Aberrant 5’-CpG Methylation of Cord Blood TNFα Associated with Maternal Exposure to Polybrominated Diphenyl Ethers

Tyna Dao1☯, Xiumei Hong2☯, Xiaobin Wang2, Wan-Yee Tang1*

1 Department of Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, United States of America, 2 Department of Human Population and Reproductive Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, United States of America

☯ These authors contributed equally to this work.
*
wtang@jhsph.edu

Abstract

Growing evidence suggests that maternal exposures to endocrine disrupting chemicals during pregnancy may lead to poor pregnancy outcomes and increased fetal susceptibility to adult diseases. Polybrominated diphenyl ethers (PBDEs), which are ubiquitously used flame-retardants, could leach into the environment; and become persistent organic pollutants via bioaccumulation. In the United States, blood PBDE levels in adults range from 30–100 ng/g- lipid but the alarming health concern revolves around children who have reported blood PBDE levels 3 to 9-fold higher than adults. PBDEs disrupt endocrine, immune, reproductive and nervous systems. However, the mechanism underlying its adverse health effect is not fully understood. Epigenetics is a possible biological mechanism underlying maternal exposure-child health outcomes by regulating gene expression without changes in the DNA sequence. We sought to examine the relationship between maternal exposure to environmental PBDEs and promoter methylation of a proinflammatory gene, tumor necrosis factor alpha (TNFα). We measured the maternal blood PBDE levels and cord blood TNFα promoter methylation levels on 46 paired samples of maternal and cord blood from the Boston Birth Cohort (BBC). We showed that decreased cord blood TNFα methylation associated with high maternal PBDE47 exposure. CpG site-specific methylation showed significantly hypomethylation in the girl whose mother has a high blood PBDE47 level. Consistently, decreased TNFα methylation associated with an increase in TNFα protein level in cord blood. In conclusion, our finding provided evidence that in utero exposure to PBDEs may epigenetically reprogram the offspring’s immunological response through promoter methylation of a proinflammatory gene.

Introduction

Polybrominated diphenyl ethers (PBDEs), known as endocrine disrupting compounds, affecting the endocrine, immune, reproductive and nervous systems [1]. PBDEs, until recently, are
ubiquitously used flame-retardants, in many consumer products, that have leached into the environment and bioaccumulated to become persistent organic pollutants (POPs) [2, 3]. There are over 209 congeners of PBDEs, but five are significant to humans, since they account for 90% of the total body burden; PBDE-47, -99, -100, -153 and -154 [2]. Human PBDE exposure occurs through multiple exposure pathways; inhalation, ingestion and dermal contact [4, 5]. Many of the lower PBDE congeners are lipophilic and accumulate in fatty tissues. They can easily cross the placenta into the fetal circulation [6]. Nursing mothers can transfer PBDEs to infants through breast milk [4]. PBDEs also accumulate in fetal lipid-rich tissues such as brain, liver and adipose. PBDEs are persistent in the body with estimated human half-lives of 2–12 years [6]. Epidemiological and experimental studies suggest that PBDEs are developmental neurotoxicants, at least partly impairing thyroid hormone homeostasis, oxidative damage or dysregulation of neurotransmitter signaling in the brain [7].

2,2',4,4'-Tetrabromodiphenyl ether (PBDE47) accounts for 50% of the PBDE body burden [7] and is the PBDE congener found at the highest levels in human serum and breast milk [8–10]. Female mice exposed prenatally or postnatally to repeated low doses of PBDE47 resulted in the maternal transfer of PBDE47 from dams to the fetus blood, brain, liver and fat tissues. The offspring showed growth retardation and behavioral deficiencies [3, 5] [11]. These animal findings are supported by epidemiological evidence of the relationship between PBDE47 levels and cognitive delay [12, 13]. Furthermore, animals exposed to a commercial PBDE mixture (PBDE47 is the dominant congener) showed decreased lymphocyte proliferation and antibody production [14], suggesting that PBDEs may pose a risk of immunotoxicity on specific target tissues. PBDE47 is postulated to disrupt thyroid function because it shares a similar structure to thyroid hormone [1]. However, studies have reported that PBDE47 does not interact with the thyroid hormone receptor [15] suggesting that alternative mechanisms may underlie the toxicity of PBDE47 in the cells.

