 7 weeks of age, splenocyte IL-2 production was decreased in Cd-exposed males while IFN- γ production was decreased from both male and female Cd-exposed offspring. The ability of the Cd-exposed offspring to respond to immunization with a *S. pneumoniae* vaccine expressing T-dependent and T-independent streptococcal antigens showed marked increases in the levels of both T-dependent and T-independent serum antibody levels compared to control animals. CD4⁺FoxP3⁺CD25⁺ (nTreg) cell percentages were increased in the spleen and thymus in all Cd-exposed offspring except in the female spleen where a decrease was seen. CD8⁺CD223⁺ T cells were markedly decreased in the spleens in all offspring at 7 weeks of age. These findings suggest that even very low levels of Cd exposure during gestation can result in long term detrimental effects on the immune system of the offspring and these effects are to some extent sex-specific.

Keywords

cadmium; prenatal exposure; thymocytes; splenocytes; cytokines; antibody

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DISCLOSURES

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INTRODUCTION

Cadmium (Cd) is a heavy metal that poses a hazard to human health due to its toxicity. There is sufficient evidence in humans to classify Cd and Cd compounds as carcinogens based on epidemiological studies demonstrating a link between Cd and lung, and possibly prostate cancers (IARC, 2004). Exposure to the heavy metal and its compounds primarily occurs in workplaces such as mining, smelting, processing, and battery manufacturing, whereas non-occupational exposures come from various foods, contaminated water, and tobacco smoke. Smokers generally have Cd blood levels 4-5 times those of non-smokers (Elinder *et al.*, 1976).

Cd levels in the environment vary widely due to its ability to be transported through air, water, and soil. Humans normally absorb Cd into the body either by ingestion or inhalation (Lauwerys *et al.*, 1986). The daily intake is estimated to be approximately 10-50 µg, but can reach levels of 200-1000 µg in highly contaminated areas (Nordberg, 2006). The average Cd intake from food in one study showed values of 38 - 300 µg/week (Olsson *et al.*, 2002), while absorption from cigarette smoke is 1-3 µg/pack/day (Faroon *et al.*, 2008). Cd levels in soils, particularly areas in which phosphate fertilizers have been applied, can range from 10 to 200 µg/g (Cook, 1995).

Humans do not have an effective Cd elimination pathway and as a result the biologic half-life of Cd in the human body is estimated to be 15-20 years (Jin *et al.*, 1998). Excessive Cd accumulation in the body often results in diseases such as kidney failure, respiratory disease, neurological disorders, and occasionally death (Waalkes *et al.*, 1992). Although pharmacokinetic studies have demonstrated that Cd does not readily reach the fetus, it accumulates in high concentrations in the placenta (Piasek *et al.*, 2001). Teratological effects associated with Cd exposure reported for humans are limited; however, maternal exposure to environmental Cd, higher placental concentration (Loiacono *et al.*, 1992), and/or fetal Cd exposure (Frery *et al.*, 1993) has been associated with lower birth weights in humans. Moreover, the teratological effects of Cd in rodents have been extensively documented (Hovland *et al.*, 1999; Scott *et al.*, 2005; Minetti and Reale, 2006; Jacquillet *et al.*, 2007).

There have been numerous studies on the immunomodulatory effects of Cd in adult humans and experimental animals; however, the findings remain controversial (reviewed by Descotes, 1992). This conflict amongst findings may be attributed to varying doses, route of administration, length of Cd exposure, and differences in the sensitivity of immune systems of different animal species. The thymus, the primary site of T-cell production, is a target organ of Cd-induced toxicity (Morselt *et al.*, 1988). Thymocytes mature through a series of stages defined by expression of cell surface markers CD4 and CD8. The most immature thymocytes are CD4⁻CD8⁻ double-negative (DN). This population gives rise to CD4⁺CD8⁺ double-positive (DP) cells, which then give rise to mature CD4⁺CD8⁻ single-positive (SP) and CD4⁻CD8⁺ SP cells. The DN population can be further subdivided in mice based on the expression of surface markers CD25 and CD44: CD44⁺CD25⁻(DN1) cells differentiate into CD44⁺CD25⁺(DN2) cells, which then develop into CD44⁻CD25⁺(DN3) cells, which differentiate into the CD44⁻CD25⁻(DN4) population. Following Cd-treatment, damage occurs to the thymus as well as changes in the proliferation rate of thymocytes in adult rats (Morselt *et al.*, 1988). In adult mice, Dong *et al.* (2001) observed a decrease in DP cells. Pathak and Khandelwal (2008) also demonstrated that Cd exposure decreased the DP population and increased the number of DN cells. In vivo studies exposing adult male rats to varying concentrations of Cd (0-100 ppm) demonstrated that lower doses of Cd inhibited humoral and cellular immune responses, while higher concentrations had a stimulatory effect (Lafuente *et al.*, 2004). Analysis of oxidative stress and apoptosis showed that splenic cells appeared more susceptible than thymus cells to the adverse effects of Cd both in vitro

(Pathak and Khandelwal, 2006a; Pathak and Khandelwal, 2006b) and in vivo (Pathak and Khandelwal, 2007).

