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A Cohort Study of Immune Cells in Children with Perinatal Exposure to Polychlorinated Biphenyls (PCBs)

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Abstract

A Cohort Study of Immune Cells in Children with Perinatal Exposure to Polychlorinated Biphenyls (PCBs)

By Kathleen Chapman Hartnett

Experimental studies have shown that polychlorinated biphenyls (PCBs) suppress immune function in animals. The aim of this study was to determine whether perinatal exposure to PCBs affects lymphocytes and other immune cells in children at age 16 months. We sampled 310 mother-child pairs from a birth cohort in the Slovak Republic. Because many women in our study lived near former PCB manufacturing plant, the mean serum level of PCB-153 was about 10 times higher than women of childbearing age in the U.S. Perinatal exposures were estimated by PCB concentrations in maternal serum sampled shortly after delivery. To obtain a measure of total PCBs, we summed the six most prevalent congeners and adjusted for serum lipids. At age 16 months, the children's blood samples were tested for cell surface expression markers. We analyzed 16 outcomes, including markers for T cells and their subsets, B cells, activated B cells, myeloid and lymphoid dendritic cells, dendritic-like cells, and macrophage-like cells. Because the Slovak and Romani mothers in our sample differed on many demographic characteristics, we stratified by ethnicity. After adjustment for potential confounders, we found lower proportions of cytotoxic T cells (CD8+) in Romani children with higher perinatal exposure to PCBs (Beta coefficient representing change in cytotoxic T cell proportion for each 1-unit change in log lipid-adjusted maternal PCBs: -3.34, 95% CI: -5.99, -0.68). The association in Slovak children was not as strong, however, and the 95% CI for the total population was imprecise (Beta coefficient:-1.14, 95% CI: -2.34, 0.06). We did not find an association between perinatal PCB exposure and any of the other 15 outcomes. Perinatal exposure in this highly-exposed population did not appear to affect lymphocyte proportions at age 16 months, except possibly cytotoxic T cells.

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Contents

Background/Literature Review

Children who are exposed *in utero* to certain environmental pollutants may be at higher risk of immune suppression and infections. Polychlorinated biphenyls (PCBs) are highly persistent chemicals that have been shown to impair immune function in animals. First produced in 1929, they were used to fill transformers, capacitors and heat exchanges, as well as in consumer products including paints and lubricants (Safe 1994). Improper storage and disposal of PCBs led to contamination of the soil, water and sediment around the factories where they were manufactured. Because PCBs are highly lipophilic and not easily metabolized, they are stored in fat and accumulate in fish and animals at the top of the food chain. People who live far from the original manufacturing sites can thus be exposed by eating contaminated food, especially large fish. Most countries banned production of PCBs in the 1970s based on evidence that they were toxic to wildlife, and levels measured in humans and animals have gradually declined since then. However, due to their persistence, PCBs remain a potential health hazard in many parts of the world.

Coplanar PCB congeners are structurally similar to 2,3,7,8-tetrachloro-dibenzodioxin (TCDD), which has been shown to be immunotoxic in animals. Like TCDD and the polychlorinated dibenzofurans (PCDFs) and dibenzodioxins (PCDDs), these PCB congeners appear to affect the immune system by binding to the aryl hydrocarbon (Ah) receptor (Safe 1994; Tryphonas 1995). High concentrations of Ah receptors are found in the thymus, a specialized organ where T lymphocytes mature and differentiate. The thymus is largest and most active during the neonatal period and plays

an important role in the early formation of the immune system. The most consistent finding across animal studies is that exposure to TCDD *in utero* causes the epithelium of the thymus to atrophy, possibly impairing the production and differentiation of T cells (Vos et al. 1997). Because the immune system matures during the perinatal period, children may be particularly vulnerable to immunotoxicants in the months before and after birth. In addition to T cells, macrophages and dendritic cells also appear to be particularly sensitive targets (Dietert 2008).

Common measures of immune function include incidence of infections, response to vaccines, measures of allergic reaction, concentrations of antibodies including immunoglobulin (Ig) M, A, and G, and changes in lymphocyte subsets, particularly cytotoxic T (CD8+) cells and helper T (CD4+) cells. Cytotoxic T cells are crucial to immune function, because they kill tumor cells and stop the replication of viruses. When activated by dendritic cells, CD4+ T cells differentiate into cells that fight specific antigens and helper T cells. These helper T cells regulate immune response by mobilizing antibodies, cytotoxic T cells, macrophages and other cells that fight pathogens. Over-activation of helper T cells results in the secretion of too many IgE antibodies, which trigger allergic reactions such as asthma (Moser and Leo 2010).

Several studies have found immune suppression associated with PCB exposure in animals. Tryphonas et al. found changes in T cell subsets in adult female rhesus monkeys exposed to PCBs (Tryphonas et al. 1991). High doses of PCBs decreased the relative numbers of T helper cells, while increasing cytotoxic T cells. There was no change in the total lymphocyte count or relative number of B cells in the monkeys, indicating that

PCBs specifically affected the T cell subsets. In association with higher levels of PCBs, researchers have also found decreases in IgG in polar bears (Bernhoft et al. 2000), declines in T-cell mediated immune responses in harbor seals (De Swart et al. 1995), and impaired lymphocyte responses in bottlenose dolphins (Lahvis et al. 1995).

Studies of humans accidentally exposed to extremely high concentrations of PCBs have also shown lower antibody concentrations and impaired T cell responses. In 1979, people living in central Taiwan were poisoned after cooking oil was accidentally contaminated with PCBs and with lower levels of PCDFs and PCDDs. The resulting symptoms were called Yu-Cheng, or oil disease. In the first year after the accident, a sample of 30 poisoned adults (Lu and Wu 1985) had lower serum levels of IgA (Mean ± sd: 185 ± 88 mg-% in poisoned adults vs. 245±70 mg-% in 23 controls) and IgM (Mean ± sd: 105 ± 58 mg-% vs. 173±48 mg-% in controls) but not IgG (Mean ± sd: 1,469 ± 566 mg-% vs. 1,377 ± 214 mg-% in controls).