It has been proposed that maternal PBDE exposure induces epigenetic reprogramming of the offspring and associates with increased disease risks in children. The "Barker Hypothesis" postulated that organs undergo developmental programming in the womb that predetermines subsequent physiological and metabolic adaptations during childhood or as adults [16, 17]. Epigenetics now underpins the developmental reprogramming by demonstrating the molecular relationship between the exposure to environmental pollutants/toxicants and gene expression changes that influence disease susceptibility [18–23]. Epigenetic mechanisms (DNA methylation, histone modifications and microRNAs) act singularly or conjointly to regulate gene expression without altering DNA sequences in response to environmental exposures. Therefore, they produce an array of unique phenotypes that control cell differentiation and organ development. DNA methylation is the covalent addition of a methyl group from S-adenosyl-methionine (SAM), by DNA methyltransferases (DNMTs), to the fifth position of cytosine in CpG dinucleotides to generate 5-methylcytosine (5-mC). DNA methylation at CpG dinucleotides mostly occurs in the 5’ flanking region and often associates with repression of gene activity. The biological contribution of DNA methylation may vary according to the position of the specific CpG sites relative to the transcription unit and the binding of transcriptional factors at the promoter [24]. Decreased global DNA methylation associates with high blood POPs levels [25]. A longitudinal birth cohort study, Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) reported increased parental exposure to PBDEs associates with decreased global DNA methylation, measured on the Alu (Arthrobacter luteus) and LINE-1 (Long Interspersed Elements) sequences, in the cord blood of newborns [26]. By stratifying the children by sex, they revealed that there is a dynamic relationship between the PBDEs exposure and global DNA methylation changes that result from differences in sex, age, exposure-dose and multiple exposures. Mice prenatally exposed to PBDE47 showed
decreased global methylation in their brains that persists into adulthood [1]. Recently, low perinatal PBDE47 exposure has been demonstrated to influence the methylation of mitochondrial cytochrome c oxidase (Mt-co2) and genes related to brain function (Bdnf and Nr3c1) [27]. All in all, it suggests exposure to PBDE47 may affect cell and tissue functions by regulating gene expression through DNA methylation.

Exposure to PBDEs in rodents was shown to impair the immune function [28, 29]. Their interaction with the circulating immune cells may be driving the immunological signals to the target tissues. It could be expected that early life exposure to PBDEs may disrupt immune cell functions via epigenetic modifications of gene regulation. Once the epigenetic program is set, epigenetic disruption of the immune system can persist throughout life, resulting in increased susceptibility to inflammation-related diseases later in life. Additionally, dysregulation of immune responses has been suggested to be associated with neurological dysfunctions. Production of cytokines like Interleukin 1 beta (IL1β) and Tumor Necrosis Factor alpha (TNFα) from the peripheral blood cells are extensively studied and suggested to be affecting the central nervous system [30–32]. However, there are no studies investigating the relationship between the PBDEs exposure and DNA methylation patterns of these two cytokines. We first utilized an in vitro system to test the epigenetic effect of PBDEs on these cytokines by exposing peripheral blood mononuclear cells (PBMCs) to PBDE47. TNFα methylation, not IL1β, showed a linear relationship to the doses of PBDEs. As a proof of concept, we utilized paired maternal and cord blood samples from the Boston Birth Cohort (BBC) and examined if maternal PBDE47 exposure was associated with cord blood TNFα promoter methylation. Our findings may provide the insight to understand the adverse effects of maternal PBDEs exposure on human health through novel epigenetic mechanisms, affecting immunity and inflammation and resulting in higher susceptibility of immune diseases like food allergy, asthma, or other metabolic symptoms [33, 34].

Materials and Methods

Boston Birth Cohort (BBC)

This study included a subset of mother-infants pairs from the BBC, a cohort consisting of multiethnic mother-infant pairs (predominantly African American) enrolled 24 to 72 hours post delivery and followed prospectively from birth onward as detailed previously [34–36]. We collected the biospecimens including maternal blood and cord blood just after delivery. We obtained written informed consent from each mother. The study protocol was approved by the Institutional Review Boards (IRB) of Boston University Medical Center and by the IRB of Johns Hopkins University Bloomberg School of Public Health.

We measured the concentrations of lipid normalized to PBDEs in peripartum maternal serum samples of the BBC (Table 1). The median level of PBDE47 (12.1 ng/g-lipid) was used as the rough cut-off for sample selection. To select samples for the high-level maternal PBDE47 exposed group, we sorted all the available samples and chose samples with PBDE47 levels higher than the median and with available clinical data and DNA samples. Similarly, to select samples for the low-level maternal PBDE47 exposed group, we sorted all the available samples and chose samples with the PBDE47 level lower than the median and with available clinical data and DNA samples. As a result, subjects in the low-level PBDE47 exposed group have maternal PBDE47 < 8.4 ng/g-lipid (about 36th percentile in the BBC) and subjects in the high-level PBDE47 exposed group have maternal PBDE47 > 20 ng/g-lipid (about 70th percentile in the BBC). The distribution of PBDE47 level in each group was illustrated in Table 2. The subjects in the low (n = 23) and high (n = 23)–level PBDE47 exposed groups also fall into the same low and high-level of the sum of all PBDEs measured.
Maternal serum samples from each subject were prepared for shipment using Center for Disease Control (CDC)’s protocols and were sent to the CDC in Atlanta for PBDE measurement using an established and validated PBDE assay. The analytical method and quality control procedures have been described previously [37]. The method used for sample processing included automatic fortification of the samples with internal standards as well as addition of formic acid and water for denaturation and dilution of the samples using a Gilson 215 liquid handler (Gilson Inc.; Middleton, WI). The samples were thereafter extracted by solid phase extraction (SPE) using a Rapid Trace (Caliper Life Sciences; Hopkinton, MA) modular SPE system. Removal of co-extracted lipids was performed on a silica/sulfuric acid column using the Rapid Trace equipment for automation. Final analytical determination of the target analytes was performed by gas chromatography isotope dilution high resolution mass spectrometry (GC-IDHRMS) employing a MAT95XP (ThermoFinnigan MAT, Bremen, Germany) instrument. The samples were analyzed for 10 PBDE congeners: PBDE-17, -28, -47, -66, -85, -99, -100, -153, -154, and -183.