Despite numerous studies demonstrating the effects of Cd on the adult immune system (Mackova *et al.*, 1996b; Liu *et al.*, 1999a; Lafuente *et al.*, 2004), there have been limited reports on the effect of Cd exposure during gestation on the immune system of the offspring. We have previously shown that prenatal exposure to Cd affects thymocyte development of newborn (<12 h old) offspring (Hanson *et al.*, 2010). The study reported herein was designed to investigate the continued effects of prenatal exposure to Cd on the immune system of the offspring. Pregnant mice were exposed to an environmentally relevant dose of CdCl₂ (10 ppm) and the effects on the immune system of the offspring were assessed at two time points following birth [2 and 7 weeks of age] to evaluate the effects in relation to developmental stage. There were numerous changes in the level of spleen cell cytokine production and serum anti-streptococcal antibody responses in the animals exposed to Cd prenatally as compared to the controls at 7 weeks of age. The relative percentage of CD4⁺FoxP3⁺CD25⁺ (nTreg) and CD8⁺CD223⁺ T cells was also markedly different between the two groups at 7 weeks of age.

MATERIALS AND METHODS

Breeding and Cd Exposure Methodology

C57Bl/6 mice at 8-10 weeks of age were obtained from Hilltop Lab Animals, Inc. (Scottsdale, PA). The C57Bl/6 strain of mouse was used for these experiments due to its reported teratogenic susceptibility to Cd exposure (Hovland *et al.*, 1999). Mice were allowed to acclimate on site for at least one week. Two females were placed in a cage with one male for 72 hours to maximize pregnancy rate. Females were inspected for a vaginal plug and if present, this day was declared as gestational day 0. Ten dams were used as controls, having free access to deionized distilled water (ddH₂O), while ten additional dams had free access to 10 ppm of CdCl₂ (Sigma-Aldrich; St. Louis, MO) dissolved in ddH₂O. The dose of 10 ppm was chosen because it is the greatest concentration that will elicit immunomodulatory effects in adult rodents without causing systemic effects (Lafuente *et al.*, 2003). In addition, 10 ppm is a relatively low and environmentally realistic concentration (Thijssen *et al.*, 2007). Cd administration was stopped at birth. Following parturition, Cd levels in the kidneys of dams were measured as previously described (Hanson *et al.*, 2009) to ensure consistent Cd dosing among dams. No significant differences in Cd burden were seen between the dams (data not shown). At 2 and 7 weeks of age, 3 offspring (at least 1 male and 1 female) selected from each of the litters were euthanized and thymi and spleens were removed. Mice born from a different set of dams were euthanized at 7 weeks of age to ensure adequate sample size. The 2 weeks of age time point was chosen to determine if any longer term effects of prenatal exposure to Cd were evident early in life and 7 weeks of age was chosen to assess the effects of prenatal Cd exposure at the mouse adult stage. The 7 week time point would approximate a younger post-pubescent human which should have a fully developed robust immune response. All offspring were weaned at 3 weeks of age and the dams were euthanized. All animal procedures were approved by the WVU Institutional Animal Care and Use Committee.

Tissue Isolation and Cell Preparation

Thymi and spleens were harvested from euthanized mice and single cell suspensions prepared. The cell preparations from each mouse were analyzed individually. Red blood cells were lysed using an ammonium chloride lysis buffer. Viable cells were enumerated using trypan blue and a hemacytometer.

Cell staining and flow cytometry

Single cell suspensions of thymocytes and splenocytes were prepared as described above. Thymocytes were stained using combinations of the following fluorochrome directly conjugated antibodies: anti-CD45-biotin (clone 30-F11; eBioscience; San Diego, CA), streptavidin-Pacific Blue (Invitrogen; Carlsbad, CA), anti-CD44-PE-Cy5 (clone IM7; eBioscience), anti-CD25-PE-Cy7 (clone PC61.5; eBioscience), anti-CD4-FITC (clone RM4-5; BD Biosciences Pharmingen; San Jose, CA), and anti-CD8-PE (clone 53-6.7; BD Biosciences Pharmingen). SP and DP cell subpopulations were identified using anti-CD4 and anti-CD8. To identify the different DN subpopulations, CD44 and CD25 expression were detected on the CD4⁺CD8⁺ population. Splenocytes were stained using combinations of the following directly conjugated Abs: anti-CD4-FITC (BD Biosciences Pharmingen), anti-CD8-PE (BD Biosciences Pharmingen), anti-F4/80-APC-Alexa Fluor 750 (clone BM8; eBioscience), anti-Gr1-Alexa Fluor 700 (clone RB6-8C5; eBioscience), and anti-B220-PE-Cy5 (clone RA3-6B2; BD Biosciences Pharmingen). Cells (1×10^6) were washed with PBSAz (phosphate buffered saline containing 2% FBS and 0.2% sodium azide), incubated with whole rat and mouse IgG (Jackson ImmunoResearch, West Grove, PA) for 30 min on ice to block Fc receptors, followed by a PBSAz wash. The cells were incubated for 30 min on ice with fluorochrome labeled antibodies, washed several times with PBSAz and fixed overnight at 4°C with 0.4% paraformaldehyde. The paraformaldehyde was removed by washing and cells resuspended in PBSAz. Data were acquired using a FACSaria and analyzed employing Diva 6.1 software (BD Biosciences Pharmingen) and FCA Express software (De Nova Software). A total of 10,000 events were collected for each sample.