In the first year after the accident, the same sample of 30 poisoned adults in Taiwan had a lower percentage of total T cells (Mean ± sd: 41.7 ± 16.3 vs. 63.3 ± 9.5 in 23 controls) active T cells (Mean ± sd: 11.3 ± 6.7 vs. 22.1 ± 4.4 in controls), and helper T cells (Mean ± sd: 21.6 ± 6.9 vs. 36.9 ± 12.1 in controls). There was no difference between poisoning patients and controls, however, in percentages of B cells (Mean ± sd: 28.9 ±7.1 vs. 26.6 ± 6.1 in controls) or suppressor T cells (Mean ± sd: 22.2 ± 8.7 vs. 24.9 ± 8.7 in controls). By the fourth year after the accident, the adult poisoning patients' total T cell percentages had returned to normal and their percentage of suppressor T cells had

increased (Lu and Wu 1985). Percentages of helper T cells, however, remained low (Mean ± sd: 36.1 ± 12.2 vs. 45.0 ± 6.4 in 27 controls).

Taiwanese mothers who had cooked with the contaminated oil when pregnant reported higher incidence of pneumonia and bronchitis in their infants. Thirty of 124 highly-exposed children had bronchitis or pneumonia before age 6 months, according to their mothers, compared to 5 out of 115 children in the comparison group (Rogan et al. 1988).

Chao et al. found a higher prevalence of middle-ear disease in 103 Yu-Cheng children than in matched controls, indicating that these children either got more ear infections or were unable to clear them as easily (Chao et al. 1997). Forty-four of the 103 Yu-Cheng children (42.7%) had at least one abnormal ear, compared with 18 of the 96 control children (18.8%).

Some Inuit populations also have unusually high levels of PCBs, due to traditional diets that are heavy in fish and can include large marine animals like whale and seal. In a cohort of Inuit infants born in northern Quebec, Dewailly et al. found a higher incidence of acute ear infections in children exposed to higher levels of certain organochlorines (Dewailly et al. 2000). The number of acute ear infections in the children's first year were consistently associated with p,p'-Dichlorodiphenyldichloroethylene (p,p'-DDE) and hexachlorobenzene (HCB) measured in fat from maternal breast milk (Relative risk for highest vs. lowest tertile of p,p'-DDE exposure: 1.52, 95% CI: 1.10-2.03; RR for HCB:1.49, 95% CI: 1.10-2.03). The association between total PCBs and ear infections in this population was not as strong,

with a confidence interval that included the null (RR for highest tertile of >873 µg/kg vs. lowest tertile of <432 µg/kg: 1.28, 95% CI: 0.92-1.77). However, because levels of all of the organochlorines were closely correlated, the authors concluded that it was not possible to determine which specific pollutants might be responsible for the increased risk. The authors found no association between prenatal organochlorine parameters and IgG, IgA, or IgM. There was no difference in lymphocyte subsets, including T helper cells or cytotoxic T cells, between breast- and bottle-fed infants at 3, 7, or 12 months (Mean ± sd for helper T cells in breastfed infants at 12-months: 2.5 ± 1.1, same as bottle-fed infants; mean±sd for cytotoxic T cells in breastfed infants at 12 months: 1.8 ± 1.2 vs. 1.7 ± 1.0 in bottle-fed infants).

In a second cohort of Inuit children born several years later, the researchers found a slightly higher incidence of four common types of infections in infants with the highest levels of prenatal exposure to PCB-153 (Dallaire et al. 2004). Infants in the highest quartile of prenatal PCB exposure (>170 µg/kg lipid-based), measured in maternal plasma at delivery, might have had more upper and lower respiratory tract infections, ear infections, and gastrointestinal infections than those in the lowest quartile (<57.6 µg/kg lipid-based). The adjusted incidence RR for all infections in the first 6 months of life, comparing the highest quartile to the lowest, was 1.27 (95% CI: 0.98-1.66). When researchers instead considered infections in the first 12 months, the RR was 1.08, with a 95% CI of 0.92-1.28. There was no association between postnatal PCB exposure, measured in infant plasma around seven months, and infection incidence.

In a third cohort of Inuit children, the researchers found higher rates of acute ear infections and lower respiratory tract infections from birth through age 5 associated with the highest levels of PCB-153 in umbilical cord blood (Dallaire et al. 2006). Incidence rate ratios comparing the most exposed quartile (150.6-653.6 µg/kg) to the least (12.3-58.2 µg/kg) were 1.25 for acute ear infections (95% CI: 1.10-1.41) and 1.40 for lower respiratory tract infections (95% CI: 1.18-1.67). However, upper respiratory tract infections were not associated with prenatal PCB exposure (RR 1.00, 95% CI: 0.89-1.12).

A study that included newborns in remote Canadian fishing villages and an urban reference group found lower proportions of naïve T cells in umbilical cord blood samples with higher levels of PCBs and methyl mercury (Belles-Isles et al. 2002). The Spearman correlation coefficient for the association between naïve T cells and total umbilical cord PCBs in 96 children was -0.22 (p=0.03). The fishing and comparison populations did not differ in total number of lymphocytes, or in the proportion of helper T cells (geometric mean: 50.9, 95% CI: 47.8-54.3 in fishing population vs. 49.0, 95% CI: 46.2-51.9 in controls), cytotoxic T cells (geometric mean: 22.8, 95% CI: 21.1-24.7 vs. 24.3, 95% CI: 22.7-26.0), total B cells (geometric mean: 8.2, 95% CI: 6.7-10.0 vs. 8.2, 95% CI: 6.9-9.7), or natural killer cells (geometric mean: 10.7, 95% CI: 8.7-13.1 vs. 11.4, 95% CI: 9.5-13.6).