PBDEs measurement in maternal serum

Maternal serum samples from each subject were prepared for shipment using Center for Disease Control (CDC)’s protocols and were sent to the CDC in Atlanta for PBDE measurement using an established and validated PBDE assay. The analytical method and quality control procedures have been described previously [37]. The method used for sample processing included automatic fortification of the samples with internal standards as well as addition of formic acid and water for denaturation and dilution of the samples using a Gilson 215 liquid handler (Gilson Inc.; Middleton, WI). The samples were thereafter extracted by solid phase extraction (SPE) using a Rapid Trace (Caliper Life Sciences; Hopkinton, MA) modular SPE system. Removal of co-extracted lipids was performed on a silica/sulfuric acid column using the Rapid Trace equipment for automation. Final analytical determination of the target analytes was performed by gas chromatography isotope dilution high resolution mass spectrometry (GC-IDHRMS) employing a MAT95XP (ThermoFinnigan MAT, Bremen, Germany) instrument. The samples were analyzed for 10 PBDE congeners: PBDE-17, -28, -47, -66, -85, -99, -100, -153, -154, and -183.

A total of 46 mother-infant pairs were selected for this study. In the low maternal PBDE47 group, PBDE47 level ranged from 0–8.4 ng/g-lipid (boys: 0–8.4 ng/g-lipid; girls: 0–7.4 ng/g-lipid). In the high maternal PBDE47 group, PBDE47 level ranged from 20.6–192.0 ng/g-lipid (boys: 20.6–192 ng/g-lipid, girls: 20.8–175.0 ng/g-lipid).

Table 1. Concentrations of lipid normalized PBDEs in peripartum maternal serum samples of the BBC.

| Congener | P25 | P50 | P75 | % Detection | Range |
|----------|-----|-----|-----|------------|-------|
| PBDE17   | LOD | LOD | LOD | 4.5        | LOD-1.8 |
| PBDE28   | LOD | 0.6 | 1.3 | 56.3       | LOD-6.9 |
| PBDE47   | 5.3 | 12.1| 24.6| 87.5       | LOD-192.0 |
| PBDE66   | LOD | LOD | LOD | 8.1        | LOD-8.2 |
| PBDE85   | LOD | LOD | 0.8 | 41.3       | LOD-7.5 |
| PBDE99   | LOD | LOD | 5.7 | 46.6       | LOD-72.3 |
| PBDE100  | 1.3 | 2.4 | 5   | 90.7       | LOD-35.6 |
| PBDE153  | 1.2 | 2.4 | 4.6 | 85         | LOD-38.6 |
| PBDE154  | LOD | LOD | 0.5 | 31.2       | LOD-6.7 |
| PBDE183  | LOD | LOD | 0.7 | 42.9       | LOD-2.4 |
| Total PBDEs | 9.75 | 20.85 | 44.75 | - | LOD-335.0 |

P, percentile; LOD, Lowest of Detection.

doi:10.1371/journal.pone.0138815.t001

Table 2. Distribution of PBDE47 level in the low vs high maternal PBDE47 exposure group.

| Sex | PBDE47 exposure group | N  | P25 | P50 | P75 | Minimum | Maximum |
|-----|-----------------------|----|-----|-----|-----|---------|---------|
| Girls | Low                  | 9  | 0   | 0   | 3.8 | 0       | 7.4     |
|      | High                 | 12 | 38.6| 57  | 104.7| 20.8    | 175     |
| Boys | Low                  | 14 | 0   | 3.1 | 7.7 | 0       | 8.4     |
|      | High                 | 11 | 31.7| 35.4| 44.9| 20.6    | 192     |
| Total| Low                  | 23 | 1.2 | 0   | 5.7 | 0       | 8.4     |
|      | High                 | 23 | 31.9| 43.8| 93.3| 20.6    | 192     |

P, Percentile.

A total of 46 mother-infant pairs were selected for this study. In the low maternal PBDE47 group, PBDE47 level ranged from 0–8.4 ng/g-lipid (boys: 0–8.4 ng/g-lipid; girls: 0–7.4 ng/g-lipid). In the high maternal PBDE47 group, PBDE47 level ranged from 20.6–192.0 ng/g-lipid (boys: 20.6–192 ng/g-lipid, girls: 20.8–175.0 ng/g-lipid).

doi:10.1371/journal.pone.0138815.t002
-100, -153, -154, -183 (comprising tri-, tetra-, penta-, hexa- and hepta-brominated congeners, but not deca).

Measurement of TNFα in cord blood serum sample

Cord blood sample was collected at delivery, using a BD Vacutainer™ Plus Plastic K20EDTA tube (purple top). The sample was centrifuged at 2500g (4°C) for 10 minutes. Cord blood serum (the supernatant) was split into 3 aliquots and stored at −80°C. Cord blood cell fraction was transferred into a single cryotube and frozen at -80°C if DNA isolation was not performed immediately after collection. Serum sample was analyzed simultaneously for TNFα by immunoassay using flowmetric Luminex xMAP technology (Luminex Corp, Austin, TX), as reported previously [38].

DNA isolation of cord blood leukocytes

Genomic DNA was extracted from cord blood leukocytes and quantified with SpectraMax M2 (www.moleculardevices.com). In brief, the cord blood cell fraction was subject to red blood cell (RBC) lysis and shaken for 10 minutes before centrifugation at 3000g (4°C) for 10 minutes. The cell pellet presented as the leukocytes and subjected to DNA isolation to collect the genomic DNA.