To detect the CD8⁺CD223⁺T cells in spleen and thymus, a combination of fluorochrome-conjugated antibodies against CD8 (FITC), CD28 (APC, clone 37.51) and CD223 (PE, clone eBioC9B7W; CD223 is also called LAG-3; eBioscience) was used as above.

CD4⁺ nTreg cells expressing the transcription factor FoxP3 were detected using antibodies (eBioscience) against membrane-associated CD4-FITC, and CD25-APC (clone PC61.5), in addition to intracellular FoxP3-PE (clone FJK-16a). Splenocyte and thymocyte surface staining with anti-CD4 and anti-CD25 performed as above followed by fixation/permeabilization for 30 min, washed and stained for 30 min with anti-FoxP3-PE antibody or the rat IgG2a isotype control-PE. Cells were washed twice, resuspended in PBSAz and analyzed on a flow cytometer.

Cytokine production

The production of cytokines by splenocytes at 2 and 7 weeks of age was measured using the Mouse TH1/TH2 Ready-SET-Go! ELISA set (eBioscience). Briefly, 1×10^6 cells/ml in RPMI media (Mediatech Cellgro; Manassas, VA) supplemented with 10% FBS were seeded in 48-well plates and stimulated with anti-CD3 (10 g/ml; clone 17A2; eBioscience) and anti-CD28 (10 g/ml; clone; 37.51; eBioscience). The culture supernatant was collected 24 h after the stimulation for the assessment of IL (interleukin)-2, and 72 h after the stimulation for the assessment of IL-4, IL-10, IL-12p70, TNF- α , Monocyte Chemoattractant Protein-1 (MCP-1), IL-6 and IFN- γ . The concentrations of the cytokines were assessed per manufacturer's protocol. The limit of detection for the cytokines shown was IL-2 (2 pg/ml), IFN- γ , (15 pg/ml), IL-10 (30 pg/ml), and IL-4 (4 pg/ml).

Preparation of *S. pneumonia* vaccine and immunization

Functional analysis of the immune system was performed by immunizing the offspring with a heat-killed vaccine of *Streptococcus pneumoniae* strain R36A antigen. *S. pneumoniae* strain R36A is an avirulent, nonencapsulated strain commonly used as a source of antigen and the kinetics of the serum antibody response and the predominant types of antibody

isotypes to phosphorylcholine (PC) and pneumococcal surface protein A (PspA) have been well characterized (Wu *et al.*, 1999; Wu *et al.*, 2000).

The vaccine was prepared as described by Salazar *et al.* (Salazar *et al.*, 2005). Briefly, *S. pneumoniae* strain R36A, was grown to mid-log phase in Todd-Hewitt broth (Difco) supplemented with 0.05% yeast extract (Difco) at 37 °C in the presence of 10% CO₂ to an OD₆₀₀ of ~0.4. They were heat killed at 600C for 2 hr, washed twice and resuspended in saline at 2×10⁹ CFU/ml. Sterility and CFU were confirmed by culture on blood agar. Heat-killed stock was stored at –80°C in 1 ml aliquots. Mice were immunized intraperitoneally with 2×10⁸ CFU in 100 µl and blood was collected 10 days following immunization.

Preparation of PspA

Plasmid UAB055, which contains the truncated PspA gene attached to a 6-His tag, was a gift of Dr. Susan Hollingshead (Department of Microbiology, UAB, Birmingham, AL). The plasmid was transformed into BL21 (DE3) pLysS (EMD Biosciences). A selected transformant colony was grown in Luria broth (Difco) supplemented with 100 g/ml ampicillin (Sigma Chemical Co, St. Louis, MO) to OD₆₀₀ of 0.5, induced with 1 mM IPTG for 4 hr and harvested. His-tagged PspA protein was purified from periplasmic extract using BugBuster HisBind purification kit (EMD Biosciences), dialyzed extensively against PBS, adjusted to 0.25 mg/ml and stored at 4°C.

Antibody quantitation

Blood samples were collected from immunized mice and sera obtained by standardized methods and stored at –20°C until assayed. For ELISA assays, Immulon 2 plates (ThermoLabsystems, Franklin, MA) were coated overnight at 4 °C with 2 µg/ml PC-BSA or 5 µg/ml PspA. Plates were washed, blocked with 1% BSA in PBS at 37 °C for 1 hr. Plates were washed with PBS and 100 µl/well serum diluted in PBS was added starting at 1/400 for the PC-BSA and 1/50 for PspA and twofold dilutions thereafter, and allowed to bind overnight at 40C. Plates were then washed and incubated with AP conjugated antibodies (Southern Biotechnology Associates, Birmingham, AL) for 3 hour at 37 °C. After washing, 100 µl/well phosphatase substrate (Sigma Chemical Co., St Louis, MO) dissolved in diethanolamine buffer, pH 9.8 was added. Absorbance values were read at 405nm (A₄₀₅) at timed intervals using a Quant spectrophotometer (Bio-Tek instruments, Winooski, VT) using KCJunior software (Bio-Tek instruments, Winooski, VT). To determine the titer, a standard pooled sera was diluted and plated on each ELISA plate. The titer for each sample was determined by comparison to the standard sera when the A₄₀₅ for the standard was 0.4 at a 1:3200 dilution to measure all anti-PC antibody isotypes and 0.2 for anti-PspA IgM and IgG isotypes. These dilutions were chosen because they were in the linear part of the curve for the respective isotypes. In some instances, the anti-PspA IgG1, IgG2a, and IgG2b titers were determined at an A₄₀₅ of 0.2 after 120 min of incubation.