In subsequent study, the researcher found no difference between the subsistence fishing population and the reference group in the proportion of T cells showing markers of activation (Bilrha et al. 2003). Based on those results and an earlier study in adults (Svensson et al. 1994), the authors concluded that environmental contaminants did not

appear to affect the differentiation, maturation or proliferation of T cells in this population. However, the researchers did find that cord blood mononuclear cells of 44 highly-exposed newborns secreted fewer cytokines in response to stimulation than those of 38 newborns in the comparison group. Secretion of tumor necrosis factor-α (TNF-α), an important pro-inflammatory cytokine, was inversely associated with levels of plasma PCBs (Pearson's R: -0.260, p=0.018), *p,p´*-DDE (Pearson's R: -0.289, p=0.008), and HCB (Pearson's R: -0.284, p=0.009).

Grandjean et al. hypothesized that early exposure to immunotoxicants might increase children's risk of asthma and allergies (Grandjean et al. 2010). The authors found a positive association between total IgE concentration, a marker for allergic diseases, and current serum PCBs in 7-year-old children from a birth cohort in the Faroe Islands. Among children with high serum total IgE concentration (>47.7kU/L), the geometric mean PCB concentration was 0.88 µg/g serum lipid (95% CI: 0.56-1.47), compared to 0.66 (95% CI: 0.35-1.30) in children with low IgE (<13.6kU/L). The p-value for the correlation of log-transformed PCBs and IgE in the 7-year-old children was 0.005. There was no association, however, between prenatal PCB exposure and IgE concentrations at age 7. The authors reported a lower prevalence of the allergic disorder atopic dermatitis in children with higher PCB exposures, especially prenatal exposure (geometric mean prenatal PCBs in children with atopic dermatitis: 0.70, interquartile range: 0.55-1.58 µg/g serum lipid vs. 1.24 with IQR 0.83-2.0 in healthy children; p-value for t-test=0.01). Children with a history of asthma were slightly more likely to have higher PCB exposures, but the association could have occurred by chance (geometric

mean prenatal PCBs in asthmatic children: 0.89, IQR: 0.49-1.70 µg/g serum lipid vs. 1.24 with IQR 0.83-2.0 in healthy children; p-value for t-test=0.17).

The authors also investigated 129 Faroese children's responses to routine vaccines as a measure of how well their immune systems were able to fight infection (Heilmann et al. 2006). They found that children with higher PCB levels had lower antibody concentrations after the diphtheria vaccine at 18 months (-24%, 95% CI for percent decrease: 1.63-41.9) and tetanus at 7 years (-16.5%, 95% CI for percent decrease: 1.51-29.3). A subsequent study with a larger sample of 587 Faroese children yielded similar results (Heilmann et al. 2010). Antibody concentrations after diphtheria and tetanus vaccines at age 7 were lower in children who had higher levels of PCBs measured in their serum at 18 months. In the strongest associations, antibody response was lower by 20% for each doubling of PCB exposure. At age 5, the odds of the children having antibody concentrations too low to provide long-term protection against diphtheria were 30% higher for each doubling of PCB level in maternal milk and at 18 months.

Other studies have examined populations with lower PCB exposures that represent typical background pollution levels in industrialized countries. In a study of 3-month-old infants in Sweden, Glynn et al. found that the percentage of cytotoxic T cells was lower in those exposed to higher levels of prenatal PCB-153, as well as total mono-*ortho* (105, 118, 156, 167) and di-*ortho* PCBs (138, 153, 180), but only in a crude analysis (Glynn et al. 2008). In models that adjusted for potential confounders, there was no association between any measure of PCB congeners and the percent of cytotoxic T

lymphocytes, total T cells, T helper cells, B lymphocytes, or natural killer cells. The sample size was small, with data on lymphocyte subsets available for only 52 infants.

In the same population, Glynn et al. found an increased risk of respiratory infections in the first three months for infants with the highest prenatal exposure to PCB congeners 28, 52, and 101 (Glynn et al. 2008). In contrast, the researchers reported a lower risk of infection in infants with the most prenatal exposure to mono-*ortho* PCBs (adjusted OR for highest category of exposure vs. lowest: 0.23, 95% CI: 0.06-0.91) and di-*ortho* PCBs (aOR for highest vs. lowest: 0.29, 95% CI: 0.08-1.0, aOR for second-highest to lowest: 0.23, 95% CI: 0.07-0.71).

In a cross-sectional study of 343 German children aged 7-10, Karmaus and Kruse did not find an association between PCB exposure and ear infections, pneumonia, whooping cough, or IgE (Karmaus et al. 2001). DDE, however, appeared to be associated with fewer infections but more allergic reactions, as measured by IgE. For children above the 50th percentile for DDE exposure, total PCBs appeared to increase the risk of ear infections (for DDE ≥0.3 µg/l, total PCBs >0.48 µg/l vs. ≤0.48 µg/l adjusted OR: 3.70, 95% CI: 1.64-8.34).

The concentration of IgM was higher in children with higher levels of total PCBs, and children with the highest PCB exposure had a reduced white blood cell count (Karmaus et al. 2005). There was no difference, however, in the number of T cells (2,156 for total PCBs >0.75; 2,286 for total PCBs ≤0.30), T helper cells (1,189 for total PCBs >0.75 µg/L; vs. 1,276 for total PCBs ≤0.30 µg/L), cytotoxic T cells (752 vs. 773),

memory T cells (325 vs. 316), natural killer cells (364 vs. 382), or B cells (469 vs. 463) across levels of PCB exposure.