Bisulfite genomic sequencing

Genomic DNA (200ng) extracted from cord blood leukocytes was bisulfite-treated (EZ DNA methylation kit, Zymo Research) before PCR. The CpG rich region of the 5’ promoter region of TNFα (NC_000006.11: 31541344–31545344) was revealed by analyses of genomic sequences at MethPrimer (http://www.urogene.org/methprimer/) (Fig 1). Primers were designed to amplify a 404 base pair fragment (-354 to +50) including a total of 12 CpG sites from bisulfite-treated DNA: hBS-TNFα-F1:5’-TTGGTTTTTAAGAAATGGGAGTA-3’; hBS-TNFα-R1: 5’-TCTCCCTCTTAACCTACTCTACTATC-3’. PCR, using GoTaq PCR mix (Promega, MO), was performed on the bisulfite-treated DNA (95°C for 10 minutes, 35 cycles of 95°C for 30 seconds, 60°C for 1 minute and 72°C for 2 minutes, followed by an extension at 72°C for 12 minutes). PCR products were purified (GeneJET Gel Extraction Kit, Thermo Scientific, NY) and subcloned into pCR 2.1 vector (Invitrogen, CA). 4–6 clones from each sample were sequenced (Macrogen, MD) to obtain direct measures of DNA methylation at each CpG site in the TNFα promoter region. Sequencing data was analyzed with the BiQ analyser. Genomic DNA with 0 and 100% methylation was used as a quality control to ensure bisulfite conversion quality on sample.

Data analysis

We analyzed the average % methylation of TNFα promoter as well as % methylation of each of the 12 CpG sites in the TNFα promoter region, separately. % methylation at each CpG site was calculated using the mean value obtained from 4–6 clones for each subject. Average % methylation of the TNFα promoter was calculated by taking the average of the methylation level of all the analyzed 12 CpG sites within the TNFα promoter region. A scatter plot with smooth fitted line was generated in R (Version 3.01) to explore the relationship between the average % methylation of TNFα promoter and log10-transformed maternal blood PBDE47 level (Fig 2). We plotted the distribution of average % methylation of the TNFα promoter in the low- and high-level PBDE47 exposed groups, separately (Fig 3). We then compared the difference between these two groups using an unpaired t-test (two-tailed p-value) with Welch’s correction. In addition, we generated the plots to describe the distribution of % methylation at each of 12
CpG sites in low- and high-level PBDE47 exposed groups (boys and girls, separately), and then compared the difference between the two groups by two-way ANOVA (Fig 4). We presented p-value without adjustment for multiple comparisons due to the fact that 1) the sample size was small; 2) these CpG sites were not independent of each other, in which, Bonferroni adjustment appeared to be very stringent. The difference of cord blood TNFα protein (in log-transformation) between low- and high-level PBDE47 exposed groups was compared using an unpaired t-test. Spearman correlation coefficient was calculated to determine the relationship between cord blood TNFα protein level and average % methylation of TNFα promoter. Graphs were generated using Prism 6 (GraphPad Software, CA).

Results
PBDE47 shows the most abundance among PBDEs congers in BBC

In the BBC cohort, a total of 247 maternal blood samples had available data for blood PBDEs level (of 10 PBDE congeners), which showed a wide range of variability (Table 1). PBDE47 is the most prevalent PBDE (accounting for 58% of the total PBDEs level) in this cohort (Table 1) and thus we focused on the epigenetic effect of PBDE47 in our study. A total of 46 mother-
infant pairs (23 in low-level maternal PBDE47 exposed group and 23 in high-level maternal PBDE47 exposed group) were selected for this study (Table 2). There was no significant difference in baby’s gender, smoking status, parity, preterm status, maternal education, maternal age and pre-pregnancy BMI between the selected 46 samples and the rest of the 201 samples (Table 3). Among the 46 selected samples, mothers with high PBDE47 exposure were significantly younger than those with low PBDE47 exposure (p<0.01, Table 3).

Fig 2. A scatter plot showing the relationship between the average % methylation of the TNFα promoter and log10-transformed maternal blood PBDE47 level. Methylation of each CpG site of the TNFα promoter was assayed in 46 cord blood DNA samples by bisulfite genomic sequencing. Average % methylation of the TNFα promoter (y-axis) was calculated by taking an average of the methylation level of a total 12 CpG sites within the TNFα promoter region.

doi:10.1371/journal.pone.0138815.g002

Fig 3. Decreased TNFα promoter methylation associates with high maternal PBDE47 exposure. Average % methylation of the TNFα promoter (y-axis) was calculated by taking an average of the methylation level of a total 12 CpG sites within the TNFα promoter region in 46 cord blood DNA samples. Results were compared between low- and high-level PBDE47 exposed groups. Each circle and square represented the average percent methylation level for each subject in low-level PBDE47 exposed group and high-level PBDE47 exposed group, respectively. Boys (in black) and girls (in red) are marked differently. The error bars represented means (±standard error of mean, SEM).