Statistical Analyses

Results are expressed as mean ± S.E.M. Data expressed in percent of total or percent of control for cell populations were analyzed using the nonparametric Mann-Whitney (Wilcoxon) test. T-test was used to analyze cytokine concentrations, PspA and PC titers, between Cd-exposed and control offspring. For Figures 1-6, each bar represents a mean of 5-8 offspring/sex/treatment group/age group. An alpha value of p 0.05 was considered significant. Experiments represented in Figures 1-3 were repeated at least three times and experiments represented in Figures 4-6 were repeated at least 2 times.

RESULTS

Prenatal Cd exposure does not change the relative percentages of thymocyte or splenocyte phenotypes in 2 and 7 week old offspring

Thymocyte phenotype of representative offspring from each litter was measured by cell surface marker expression using flow cytometry. Total thymocyte number was not significantly different between Cd-exposed and control offspring at 2 weeks of age ($25.9 \pm 3.4 \times 10^7$ vs. $23.0 \pm 3.0 \times 10^7$, respectively) or at 7 weeks of age ($25.4 \pm 2.2 \times 10^7$ vs. $21.2 \pm 1.8 \times 10^7$, respectively). No phenotypic changes in the thymocyte populations were detectable at 2 weeks of age (Figure S1, Panel A and B) or at 7 weeks of age for either sex (Figure 1, panel A and B).

Splenocyte phenotype of representative offspring from each litter was determined by flow cytometry using cell markers specific for CD4 and CD8 T cells, B cells, macrophages, and granulocytes. Total splenocyte number was not significantly different between Cd-exposed and control offspring ($1.1 \pm 0.8 \times 10^8$ vs. $1.0 \pm 0.93 \times 10^8$, respectively). At 2 weeks of age, neither sex showed any differences in the percentage of these cell types (Figure S2). The only cell type that showed a significant difference between the Cd-exposed offspring and control animals was reproducible decrease in macrophages in males at 7 weeks of age (Figure 2).

Prenatal Cd exposure caused marked changes in spleen cells ex vivo cytokine production at 2 and 7 weeks of age

In order to determine the effects of prenatal Cd exposure on the immune response of offspring at 2 and 7 weeks of age, production of IL-2, IL-4, IL-10, IL-12p70, TNF- α , MCP-1, IL-6 and IFN- γ by splenic T cells was measured following stimulation with anti-CD3 and anti-CD28. At 2 weeks of age, Cd-exposed female offspring produced significantly less IL-2 than control female offspring (Cd-treated, 2.31 ± 0.77 g/ml vs. control, 7.40 ± 1.41 g/ml ($p < 0.01$; Figure S3). IL-4 production was also significantly decreased in Cd-exposed female offspring compared to control female offspring (Cd-treated, 202.42 ± 7.15 pg/ml vs. control, 299.76 ± 17.86 pg/ml; $p < 0.05$; Figure S3). In addition, IFN- γ was markedly lower in Cd-exposed female offspring ($p < 0.06$) (Figure S3). Cd-exposed male offspring did not demonstrate any statistically significant difference in cytokine production at 2 weeks of age. There were no significant differences in the levels of IL-12p70, TNF- α , MCP-1, and IL-6 between the Cd-exposed offspring and the controls at 2 or 7 weeks of age (data not shown).

At 7 weeks of age, IL-2 production rebounded in Cd-exposed females compared to control females (Cd-exposed, 11.4 ± 1.9 μ g/ml vs. control, 8.21 ± 1.22 μ g/ml; $p < 0.05$) while males showed significant decreases in IL-2 at this age (Cd-treated, 3.5 ± 0.9 μ g/ml vs. control, 8.1 ± 1.9 μ g/ml; Figure 3). IFN- γ was significantly decreased in both Cd-exposed females (Cd-exposed, 0.47 ± 0.10 μ g/ml vs. control, 12.52 ± 2.48 μ g/ml) and males (Cd-exposed, 3.69 ± 0.98 μ g/ml vs. control, 9.29 ± 2.54 μ g/ml) compared to control animals (Figure 3). There were no differences in IL-10 or IL-4 production in either Cd-exposed males or females at 7 weeks of age. In summary, the data suggest that in Cd-exposed female offspring the levels of cytokines produced by Th1 cells (IFN- γ producing) were compromised, while Th2 cells (IL-10 and IL-4 producing) did not appear to be affected. In the male offspring, at 7 weeks of age, a similar bias against Th1 cell derived cytokines was evident.