Nagayama et al. did find differences in T cell proportions in a sample of 36 breastfed infants in Japan. The authors estimated the total postnatal intake of co-planar PCBs, PCDDs and PCDFs, calculated from concentrations in maternal milk sampled three months after birth (Nagayama et al. 1998). Higher total intakes were associated with a higher ratio of helper to cytotoxic T cells ($p=0.025$ for the correlation).

In a longitudinal cohort of children born in the Netherlands between 1990 and 1991, ten Tusscher et al. measured PCDD/Fs in maternal milk shortly after birth (ten Tusscher et al. 2003). The authors reported a negative association between allergies and prenatal and postnatal dioxin exposure in 8-year-old children. The authors also found increases in T helper cells (slope: 0.083; 95% CI: 0.026-0.140) and in the CD45RA+ (naïve T) cell count (slope: 0.078; 95% CI: 0.014-0.141) associated with postnatal exposure, a measure that they extrapolated from the concentration in maternal milk and duration of breastfeeding (ten Tusscher et al. 2003). There was no association, however, between dioxin exposure and a history of ear infections, chicken pox, or pneumonia.

When children in the cohort were ages 14-19, the researchers measured dioxin-like PCBs in the serum of 29 participants (Leijs et al. 2009). Higher levels of dioxin-like PCBs in these adolescents were associated with lower concentrations of polymorphic neutrophils.

In a separate cohort of Dutch infants, Weisglas-Kuperus et al. calculated prenatal and postnatal exposures using the total toxic equivalence factor (TEQ) of dioxins and PCBs (Weisglas-Kuperus et al. 1995). The researchers found no association between prenatal or postnatal TEQ and rhinitis, bronchitis, tonsillitis or ear infections during the first 18 months after birth. There were also no significant associations between TEQ and levels of antibodies to mumps, measles and rubella at 18 months. However, there was a positive association between the T cell receptor TcR$\gamma\delta^+$ and PCBs in the children at birth. At 18 months, there was a positive association between total prenatal PCBs and the total number of cytotoxic T cells n 48 infants (Spearman rank correlation coefficient=0.38; p=0.01). A higher total TEQ level was positively associated with the T cell receptor TcR$\alpha\beta^+$ (Spearman rank correlation coefficient: 0.57; $p \leq 0.05$).

In a subgroup of 85 children at age 3 and a half, the researchers found that prenatal PCB exposure was positively associated with the total number of lymphocytes, T cells (Pearson correlation coefficient for total maternal PCBs and absolute T cell count=0.25; p=0.02), cytotoxic T cells (Pearson correlation coefficient=0.27; p=0.01), memory T cells (Pearson correlation coefficient=0.25; p=0.02), T cell receptor $\alpha\beta^+$ (Pearson correlation coefficient=0.25; p=0.02) and activated T cells (Pearson correlation coefficient=0.26; p=0.02) (Weisglas-Kuperus et al. 2000). The researchers also found fewer allergies in children with a higher current body burden of PCBs. Preschoolers with the highest current PCB body burden, however, had more recurrent middle-ear infections (OR: 3.06; 95% CI: 1.17-7.98) and chicken pox (OR: 7.63; 95% CI: 1.21-48.54).

When the children reached school age, a higher prevalence of middle ear infections was associated with postnatal PCB exposure through breastfeeding, but not with the total TEQ (Weisglas-Kuperus et al. 2004). The children with higher prenatal PCB exposure had a lower prevalence of chicken pox from age 3 to 7 (OR: 0.53; 95% CI: 0.30-0.94), and less shortness of breath with wheeze (OR: 0.59; 95% CI: 0.36-0.97). Chicken pox and symptoms of asthma, however, were not associated with cord PCBs or postnatal exposure through breast milk.

In summary, the evidence is mixed on how perinatal PCB exposure may suppress immune response in children, particularly as measured by concentrations of lymphocytes and their subsets (Table A). No studies in children have found any association between PCBs and natural killer or B cells. But some studies have reported differences in T cell populations. Weisglas-Kuperus et al. found higher concentrations of total T cells in 3-year-old children exposed to PCBs, while two other studies (Karmaus et al., N=331 and Glynn et al., N=52) reported null results. Weisglas-Kuperus et al. were also the only researchers to find higher concentrations of cytotoxic T cells in 18-month-old and 3-year-old children exposed to higher PCB levels; four other studies found no association (Belles-Isles et al. N=120, Dewailly et al., N=96, Glynn, and Karmaus). Weisglas-Kuperus et al. also found higher concentrations of memory T cells, while Karmaus et al. found no association. Ten Tusscher et al. found a positive association between PCB exposure and T-helper (CD4+) cell concentrations in a sample of 27 children, but four larger studies (Belles-Isles, Dewailly, Glynn, and Karmaus) were null. The two teams of researchers that examined the association between naïve T cells and perinatal PCB

exposure came to opposite conclusions, with Belles-Isles et al. reporting lower concentrations in the highly exposed, and ten Tusscher reporting higher concentrations.

Although most industrialized countries banned the manufacture of PCBs in the 1970s, PCB production in the former Czechoslovakia continued until the mid-1980s. In the Michalovce district of what is now eastern Slovakia, the Chemko Company manufactured PCBs from 1959 until 1984 (Kocan et al. 1994). The company released effluent into a canal that flowed into the Laborec River and a local reservoir, contaminating the water, underlying sediment, and surrounding soil (Kocan et al. 2001). Studies in the 1980s and 1990s showed that adipose tissue levels of PCBs in Michalovce residents were up to three times higher than in other districts sampled in the former Czechoslovakia. And from the 1970s through the mid-1990s, residents of districts throughout Czechoslovakia had adipose tissue PCB levels several times higher than in other industrialized nations (Kocan et al. 1994).