doi:10.1371/journal.pone.0138815.g003
Fig 4. Decreased CpG site-specific methylation of TNFα in cord blood associates with high maternal PBDE47 exposure in girls (lower panel) not boys (upper panel). Percentage of methylation of each CpG site of the TNFα promoter represented as mean ± SEM in low- (gray open circles) and high-level (black squares) PBDE47 exposed group. The difference in % methylation in these 12 CpG sites between the two groups was compared by two-way ANOVA. * p<0.05, meant % methylation at a CpG site was significantly different between low- and high-level PBDE47 exposed groups (based on t-test).

doi:10.1371/journal.pone.0138815.g004
Decreased cord blood TNFα promoter methylation associated with high maternal PBDE47 exposure

We first utilized an in vitro system to test the epigenetic effect of PBDE47 on these cytokines by exposing peripheral blood mononuclear cells (PBMCs) to PBDE47 (0.1–100nM). TNFα methylation, not IL1β, showed a linear relationship to the doses of PBDEs (data in S1 Fig). DNA methylation patterns of a total of 12 CpG sites located at the promoter region of TNFα (Fig 1) were analyzed in cord blood leukocytes. Previous studies have shown the significance of CpG site-specific DNA methylation in TNFα transcription although the TNFα gene does not have traditional CpG islands (GC% >60%) at its promoter region [39]. We first examined the linear relationship between average % methylation of the TNFα promoter in cord blood and maternal blood PBDE47 level (log10-transformed). As shown in Fig 2, their relationship was not linear. Instead, TNFα methylation remained unchanged when maternal blood PBDE47 level (log10-transformed) was less than 0.7 (or PBDE level <5 ng/g-lipid) but TNFα methylation decreased with increasing maternal PBDE47 exposure when maternal blood PBDE47 level (log10-transformed) is greater than 0.7 (or PBDE level >5ng/g-lipid).

Compared to that in the low-level PBDE47 exposed group, average % methylation of the TNFα promoter was lower in the high-level PBDE47 exposed group (p = 0.046) (Fig 3). When stratified by the gender of offspring, a similar association was found in girls (p = 0.004), but not in boys (p > 0.99) (Fig 3). To determine if one or more CpG sites at the TNFα promoter were more sensitive to PBDE47 exposure, we compared % methylation at each individual CpG site between low- and high-level PBDE47 exposed groups. In boys, there was no significant difference in % methylation of any CpG sites between low- and high-level PBDE47 exposed groups. In girls, three CpG sites, CpG_-172 (p = 0.04), CpG_-75 (p = 0.05), and CpG_+8 (p = 0.05) showed significantly decreased % methylation in the high-level PBDE47 exposed group as compared to that of low-level PBDE47 exposed group (Fig 4).

Table 3. Population Characteristics.

|                      | Low PBDE47 group | High PBDE47 group | p-value* | Enrolled in this study | Non-enrolled in this study | p-value* |
|----------------------|------------------|-------------------|----------|------------------------|---------------------------|----------|
| N                    | 23               | 23                | 0.554    | 25(54.3)               | 99(49.3)                  | 0.646    |
| Boys, N(%)           | 14(60.9)         | 11(47.8)          |          | 15(32.6)               | 82(40.8)                  | 0.391    |
| Maternal smoking during pregnancy, N(%) | 2(8.7) | 2(8.7) | 1        | 4(8.7)               | 28(13.9)                  | 0.477    |
| Nulliparty, N(%)     | 6(26.1)          | 9(39.1)           | 0.529    | 17(36.0)               | 54(27.8)                  |          |
| Cesarean section, N(%) | 8(36.4) | 4(17.4) | 0.271    | 12(26.7)              | 61(31.8)                  | 0.625    |
| Preterm birth, N(%)  | 8(34.8)          | 9(39.1)           | 1        | 17(37.0)               | 106(52.7)                 | 0.077    |
| Maternal education   | 0.406            |                   |          |                        |                           | 0.473    |
| < high school, N (%) | 8(34.8)          | 9(39.1)           |          | 17(36.0)               | 54(27.8)                  |          |
| High School, N (%)   | 6(26.1)          | 9(39.1)           |          | 15(32.6)               | 74(38.1)                  |          |
| College and above, N (%) | 9(39.1) | 5(21.7) | <0.01    | 27.7±7.0              | 28.0±6.9                  | 0.78     |
| Maternal age, year (mean±SD) | 31.0±6.6 | 24.3±5.7 | <0.01    | 25.3±4.8              | 25.9±5.5                  | 0.531    |
| Maternal BMI, kg/m² (mean±SD) | 25.8±5.5 | 24.9±4.0 | 0.5       | 25.3±4.8              | 25.9±5.5                  |          |

SD, standard deviation.
*p values were calculated using Chi-square test and t-test was performed to test group difference for categorical and continuous variables, respectively.

doi:10.1371/journal.pone.0138815.t003

PBDEs and DNA Methylation of TNFalpha

PLOS ONE | DOI:10.1371/journal.pone.0138815 | September 25, 2015
Inverse relationship between cord blood TNFα promoter methylation and cord blood TNFα protein level in girls