Prenatal Cd exposure increased the specific anti-PC and anti-PspA antibody response at 7 weeks of age

An assessment of the functionality of the immune system was performed by measuring the T-independent and T-dependent antibody responses after vaccination with *P. pneumoniae*

vaccine. The T-independent response, anti-PC, provides a functional assessment of the ability of the B cell to be stimulated by antigen and differentiate into IgM producing plasma cells. In addition, type II T-independent antigens, such as PC, can induce switching via dendritic cells to produce IgG antibodies (Colino *et al.*, 2002). The level of anti-PC antibody of all isotypes was significantly higher in offspring prenatally exposed to Cd (Figure 4A and Table 1). For the males, the antibody titers were approximately three-fold higher in the Cd-exposed animals over the control male offspring (Figure 4A and Table 1). Similar or higher fold differences were also seen in the female offspring except for the IgM, which showed only a small but significant increase over controls (Figure 4A and Table 1).

The T-dependent response requires the cooperative participation of antigen-presenting cells, T-helper cells and B cells. Thus, this measurement provides a very comprehensive picture of the ability of the animal to respond to an antigenic challenge. The T-dependent antigen PspA induced a robust IgG response in both Cd-exposed and control offspring, with the exception of the female controls, which produced no detectable anti-PspA IgG2a antibody (Figure 4B and Table 1). The anti-PspA IgG response was always an order of magnitude higher in those animals exposed to Cd prenatally. IgM titers were also measured (Table 1) and showed no significant difference between Cd-exposed and control animal titers for either sex.

Prenatal Cd exposure altered the percentages of natural T regulatory cell populations at 7 weeks of age

Changes in nTreg cell levels in the Cd-exposed offspring could provide a possible explanation for the increase in anti-*S. pneumoniae* antibodies. Therefore, nTreg cells were measured in the spleen as well as the thymus in both males and females. These cells were enumerated by flow cytometric analysis after staining with nTreg-specific markers in addition to the traditional CD4 markers, the CD4⁺CD25⁺FoxP3⁺ phenotype. In the male offspring, the percentages of nTreg cells were statistically higher in the Cd-exposed offspring in both the spleen and thymus (Figure 5). In the female offspring, the nTreg cells in Cd-exposed animals were statistically lower than the comparable controls in the spleen (Figure 5). However, the opposite result occurred in the thymus where Cd-exposed offspring showed statistically higher percentages of nTreg cells (Figure 5).

Prenatal Cd exposure altered the percentages of CD223⁺ (LAG-3) CD8⁺ cell populations

A CD8⁺CD223⁺ T cell has been identified that shows increased expression on antigen-stimulated CD8⁺ T cells (Blackburn *et al.*, 2009) and on CD8⁺ cells that demonstrate immune 'exhaustion' as a result of chronic virus infection (Blackburn *et al.*, 2009) and loss of self-tolerance (Grosso *et al.*, 2007). In the spleen, CD8⁺CD223⁺ T cells were approximately three-fold lower in the Cd-exposed male offspring and approximately two-fold lower in the female Cd-exposed offspring as compared to their sex-matched control counterparts (Figure 6). These cells were harvested from non-immunized animals and not experimentally stimulated with antigen. The numbers of these CD8⁺CD223⁺ T cells were not significantly different in the thymus between Cd-exposed and controls of either sex (data not shown).

DISCUSSION

Immunotoxicity after Cd exposure in adult animals is well documented; however, reports concerning the effect of Cd exposure during gestation on the immune system are limited. We have previously demonstrated that prenatal Cd exposure alters thymocyte development in offspring at postnatal day 0 (PND0) (Hanson *et al.*, 2010). Soukupova *et al.* (1991) reported that the proliferative responses of spleen cells to mitogens was increased at 8 weeks of age and while the oxidative burst activity of peritoneal macrophages was increased at 4

weeks of age, it was reduced by 8 weeks of age. Soukupova et al. (1991) also showed that the delayed type hypersensitivity to sheep red blood cells after immunization was decreased in prenatal Cd-exposed offspring at 8 weeks of age. Due to organogenesis of the immune system occurring primarily at the prenatal, and to a lesser extent, at the early postnatal stage, the perinatal period is not only more sensitive to deleterious effects of immunotoxicants, but alterations in the immune system can result in persistent effects (Holladay and Smialowicz, 2000). Exposure to toxic agents such as halogenated aromatic hydrocarbons (HAHs), polycyclic aromatic hydrocarbons (PAHs), hormonal substances, and heavy metals during the developmental period can result in a range of functional defects in adulthood, including suppression of the immune system (Blaylock *et al.*, 1992; Keil *et al.*, 2008), hypersensitivity (Miller *et al.*, 1998), and autoimmune disease (Snyder *et al.*, 2000; Mustafa *et al.*, 2008). In vitro studies have demonstrated that Cd causes oxidative stress and apoptosis in adult mouse T- and B-cells (Pathak and Khandelwal, 2006b; Pathak and Khandelwal, 2006a), as well as macrophages (Kim and Sharma, 2006). The thymus is an important primary lymphoid organ where successive stages of cell development and selection produce functionally competent T cells from immature precursor cells. Several in vivo studies demonstrated that adult exposure to a wide range of Cd doses is able to cause significant weight decrease or atrophy of the thymus in mice (Borgman *et al.*, 1986; Mackova *et al.*, 1996a; Liu *et al.*, 1999b). In addition, in vitro studies have demonstrated that exposure to Cd results in apoptosis and phenotypic changes in thymocytes from adult mice (Dong *et al.*, 2001; Pathak and Khandelwal, 2008). The present analysis of offspring thymocyte phenotype following prenatal Cd exposure demonstrates no significant changes in the DN population. Examination of the four DN subpopulations after prenatal Cd exposure at both 2 and 7 weeks of age also showed no significant persistent phenotypic changes. We have previously shown a link between prenatal Cd exposure and dysregulation of the sonic hedgehog (Shh) and Wnt/ β -catenin signaling pathways in the thymus at PND0 (Hanson *et al.*, 2010). Both of these pathways are needed for differentiation of DN1 cells to DN2 cells, and while a continued perturbation of the phenotypes at 2 and 7 weeks of age was expected, we do not believe that the lack of phenotypic differences necessarily negates a probable role of the Shh and Wnt/ β -catenin pathways at later time periods. Studies using Shh or Wnt/ β -catenin knockout mice cannot be referenced to verify this since embryonic Shh^{-/-} and Wnt/ β -catenin^{-/-} is lethal and only conditional KO studies have been conducted on Shh and Wnt/ β -catenin postnatally.