An analysis of environmental samples in 1998 found that PCB levels remained high in Michalovce's air, reservoir water, soil, sediment, and fish (Kocan et al. 2001). Michalovce residents, particularly those who eat locally produced meats and dairy products, continue to have elevated levels of PCBs. Residents who reported eating locally-grown products with high fat content, such as pork, butter, and milk, have the highest levels (Sonneborn et al. 2008). In a sample from our cohort of mother-infant pairs recruited between 2002 and 2004, maternal levels of the most prevalent congener, PCB-153, were 10 times higher than in U.S. women of childbearing age during the same time (Jusko et al. 2011). Polychlorobiphenyl (OH-PCB) metabolite levels in the Michalovce

mothers were higher than in the Netherlands and Sweden, comparable to Faroe Island women who eat little fish, and about half as high as Inuit women in northern Canada (Park et al. 2007).

Previous studies in a cohort of infants recruited at birth from Michalovce and a comparison district in Slovakia have shown possible immune effects of PCBs. Using ultrasound to estimate the thymus volume of 982 newborns on their third or fourth day after birth, Park et al. found that the size was inversely associated with prenatal PCB exposure (Park et al. 2008). Children at the 90th percentile of serum PCB concentration had a 7% smaller thymic index than children at the 10th percentile. The findings suggest that *in utero* PCB exposure might cause thymic atrophy in humans, as it seems to in animals.

In a subset of 384 infants from the cohort, Jusko et al. analyzed post-vaccination antibody response, a T-cell dependent measure of immune function, at age 6 months (Jusko et al. 2010). In contrast to the Dutch and Faroese cohorts, there was no association between PCB exposure and vaccine antibodies in Slovak infants. For anti-haemophilus influenza type b, the estimated percent change for an increase in wet-weight total maternal PCBs was -6.8 percent, with a 95% CI of -23.4-13.3. Confidence intervals for tetanus toxoid (95% CI: -19.5-20.1) and diphtheria toxoid (95% CI: -18.9-12.5) were also null and imprecise. The results were similarly null for PCBs in umbilical cord serum and the infants' own serum at age 6 months. Jusko et al. also found no relationship between maternal PCBs and measures of the infants' IgG (% change: 1.2, 95% CI: -4.2-7.0%), IgA (% change -2.0, 95% CI: -9.9-6.5%), IgM (% change: 0.1, 95% CI: -6.1-6.6%), or

IgE (% change: 7.4, 95% CI: -8.7-26.5). There was also no association between PCBs in cord or infant serum and IgG, A, M, or E (Jusko et al. 2011).

The possible effects of PCBs in this highly-exposed population thus remain a puzzle. Although there is evidence of thymus atrophy at birth, pre- and postnatal PCB exposure did not appear to result in immune suppression by age 6 months. In order to better understand how perinatal PCB exposure may affect the developing immune system, we analyzed concentrations of lymphocyte subsets in a sample of the children at age 16 months.

Materials and Methods

Sample selection. Between 2002 and 2004, women in the more heavily polluted district of Michalovce and the comparison area of Svidnik and Stropkov were recruited into the study when they came to local hospitals to give birth. A total of 1,134 mother-infant pairs enrolled in the cohort, including 811 from Michalovce and 323 from the comparison area. Women were excluded from the study if they were younger than 18, had more than four previous births, had lived in their district for less than 5 years, or had a major illness while pregnant. Infants with birth defects were also excluded. After the women gave informed consent, research staff collected maternal and umbilical cord blood. The mothers provided answers to a questionnaire that included years of school completed, home environment, use of alcohol and tobacco, and a medical history that included past pregnancies. The infants' birth weight and gestational age were abstracted from medical records. From this cohort, the research team selected 310 children to return at age 16 months. These children provided a serum sample that was tested for CD marker expression. The 310 children in our subset selected for lymphocyte analysis at age 16 months were not chosen from the full cohort by simple random sample; children at the highest levels of PCB exposure were overrepresented. To account for this lack of random sampling, we applied weights that were inversely proportional to the sampling probabilities in each stratum of PCB exposure.

This study was approved by the Institutional Review Board at the University of California, Davis. The Institutional Review Board at Emory University gave approval for the secondary data analysis. Previous papers describe sample selection, consent protocols, and data collection in more detail.(Jusko et al. 2010)

PCB and lipid measurement. Concentrations of PCBs were measured in maternal serum at The Department of Toxic Organic Pollutants at the Slovak Medical University in Bratislava. The serum was tested for 15 PCB congeners: International Union of Pure and Applied Chemistry (IUPAC) numbers 28, 52, 101, 105, 114, 118, 123^{+149}, 138^{+163}, 153, 156^{+171}, 157, 167, 170, 180, and 189. Total serum lipids were calculated using the enzymatic summation formula from Akins et al (Akins et al. 1989). and Takayama et al (Takayama et al. 1977). We used lipid-adjusted total maternal PCBs at the time of birth as a marker of prenatal exposure. Congeners that were below the limit of detection (LOD) in more than 20% of samples were excluded. To calculate total maternal PCBs, we took the arithmetic sum of the six congeners with fewer than 20% of samples below the LOD, which included PCB 118, 138^{+163}, 153, 156^{+171}, 170, and 180.

Cell surface expression. Research staff in Slovakia measured T lymphocytes (CD3+), and the following T subsets: T helper cells (CD4+), cytotoxic T cells (CD8+), natural regulatory T cells (CD4+CD25+), memory T cells (CD4+CD45RO+CD45RA-), naïve T cells (CD4+CD45RO-CD45RA+), suppressor inducer T cells (CD4+CD62L+), truly naïve helper/inducer T cells (CD4+CD62L+CD45RA+), and terminally differentiated effector memory T cells (CD4+CD62L-CD45RA+). The Slovak research team also measured natural killer cells (CD3-CD(56+16)+), B lymphocytes (CD19+), activated B lymphocytes (HLADR+CD19+), myeloid dendritic cells (CD19-CD11c+CD11b+), lymphoid dendritic cells (CD19-CD11c+CD11b-), dendritic-like cells (CD83+CD19+), and macrophage-like cells (CD19-CD11c-CD11b+).