To examine if the decreased cord blood TNFα promoter methylation contributed to increased gene expression, we measured TNFα protein level in 23 (out of 46 selected samples) cord blood serum as RNA sample was not available. The 23 selected samples were not chosen by either maternal PBDE47 level or cord blood methylation status. The analysis of TNFα protein level in serum was primarily designed to study preterm birth and related traits in other BBC-related studies. Therefore, we would expect more cases of preterm birth among the 23 selected samples we measured cord blood TNFα protein. When compared with those unselected samples (without TNFα protein analysis), these 23 selected samples were more apt to preterm birth (65.2% vs 8.7%, p = 0.0003). Nonetheless, they were comparable with unselected samples on other variables, such as, gender, nulliparity, cesarean section, maternal education, maternal age and maternal BMI. The distribution of cord blood TNFα protein level in each PBDE47 exposed group was shown in Table 4. The median of TNFα protein level in the low-level PBDE47 exposed group (n = 13) (28.2 pg/mL) was higher than that of high-level PBDE47 exposed group (n = 10) (19.3 pg/mL). A similar trend was found in boys (n = 15). In girls (n = 8), cord blood TNFα protein level tended to be higher in the high-level PBDE47 exposed group than in the low-level PBDE47 exposed group. However, none of the differences were statistically significant.

As shown in Table 5, average % methylation of the TNFα promoter was not significantly correlated to cord blood TNFα protein level in 23 samples, as indicated by the Spearman’s rank coefficient (ρ, rho). When we focused this analysis to each specific CpG site at TNFα promoter, we did not identify any significant association in the total 23 samples and in boys (n = 15). In girls (n = 8), cord blood TNFα protein level tended to be higher in the high-level PBDE47 exposed group than in the low-level PBDE47 exposed group. However, none of the differences were statistically significant.

Discussion

Our results suggested that maternal PBDE47 exposure altered CpG site specific DNA methylation at the TNFα promoter, which may contribute to the aberrant TNFα gene expression in the offspring. There are intensive studies that have reported the toxic effect of PBDEs on neural and thyroid systems [7, 12–13]. Strikingly, PBDEs can also influence the functions of the immune system [14, 28–29]. Our previous studies reported a number of CpG sites (located

| TNFα (pg/mL)*, Median (P25-P75) | Low PBDE47 group | High PBDE group | p-valueb |
|-------------------------------|------------------|-----------------|---------|
| Total Sample (nª = 13 / 10)   | 28.2 (13.5–36.1) | 19.3 (13.4–31.8) | 0.75    |
| Boys (nª = 10 / 5)            | 31.5 (16.5–40.5) | 13.9 (13.4–26.0) | 0.18    |
| Girls(nª = 3 / 5)            | 9.0 (6.6–22.6)   | 23.7 (14.8–37.2) | 0.39    |

*The 23 selected samples were not chosen by either maternal PBDE47 level or cord blood methylation status. The analysis of TNFα protein level in serum was primarily designed to study the preterm birth and related traits in other BBC-related studies.

ª Sample size in low-level PBDE47 exposed group /in high-level exposed PBDE47 group

b p values were estimated based on Welch two-sample t-test for log-transformed cord blood TNFα levels between low- and high-level PBDE47 exposed groups.
within the genes with immunological functions) showing significant longitudinal changes in methylation levels [34]. Recently, we have demonstrated that pre-pregnancy maternal BMI associated with the epigenetic alterations in offspring’s genes related to the inflammatory and lipid metabolism response [40]. Here, we provide the evidence showing the relationship between maternal PBDE47 exposure and gene-specific methylation of a proinflammatory gene at birth. TNFα is a proinflammatory cytokine produced mainly by macrophages but also found in T cells, B cells and fibroblasts. This cytokine has been implicated in a variety of diseases including immune-related disease [41–43] and cancer [44]. The proximal region of the TNFα promoter (~200 bp upstream of the TSS) regulates the transcription of TNFα in multiple cell types that respond to various stimuli including the T cell and B cell activation, infection and cytokines [45]. Sullivan et al demonstrated that TNFα production correlated to the CpG site-specific methylation of the TNFα promoter in monocytes and THP-1 cells in the presence of lipopolysaccharide (LPS) [39]. By using a subset of the BBC, we showed that TNFα hypomethylation in cord blood correlated to high maternal PBDE47 exposure. Remarkably, we found that girls are more susceptible to TNFα promoter hypomethylation in response to maternal PBDE47 exposure. Further, we showed, in girls, % methylation at the specific CpG site (CpG_-172, CpG_-75 and CpG_+8) as previously described by Sullivan et al [39], significantly associated with maternal PBDE47 exposure. In addition, methylation status of CpG_+8 (girls) inversely correlated to TNFα protein level in cord blood. It suggests that maternal PBDE47 exposure might alter TNFα production in the offspring via CpG site-specific DNA methylation. We did not note a statistically significant correlation between cord blood TNFα protein level and maternal PBDE47 exposure in girls, although we found the median of TNFα protein level in the high-level PBDE47 exposed group was higher than that of low-level PBDE47 exposed group. We acknowledged that this TNFα protein analysis is limited by a relatively small sample size and so these findings warrant further validation in other samples enrolled in BBC.