In vitro and in vivo studies in adult mice have shown that splenocytes are more sensitive to Cd exposure than are thymocytes (Pathak and Khandelwal, 2006b; Pathak and Khandelwal, 2006a; Pathak and Khandelwal, 2007). Although we did not observe a significant decrease in the total number of splenocytes in prenatally Cd-exposed offspring, an analysis of splenocyte phenotype demonstrated that the macrophage population was decreased in Cd-exposed offspring. More strikingly, this effect was only present in male offspring. In vivo studies by others indicate that chronic Cd exposure alters the redox balance in adult male mice, inducing changes in lipid metabolism in macrophages, ultimately leading to apoptosis (Ramirez and Gimenez, 2002). In addition to being antigen presenting cells and phagocytes, macrophages can recognize tumor cells and induce cell death by releasing cytotoxic factors such as reactive oxygen and/or nitrogen intermediates, as well as cytokines; thus, the decrease in macrophage number in prenatal Cd-exposed male offspring might indicate an increased susceptibility of males to tumor incidence. Further studies are warranted to understand the mechanism of this cell type-, sex-specific effect.

Since cytokines influence or control most immune responses, cytokine production by spleen cells after T cell-specific stimulation was determined. In vitro studies have suggested that Th1 type cytokines (IL-2, IFN- γ) are depressed to a higher degree than Th2 type cytokines (IL-10, IL-4) following Cd exposure (Krocova *et al.*, 2000; Hemdan *et al.*, 2006; Pathak and

Khandelwal, 2008). Studies demonstrating that a decrease in Shh signaling in peripheral CD4⁺ T cells down-regulates the synthesis of IL-2 and IFN- γ (Stewart *et al.*, 2002) and that prenatal Cd exposure decreases Shh signaling in the thymus of offspring at PND0 (Hanson *et al.*, 2010), led to the hypothesis that Th1 cells in the spleen would be more sensitive to prenatal Cd exposure than Th2 cells. In the present study, analysis at 2 weeks of age demonstrated that while cytokine production (IL-2 and IL-4) was only decreased in females, by 7 weeks of age a dramatic decrease in the ability of splenocytes from Cd-exposed male offspring to produce cytokines characteristic of the Th1 type was evident. Furthermore, a decrease in IFN- γ secretion by spleen cells from female offspring was also evident.

Mouse studies evaluating sex differences following Cd exposure are sparse. In humans, although no studies have been designed to investigate sex differences directly, related studies have shown that Cd-associated health effects are more frequent among women than men (Vahter *et al.*, 2002). This may be due to a higher Cd body burden in women, reflected as higher Cd levels in blood, urine, and kidney cortex (Vahter *et al.*, 2007). The main reason for the higher body burden in women is increased intestinal absorption of dietary Cd at low iron stores (Akesson *et al.*, 2002; Kippler *et al.*, 2007). Cd and iron compete with one another for transport into the mucosa cell via the divalent metal transporter 1 (DMT-1). When a woman is pregnant, enterocytes have an increased divalent metal transporter-1 density at the apical surface to increase absorption of micronutrients, thus Cd absorption is increased during pregnancy (Akesson *et al.*, 2002; Vahter *et al.*, 2007). It has been reported that 90% of patients with Itai-Itai disease, the most severe form of chronic Cd intoxication in humans, were postmenopausal women (Jarup *et al.*, 1998). This is due to Cd's ability to disrupt calcium homeostasis caused by estrogen depletion during menopause or following ovariectomy (Jarup *et al.*, 1998). Another reason for sex-differences in susceptibility to Cd-induced toxicity may be attributed to Cd having estrogenic effects (Garcia-Morales *et al.*, 1994; Stoica *et al.*, 2000; Sogawa *et al.*, 2001; Choe *et al.*, 2003; Johnson *et al.*, 2003). Following in utero exposure to Cd, female offspring experienced an earlier onset of puberty and an increase in the epithelial area and the number of terminal end buds in the mammary gland (Johnson *et al.*, 2003). In addition, in vivo studies using adult female rats suggest that females may be at a greater risk than males for Cd-induced immunomodulation due to interactions between estrogen and Cd (Pillet *et al.*, 2006). Pathak and Khandelwal (2008) demonstrated that IFN- γ is inhibited at a lower Cd concentration than IL-2 in adult male mice, so if female offspring have a higher body burden or are more sensitive to prenatal Cd exposure, then a higher dose of Cd may be necessary to elicit a decrease in IL-2 in male offspring, thus explaining the lack of effect on IL-2 production, but decreased IFN- γ production in male offspring in the present study.