Laboratory methods are described in more detail elsewhere.(Horvathova et al. 2011) CD4+ T cells were gated through lymphocytes, while the gate for dendritic cells, dendritic-like cells and macrophage-like cells included all mononuclear cells. For multiparametric analysis, research staff took percent positive values from quadrants with isotype control antibodies. The antigen cell surface density is the percentage of positive events within a region.

Data analysis. We used SAS version 9.2; SAS Institute Inc., Cary, NC, USA for all statistical analyses. Because Roma and Slovak/Eastern European mothers differed on key characteristics including age at delivery, level of education, number of previous births, and exposure to cigarette smoke, we stratified our analyses by ethnicity. We identified potential confounders through literature review and a Directed Acyclic Graph (DAG). We expected the causal relationships would be similar across lymphocyte subsets, so used one DAG for all 16 CD marker outcomes. Our final model included the following covariates: maternal age at delivery (years), smoking before or during pregnancy (yes/no), maternal passive smoke exposure (no/rarely/sometimes/often), maternal education level (basic schooling/some high school/high school graduate/college or higher) and child sex.

A total of 310 children had their serum tested for lymphocyte subsets at 16 months. For our exposure, we used the weight wet concentration of maternal PCBs measured in units of nanograms per milliliter (equivalent to parts per billion). Maternal PCB levels followed a log-normal distribution, so we used the log-transformed concentrations, adjusted for serum lipids.

Five of the 310 children did not have measurements for serum lipids, so were excluded from the model. Seven of the outcomes – total T cells, helper T cells, naïve T cells, terminally differentiated effector memory T cells, cytotoxic T cells, B cells, and activated B cells - were normally distributed. Because the other nine outcomes were skewed, we transformed them using the natural log before including them in the models.

We fit regression models using the SURVEYREG procedure, which provides a larger estimate of standard error to account for the possibility that the sampled children may have more in common than those chosen at random.

In order to evaluate whether possible associations between PCB exposure and lymphocyte subsets were influenced by extreme values, we used the leverage statistic to identify highly influential outliers. For each immune outcome that appeared associated with PCBs, we ran regression models with the highly influential outliers (absolute value of the leverage statistic >3) removed.

Results

Table 1 presents demographic characteristics of the 305 mother-child pairs in our study, classified above and below-median maternal PCBs. Most of the children in our subset lived in the more heavily polluted area of Michalovce (83.9%) and were born at term (91.9%). Exposure to cigarette smoke in this population was high, with 37.7% of mothers saying that they smoked during pregnancy and 65.8% saying they were exposed to passive smoke around the time the child was born.

Compared to Slovak and other Eastern European mothers, Romani mothers were less educated, had more children, had lower birth weight babies, and were more likely to be exposed to cigarette smoke during pregnancy. None of the 52 Romani mothers in this sample reported finishing high school, while 47.1% of the Slovak/Eastern European women were high school graduates and 5.2% had a college degree. Among Romani mothers, 73.1% said they smoked during pregnancy, and 86.5% percent reported exposure to passive smoke, compared to 37.7 percent and 65.8 percent of Eastern European mothers, respectively. Only 13.5% of Romani infants weighed at least 3,500 grams at birth, compared to 39% of Slovak/Eastern European mothers.

Median lipid-adjusted total maternal PCB concentrations in Michalovce were more than twice as high as those in the comparison districts of Svidnik and Stropkov (715.4 ng/ml vs. 312.8, $p<0.0001$). Serum PCBs for the 257 mothers in Michalovce ranged from 138.8 to 11,707.5 ng/ml (25^{th} percentile: 466.1; 75^{th} percentile: 1,105.3).

Mean T cell proportions were slightly lower in children of Roma ethnicity (mean: 61.8, 95% CI for mean: 59.4-64.1 in Romani vs. mean 64.8, 95% CI for mean: 63.9-65.8 for Slovak and other). T cell proportions also differed by district, with a mean of 65.0 in

Michalovce and 60.9 in Svidnik. T cells were not associated with any of the other covariates. B cell proportions did not appear to be associated with any of the covariates.

Higher maternal PCBs were associated with lower proportions of cytotoxic T cells in Romani children (Table 3) at age 16 months. The inverse relationship between PCB exposure and cytotoxic T cells was weaker in the total population, and the 95% CI included the null value. Two children were highly influential outliers in the model of PCBs and cytotoxic T cells. Removing these children from the analysis did not change the results (Beta coefficient: -1.14, 95% CI: -2.34-0.06 in total sample vs. -1.05, 95% CI: -2.21-0.11 with the two highly influential outliers removed).

Slovak and Romani children with higher exposures to maternal PCBs also had lower proportions of total T cells, although the confidence intervals were imprecise (Table 3). For most of the immune outcomes, the direction of association was the same in Slovak and Romani children. However, Slovak children with higher PCB exposures had lower proportions of terminally differentiated effector memory T cells at age 16 months, while the Romani children had higher proportions. The confidence intervals were imprecise, however, so the apparent difference in direction of effect may be the result of random error.

Discussion

We found lower proportions of cytotoxic T cells in 16-month-old Romani children who were exposed to higher levels of PCBs *in utero*. This association was weaker in Slovak and other Eastern European children, however, and the estimate for the total population was imprecise. Total T cell proportions may have also been lower in children with higher perinatal PCB exposure.