Table 5. Spearman correlation between cord blood TNFα promoter methylation and cord blood TNFα protein level.

| CpG loci from TSS | Genome Coordinate at NC_000006.12 | Total = 23 | Boys, N = 15 | Girls, N = 8 |
|------------------|-----------------------------------|--------|-------------|-------------|
|                  |                                   | rho*   | p-value*    | rho*        | p-value*    | rho*   | p-value*    |
| CpG_-306        | 31575258                          | 0.333  | 0.12        | 0.311       | 0.259       | 0.279  | 0.503       |
| CpG_-247        | 31575317                          | -0.242 | 0.266       | -0.045      | 0.874       | -0.504 | 0.203       |
| CpG_-241        | 31575323                          | -0.132 | 0.575       | -0.207      | 0.46        | -0.055 | 0.898       |
| CpG_-172        | 31575392                          | 0.032  | 0.886       | 0.199       | 0.476       | -0.419 | 0.301       |
| CpG_-166        | 31575398                          | 0.015  | 0.945       | 0.096       | 0.732       | -0.074 | 0.862       |
| CpG_-164        | 31575400                          | 0.048  | 0.828       | 0.215       | 0.442       | -0.388 | 0.342       |
| CpG_-149        | 31575415                          | 0.203  | 0.352       | 0.141       | 0.616       | 0.077  | 0.857       |
| CpG_-122        | 31575442                          | 0.039  | 0.858       | -0.017      | 0.953       | -0.026 | 0.952       |
| CpG_-75         | 31575489                          | -0.19  | 0.386       | -0.14       | 0.62        | -0.25  | 0.55        |
| CpG_-52         | 31575512                          | -0.152 | 0.49        | -0.21       | 0.452       | -0.024 | 0.954       |
| CpG_-41         | 31575523                          | -0.06  | 0.786       | -0.055      | 0.846       | -0.146 | 0.729       |
| CpG_+8          | 31575572                          | -0.2   | 0.36        | 0.112       | 0.691       | -0.733 | *0.039      |
| Total CpGs      | 31575258–572                      | 0.005  | 0.98        | 0.075       | 0.79        | -0.238 | 0.57        |

*The Spearman’s rank correlation coefficient (p, rho) was calculated to measure the strength of the relationship between the percent of methylation of TNFα promoter and TNFα protein level in cord blood samples. Negative values indicated inverse relationship between cord blood TNFα promoter methylation and cord blood TNFα protein level. The larger the negative value meant greater negative correlation. Statistical significance at individual CpG site was determined with p<0.05.

doi:10.1371/journal.pone.0138815.t005
Studies showed that transcriptional control at the TNF\(\alpha\) promoter associated with its production and with the disease risk. In Jurkat T cells or human monocytes, promoter methylation [39, 46], histone acetylation and methylation [47–49] together with other transcription factors [50] could regulate transcription of TNF\(\alpha\) gene. Long noncoding RNAs and their binding proteins were shown to regulate gene expression of TNF\(\alpha\) [51]. Histone modifications at the TNF promoter also associated with the disease states of diabetes [52] and systemic lupus erythematosus [53]. Women with higher adiposity showed decreased methylation of the TNF\(\alpha\) promoter and higher plasma TNF\(\alpha\) protein level in their peripheral white blood cells [54]. Further, epigenetic regulation of the monocyte and T cell lineages by TNF\(\alpha\) production showed to play a key role in immunological diseases [55]. These results suggested that epigenetic regulation of TNF\(\alpha\) may contribute to the modulation of inflammatory responses.

No studies have examined the association between the maternal PBDE exposure and TNF\(\alpha\) promoter methylation in the developing fetus. Newborns with growth restriction showed increased TNF\(\alpha\) expression in the placenta [56]. It suggests that TNF\(\alpha\) cytokine expression may influence developmental outcomes of the offspring. Our study, although limited by a small sample size and no adjustment for multiple testing, may represent the first step to demonstrate the potential influence of prenatal PBDE exposure on the fetal epigenome. We acknowledged that cord blood leukocytes utilized in the current study consist of many functionally and developmentally distinct cell populations in varying proportions. Because DNA methylation is cell-type specific, it is possible that cellular heterogeneity may confound our methylation measurements. It suggests that differential cell counting and/or sorting of cells from whole blood is required to adjust the findings from the methylation patterns by regression model (using the estimated cell type composition as a covariate in the regression model [57]) or access the methylation patterns in different types of blood cells [58, 59]. Unfortunately, it is not feasible to obtain the cell distribution of the immune cells in the archived samples we utilized in this study. Given the fact that cord blood leukocytes may contain stem cells that can populate the brain in later life [60] and also provide a rich source of immune cells which are important producers of cytokines [61], it suggests that cord blood leukocytes (if only archived cord blood samples are available) may provide a reasonable surrogate for our target organ/tissues (brain/immune cells). Our previous study demonstrated the feasibility of using cord blood DNA methylation as a surrogate biomarker to associate the risk of childhood asthma [21, 23]. All-in-all, in the future, we will take this step further by performing replicated studies in a larger sample pool and by following up the developmental outcomes of the offspring from infancy, childhood, to adolescence. We will assess the disease phenotypes like food allergy, asthma as well as metabolic and neurological disorders. If we further confirm our findings on a relatively large sample size at the BBC, we may be able to evaluate the use of cord blood TNF\(\alpha\) methylation patterns as biomarkers for the susceptibility of immunological and neurological diseases. Or we can measure cord blood TNF\(\alpha\) methylation to assess the adverse health effects of maternal PBDEs exposure.