Specific anti-streptococcal antibodies were uniformly greater in the Cd-exposed offspring. In most cases, the increase was markedly higher and covered all of the IgG subtypes. The anti-PC IgM levels were only slightly (but significantly) higher in the females. These increased antibody levels (Figure 5) appear to be at odds with the increased nTreg population in the spleen and thymus in the male offspring. Further, the decrease in the splenic nTreg cells in the female offspring does not seem to be of sufficient magnitude to cause the marked increases in antibody levels (Figure 5). However, it is important to note that the Treg assays were performed on the spleen and thymus cell populations whereas the bone is a major source of plasma cells that produce serum antibody (Winter *et al.*, 2010). Thus, one explanation for these data is that the serum antibody levels are a product of plasma cells resident in the bone marrow and speculatively, if the number of nTreg cells in the bone marrow was lower in Cd-treated offspring, then the effect would be an increase in serum antibody levels.

A reduction of 64% and 57% in males and females over control levels of splenic CD8⁺CD223⁺ cells (Figure 6) due to prenatal xenobiotic exposure has not been previously reported to our knowledge. An increase in CD8⁺CD223⁺ cells has been described in the literature as being directly correlated with an increase in autoimmune disorders (Grosso *et al.*, 2007) and T cell exhaustion (Shin and Wherry, 2007; Blackburn *et al.*, 2009). At first blush, our measured decrease in CD8⁺CD223⁺ cells may indicate a decreased susceptibility of the Cd-exposed animals to autoimmune disorders as described by (Grosso *et al.*, 2007), however, prenatal Cd-exposure caused a dramatic increase in anti-streptococcal antibodies which might be indicative of an actual increase in autoimmune susceptibility. Shin and Wherry (2007) reported that IL-2 production drops with T cell exhaustion which matches our results in the males, however, Blackburn *et al.* (Blackburn *et al.*, 2009) showed increased expression of CD8⁺CD223⁺ with T cell exhaustion due to chronic virus infection. This is a contradiction to our results. Without experiments specifically designed to determine whether these changes in CD8⁺CD223⁺ populations have functional consequences, it would be overly speculative to place a meaning on the changes noted in the CD8⁺CD223⁺ cells. However, reductions in the number of cells expressing this marker in the Cd-exposed offspring that exceed ~60% on un-manipulated cells in a population of splenic CD8⁺ T cells previously shown to be active in autoimmunity and tumor surveillance (Grosso *et al.*, 2007) as well as anti-viral defenses (Blackburn *et al.*, 2009) is of concern. These results also serve to highlight that animals exposed prenatally to a xenobiotic often show a phenotype that differs from the phenotype produced by the same xenobiotic administered during adult life.

The immunomodulatory effects of prenatal Cd exposure observed in this study may provide insight as to why children of women who smoke during pregnancy have an increased risk for developing cancer later in life. Several studies have indicated that smoking while pregnant increases the risk of certain types of childhood cancers, such as leukemia, lymphoma, and central nervous system tumors, in the prenatally exposed offspring (Filippini *et al.*, 1994; Filippini *et al.*, 2000; Schuz *et al.*, 2001; Brooks *et al.*, 2004). In the U.S., more than a million newborns are exposed to cigarette smoke during gestation (DHHS, 2000). The amount of Cd absorbed from smoking a pack of cigarettes is about 1-3 µg per day (Faroon *et al.*, 2008). The dams in our study ingested an estimated 15 µg of Cd per day, based upon the average daily water intake determined by Bachmanov *et al.* (2002). Considering that 10% of Cd ingested is absorbed (Faroon *et al.*, 2008), the body burden of these dams would be 1.5 µg per day. Therefore, without regard to differences in body surface area and route of exposure, the concentration of Cd being ingested by mice in the present experiment is equivalent to the concentration of Cd that would be inhaled from smoking 1 pack of cigarettes per day. A study in which pregnant mice were exposed to a low concentration of mainstream cigarette smoke throughout gestation increased the incidence and growth rate of EL-4 cells (mouse T-cell lymphoma) - induced tumors in male offspring, as well as reduced cytotoxic T lymphocyte activity in male offspring at 5 and 10 weeks after birth (Ng *et al.*, 2006). Ng *et al.* (2006) findings support epidemiologic data indicating that children of mothers who smoke during pregnancy have a greater risk of developing cancer later in life. The decreased macrophage number, as well as decreased IFN-γ production in male offspring in the present study may explain the increased susceptibility to tumor incidence and growth in male offspring exposed to cigarette smoke in utero.