CD8+ expressing cytotoxic T cells play a key role in killing dysfunctional cells, including tumor cells and those infected with a pathogen. More recent studies have shown that they can inhibit replication of viruses without damaging the integrity of healthy cells (Moser and Leo 2010). Cytotoxic T cells may be especially important in fighting new strains of viruses such as influenza, when the antibody response may be inadequate (Kohlmeier and Woodland 2009).

In animal studies, exposure to TCDD reduces the differentiation and proliferation of cytotoxic and other T cells, reducing their ability to fight infection (Lawrence et al. 2006). The evidence on whether perinatal PCB exposure affects the cytotoxic T cell response in children remains inconclusive. In contrast to our results, Weisglas-Kuperus et al. found a positive association between total maternal PCBs and cytotoxic T cells in a sample of Dutch children at ages 18 months (Spearman rank correlation coefficient=0.38; p=0.01) and 3 years (Pearson correlation coefficient=0.27; p=0.01). In a cross-sectional study of 331 German children, Karmaus et al. found no association between the children's PCB levels at ages 7 to 10 and cytotoxic T cells (cell count 752 for total PCBs > 0.75 µg/L vs. 773 for total PCBs ≤ 0.30 µg/L). However, these study participants were

much older than the 16-month-olds in our sample, and the PCB levels measured may not reflect *in utero* exposures. Three smaller studies also found no association, but may have been underpowered to detect small differences.

Our findings may be consistent, however, with those of Nagayama et al., who found that the total TEQ of PCBs, PCDDs and PCDFs in breast milk was associated with a significantly higher ratio of helper T cells to cytotoxic T cells in a sample of 36 infants at age 3 months. Considered separately, helper T cells appeared to increase and cytotoxic T cells appeared to decrease with higher PCB exposures.

Fan et al. proposed that an inverse U-shaped relationship between these environmental contaminants and immune outcomes might explain why observational studies have reached such different conclusions In a study of rats, the authors found that high doses of 2,3,7,8-TCDD suppressed immune function, while lower levels actually improved cell-mediated immunity (Fan et al. 1996). Indeed, studies of populations with high exposures, such as the adult victims of the rice oil poisoning and the Inuit in northern Canada, have shown both decreases in T cell subsets and a high rate of infection. By contrast, European studies of lower exposure levels more typical of those in developed countries have more often found null or positive associations between PCBs and immune cell outcomes. These differences could also be the result of a threshold effect whereby lower levels of PCB exposure do not result in adverse outcomes. The mothers in our study were exposed to higher levels of PCBs than most European women of childbearing age, but were only about half as high as those of the Inuit women.

Given the possible decline in cytotoxic T cells, it is surprising that we did not see any evidence that PCB exposure was associated with changes in dendritic cells. These

specialized antigen-presenting cells play a key role in activating T cells, and evidence has accumulated in recent years that TCDD suppresses T-cell responses by interfering with dendritic cell signaling or survival (Nguyen et al. 2010; Bankoti et al. 2010; Ruby et al. 2005; Vorderstrasse et al. 2003; Frawley et al. 2011). Jin et al. recently reported that exposure to TCDD limits the ability of dendritic cells to activate naïve cytotoxic T cells. (Jin et al. 2010).

To our knowledge, this is the first study to examine the association between PCB exposure and dendritic cell proportions in children. Future studies should seek to clarify the relationship between dendritic cell and T-cell function in children exposed to dioxin and PCBs.

One major strength of our study is its size. To date, this is the largest longitudinal study of perinatal PCB exposure on immune cell outcomes. We did not, however, have measures of lymphocyte subsets past the age of 16 months. It is unclear how much these measures may fluctuate in both the short and long-term. Future studies should determine whether exposure to environmental pollutants can cause permanent damage to children's immune function, or whether these changes may be more transient. It is also important to assess whether changes in these lymphocyte subsets predict increases in infection rates. Although some studies have analyzed related outcomes such as lymphocyte proportions, vaccine response, and infection rates, few have examined the extent to which these outcomes are correlated.

In summary, perinatal PCB exposure was associated with a possible reduction in cytotoxic T cells but not with dendritic or other immune cells. Because the declines in cytotoxic T cells were most pronounced among the Romani children, future studies

should determine whether vulnerable populations may be more susceptible to environmental contaminants. Researchers should also investigate whether changes in immune cell proportions can increase children's risk of infection.

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Tables

Table A. The association between PCB exposure and immune cell counts or proportions in previous studies.

First Author	N	Natural killer	Total T	T helper	Naïve T	Memory T	Cytotoxic T	B
Belles-Isles	96	none		none	inverse		none	none
Dewailly	120			none			none	
Glynn	52	none	none	none			none	none
Karmaus	331	none	none	none		none	none	none
Nagayama	36			positive			inverse	
Weisglas-Kuperus	85		positive			positive	positive	
ten Tusscher	27			positive	positive			

Table 1. Characteristics of mothers and children selected for lymphocyte analysis at age 16 months, by lipid-adjusted maternal PCB levels.

	Total (n=305)		< median PCBs		≥ median PCBs	
Characteristic	N	%	N	%	N	%
District						
Michalovce	257	84.3	111	73.0	146	95.4
Svidnik and Stropkov	48	15.7	41	27.0	7	4.6
Child gender						
Male	151	49.5	69	45.4	82	53.6
Female	154	50.5	83	54.6	71	46.4
Maternal age at delivery						
<20	19	6.2	10	6.6	9	5.9
20-29	209	68.5	116	76.3	93	60.8
30 and older	77	25.2	26	17.1	51	33.3
Maternal education						
Basic schooling	62	20.3	28	18.4	34	22.2
Some high school	76	24.9	32	21.1	44	28.8
High school graduate	144	47.2	80	52.6	64	41.8
College or higher	16	5.2	8	5.3	8	5.2
Missing	7	2.3	4	2.6	3	2.0
Parity						
0	134	43.9	78	51.3	56	36.6
1	92	30.2	45	29.6	47	30.7
2	57	18.7	22	14.5	35	22.9
3	20	6.6	7	4.6	13	8.5
4	1	0.3	0	0.0	1	0.7
Missing	1	0.3	0	0.0	1	0.7
Gestational age (weeks)						
<37	9	3.0	4	2.6	5	3.3
37-41	281	92.1	139	91.4	142	92.8
42	11	3.6	6	3.9	5	3.3
Missing	4	1.3	3	2.0	1	0.7
Birth weight (grams)						
<2500	17	5.6	9	5.9	8	5.2
2500-3499	166	54.4	87	57.2	79	51.6
3500+	120	39.3	55	36.2	65	42.5
Missing	2	0.7	1	0.7	1	0.7
Number of children younger than 6 living with study child						
0	222	72.8	106	69.7	116	75.8
1	68	22.3	39	25.7	29	19.0
2 or more	15	4.9	7	4.6	8	5.2

Table 1. continued

Maternal smoking before or during pregnancy						
No	179	58.7	99	65.1	80	52.3
Yes	117	38.4	52	34.2	65	42.5
Missing	9	3.0	1	0.7	8	5.2
Maternal passive smoke exposure						
No	94	30.8	61	40.1	33	21.6
Yes	203	66.6	90	59.2	113	73.9
Missing	8	2.6	1	0.7	7	4.6

Table 2. Median and Interquartile Range (IQR) for each of the 16 immune outcomes in 310 children.

Immune cell type	Median	25^{th} percentile	75^{th} percentile
Natural killer cells	4.7	3.0	7.5
T lymphocytes	65.6	59.8	69.3
T helper cells	41.2	35.5	45.8
Natural regulatory T cells	0.6	0.3	1.2
Naïve/resting T cells	80.5	73.3	85.2
Truly naïve helper/inducer T cells	0.9	0.2	3.7
Suppressor inducer T cells	2.2	0.6	6.5
Memory T cells	13.9	10.3	17.9
Terminally differentiated effector memory T cells	78.6	71.9	83.6
Cytotoxic T cells	18.4	14.7	21.4
B cells	26.0	21.6	31.4
Activated B cells	25.6	21.7	31.0
Myeloid dendritic cells	8.8	5.1	14.3
Lymphoid dendritic cells	3.9	1.9	9.1
Dendritic-like cells	0.1	0.0	0.3
Macrophage-like cells	1.1	0.2	2.5

Table 3. Multiple linear regression model of the association between log- and lipid-adjusted total maternal PCBs and lymphocyte subsets at age 16 months.

	Slovak and other					Romani					Total Population				
Cell type	N	β	95% CI		p	N	β	95% CI		p	N	β	95% CI		p
Natural killer+	238	0.04	-0.11	0.20	0.58	51	0.16	-0.14	0.46	0.28	289	0.11	-0.03	0.24	0.12
T	238	-1.13	-3.13	0.87	0.27	51	-1.74	-5.44	1.96	0.35	289	-1.16	-2.84	0.52	0.18
T helper	238	0.06	-2.15	2.27	0.96	51	1.12	-2.92	5.16	0.58	289	0.08	-1.82	1.98	0.93
Natural regulatory T+	238	0.01	-0.23	0.24	0.96	51	-0.19	-0.64	0.27	0.41	289	-0.01	-0.21	0.20	0.95
Naïve/resting T	238	0.23	-2.08	2.55	0.84	50	0.28	-4.84	5.39	0.91	288	0.06	-1.86	1.98	0.95
Truly naïve helper/inducer T+	238	0.00	-0.31	0.31	0.99	51	-0.14	-0.81	0.53	0.67	289	-0.01	-0.28	0.26	0.95
Suppressor inducer T+	238	0.12	-0.19	0.42	0.45	51	-0.37	-0.74	0.00	0.05	289	0.03	-0.22	0.29	0.81
Memory T+	238	0.00	-0.09	0.10	0.93	51	-0.13	-0.40	0.14	0.34	289	-0.01	-0.11	0.08	0.78
Terminally differentiated effector memory T	238	-1.84	-5.27	1.59	0.29	51	2.17	-1.94	6.27	0.29	289	-1.30	-4.07	1.48	0.36
Cytotoxic T	237	-0.94	-2.23	0.35	0.15	51	-3.34	-5.99	-0.68	0.01*	288	-1.14	-2.34	0.06	0.06
B	233	0.73	-0.98	2.44	0.40	51	-1.73	-5.36	1.89	0.34	284	-0.43	-2.01	1.14	0.59
Activated B	238	0.31	-1.39	2.01	0.72	51	-1.90	-5.43	1.63	0.28	289	-0.65	-2.18	0.87	0.40
Myeloid dendritic+	238	-0.06	-0.24	0.11	0.48	51	0.12	-0.24	0.47	0.51	289	-0.01	-0.15	0.14	0.92
Lymphoid dendritic+	238	-0.15	-0.43	0.14	0.32	51	0.02	-0.42	0.45	0.94	289	-0.10	-0.34	0.13	0.39
Dendritic-like+	238	0.05	-0.19	0.29	0.70	51	-0.26	-0.67	0.16	0.22	289	0.00	-0.20	0.21	0.98
Macrophage-like+	238	-0.13	-0.38	0.13	0.33	51	-0.05	-0.56	0.46	0.85	289	-0.07	-0.31	0.18	0.60

+Outcome is log-adjusted

Covariates include maternal age at delivery, smoking before or during pregnancy, maternal passive smoke exposure, maternal education, child gender, and district. The model for the total population includes an indicator variable for Romani ethnicity (yes/no).

*p-value <0.05