In summary, we have demonstrated that prenatal exposure to environmentally relevant Cd levels causes persistent immunomodulatory effects in murine offspring. Thymocyte phenotype analysis determined that these effects are cell type-specific, whereas analysis of splenocyte phenotype demonstrates a cell type- and sex-specific effect. The cytokine profiles suggest an effect on peripheral Th1 cells in females and to a lesser degree in males. The decrease in Th1 type cytokine production in females and the decreases in IFN-γ production and macrophage cell number in males may lead to increased susceptibility of the

offspring to infections and tumor growth. The two major findings that defy simple explanation are a marked increase in anti-bacterial antibody levels and a marked decrease in the numbers of splenic CD8⁺CD223⁺ cells. These findings suggest that even very low exposure to Cd during gestation may result in long term detrimental effects on the immune system of the offspring, possibly resulting in cancer at adulthood, thus reinforcing that exposure to Cd during pregnancy should be limited.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Prenatal exposure to Cd causes no thymocyte phenotype changes in the offspring
- Analysis of the splenocyte phenotype demonstrates a macrophage-specific effect only in male offspring
- The cytokine profiles suggest an effect on peripheral Th1 cells in female and to a lesser degree in male offspring
- There was a marked increase in serum anti-streptococcal antibody levels after immunization in both sexes
- There a marked decrease in the numbers of splenic CD8⁺CD223⁺ cells in both sexes

Figure 1.

Prenatal Cd exposure failed to induce lasting changes to the thymocyte phenotype of 7 week-old offspring. Thymocytes were isolated from mice that were exposed to 10 ppm Cd and analyzed by flow cytometry analysis as described in the Materials and Methods. Each bar represents the mean SE. Data are representative of 3 experiments where N=4 in each group. (A) Thymocyte phenotype was determined based on CD4 and CD8 cell surface expression. (B) DN subpopulation phenotype was determined based on CD44 and CD25 cell surface expression.

Figure 2.

Prenatal Cd exposure induced minimal changes to the splenocyte phenotype of 7 week-old offspring. Spleen cells were isolated from mice that were exposed to 10 ppm Cd and analyzed by flow cytometry analysis as described in the Materials and Methods. Flow cytometric analysis was performed by staining splenocytes with anti-CD4, anti-CD8, anti-B220 (B cells), anti-F4/80 (macrophages), and anti-Gr-1 (granulocytes). Each bar represents the mean SE. Data are representative of 3 experiments where N=5-8 offspring/sex/treatment group/age group. *p<0.01

Figure 3.

Cytokine production by spleen cells from 7 week-old offspring was markedly altered by prenatal Cd exposure. Spleen cells were isolated and stimulated with anti-CD3/CD28 for 24h (IL-2) or 72h (IFN- γ , IL-10 and IL-4). Supernatants were analyzed for cytokine expression by ELISA kits. Limit of detection: IL-2 (2 pg/ml), IFN- γ , (15 pg/ml), IL-10 (30 pg/ml), IL-4 (4 pg/ml). Each bar represents the mean SE. Data are representative of 3 experiments where N=5-8 offspring/sex/treatment group. *p<0.05

Figure 4.

T-independent and T-dependent serum antibody production at 7 weeks of age was markedly altered by prenatal exposure to Cd. Seven week-old mice that were exposed to 10 ppm Cd in utero were immunized with *S. pneumoniae* heat-killed vaccine. Sera were collected 10 days after immunization for anti-PC antibody (Figure 4A) and anti-PspA antibody (Figure 4B). Antibody levels were determined by ELISA as described in the Materials and Methods. Each bar represents the mean SE. Data are representative of 2 experiments where N=5 offspring/sex/treatment group/age group. *p<0.05

Figure 5.

Prenatal Cd exposure altered the relative percentage of a population of CD4+ natural T regulatory cells (nTreg) in the spleens and thymi of 7 week-old offspring. Single cell suspensions were prepared for flow cytometry analysis by standard methods. Aliquots of unstimulated cells were stained with anti-CD4, anti-CD25 and anti-FoxP3 to quantify nTreg cells as described in the Materials and Methods. Each bar represents the mean SE. Data are representative of 2 experiments where N=5 offspring/sex/treatment group/age group.

*p<0.05

Figure 6.

Prenatal Cd exposure altered the relative percentage of CD8⁺CD223⁺ cell populations in the spleens of 7 week-old offspring. Flow cytometric analysis was performed by staining splenocytes or thymocytes with anti-CD8, anti-CD28 and anti-CD223. Each bar represents the mean SE. Data are representative of 2 experiments where N=5 offspring/sex/treatment group/age group. *p<0.05

Table 1

Serum anti-PC and anti-PspA titers in 7 week-old offspring 10 days after immunization with *S. pneumoniae* heat-killed vaccine

Antibody	Sex	Cd-treated	Control
Anti-PC			
IgM	Male	6720±2322 *	1560±163
IgG2b	Male	5660±1349 *	1500±625
IgG3	Male	7520±2298 *	1940±430
IgM	Female	2720±495 *	1940±750
IgG2b	Female	6160±1824 *	2360±1306
IgG3	Female	8720±2369 *	1540±1261
Anti-PspA			
IgM	Male	1010±599	595±100
IgG1	Male	360±163 *	50±40
IgG2a	Male	2540±936 *	175±159
IgG2b	Male	2360±724 *	77.5±38
IgM	Female	590±139	690±120
IgG1	Female	560±311 *	55±26
IgG2a	Female	1680±605 *	ND
IgG2b	Female	1800±594 *	10±5.5

ND, none detected

*
p<0.05;