In the present study, we adjust the BBC samples by mother’s age and birth implications and outcomes (low birth weight). Nevertheless, profound factors for TNF\(\alpha\) methylation may include social-economics status, psychological stress, ethnicity, and exposures to other endocrine disrupting chemicals and PBDEs congers. PBDE47 showed sex-, age-, species- and dose-dependent effects on its metabolism and elimination in experimental animal studies [62–65]. Therefore, we must carefully choose proper data modeling and multiple testing when we employ the future epigenetic analyses. We will need a set of follow-up studies, in vitro or in vivo, to show the epigenetic effect of the PBDEs toxicity in humans. Taken together, our findings provide evidence that maternal exposure to PBDEs may induce epigenetic reprogramming of the offspring’s immune response by aberrant DNA methylation of a proinflammatory gene.
Supporting Information

S1 Fig. Exposure to PBDE47 decreased TNFα but not IL1ß methylation status of peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs, HemaCare, CA) were exposed to a range of PBDE47 (AccuStandard, NH) exposures for 3 days. DNA isolated from cells was undergone bisulfite treatment before PCR. Promoter methylation status for TNFα and IL1ß was assayed by bisulfite genomic sequencing. Results were collected from three independent sets of experiment and analyzed by 2-tailed t test. Error bar represented standard error of mean. p<0.05 presented result was statistically significant in PBDE47-exposed group when compared with that of untreated control (PBDE47_0nM).

TIFF

Author Contributions

Conceived and designed the experiments: WYT XBW. Performed the experiments: TD XMH. Analyzed the data: XMH WYT. Contributed reagents/materials/analysis tools: WYT XBW. Wrote the paper: TD XMH XBW WYT.

References

1. Woods R, Vallero RO, Golub MS, Suarez JK, Ta TA, Yasui DH, et al. Long-lived epigenetic interactions between perinatal PBDE exposure and MeCP2308 mutation. Human molecular genetics. 2012; 21(11):2390–411. doi:10.1093/hmg/dds046 PMID:22343140
2. Costa LG, de Laat R, Tagliaferri S, Pellacani C. A mechanistic view of polybrominated diphenyl ether (PBDE) developmental neurotoxicity. Toxicology letters. 2014; 230(2):282–94. doi:10.1016/j.toxlet.2013.11.011 PMID:24270005
3. Ta TA, Koenig CM, Golub MS, Pessah IN, Qi L, Aronov PA, et al. Bioaccumulation and behavioral effects of 2,2’,4,4’-tetrabromodiphenyl ether (BDE-47) in perinatally exposed mice. Neurotoxicology and teratology. 2011; 33(3):393–404. doi:10.1016/j.nnt.2011.02.003 PMID:21334437
4. Abdallah MA, Harrad S. Polybrominated diphenyl ethers in UK human milk: implications for infant exposure and relationship to external exposure. Environment international. 2014; 63:130–6. doi:10.1016/j.envint.2013.11.009 PMID:24291663
5. Koenig CM, Lango J, Pessah IN, Berman RF. Maternal transfer of BDE-47 to offspring and neurobehavioral development in C57BL/6J mice. Neurotoxicology and teratology. 2012; 34(6):571–80. doi:10.1016/j.nnt.2012.09.005 PMID:23022914
6. Geyer HJ, Schramm K, Damerdz JD, Aune M, Feicht EA, Fried KW, et al. Terminal elimination half-lives of the brominated flame retardants TBBPA, HBCD, and lower brominated PBDEs in humans. Organohalogen Compounds. 2004; 66:3820–5.
7. (EPA) USEPA. An Exposure Assessment of Polybrominated Diphenyl Ethers. Washington, DC 2010.
8. Koh TW, Chih-Cheng Chen S, Chang-Chien GP, Lin DY, Chen FA, Chao HR. Breast-milk levels of polybrominated diphenyl ether flame retardants in relation to women’s age and pre-pregnant body mass index. Int J Hyg Environ Health. 2010; 213(1):59–65. doi:10.1016/j.ijh.2009.09.004 PMID:19781992
9. Costa LG, Giordano G. Developmental neurotoxicity of polybrominated diphenyl ether (PBDE) flame retardants. Neurotoxicology. 2007; 28(6):1047–67. PMID:17904639
10. Suworov A, Girard S, Lachapelle S, Abdelouahab N, Sebire G, Takser L. Perinatal exposure to lowdose BDE-47, an emergent environmental contaminant, causes hyperactivity in rat offspring. Neonatology. 2009; 95(3):203–9. doi:10.1159/000155561 PMID:18798992
11. Suworov A, Takser L. Delayed response in the rat frontal lobe transcriptome to perinatal exposure to the flame retardant BDE-47. Journal of applied toxicology: JAT. 2011; 31(5):477–83. doi:10.1002/jat.1667 PMID:21394737
12. Herbstman JB, Sjodin A, Kurzon M, Lederer SA, Jones RS, Rauh V, et al. Prenatal exposure to PBDEs and neurodevelopment. Environmental health perspectives. 2010; 118(5):712–9. doi:10.1289/ehp.0901340 PMID:20056561
13. Eskenazi B, Chevrier J, Rauch SA, Kogut K, Harley KG, Johnson C, et al. In utero and childhood polybrominated diphenyl ether (PBDE) exposures and neurodevelopment in the CHAMACOS study. Environmental health perspectives. 2013; 121(2):257–62. doi:10.1289/ehp.1205597 PMID:23154064
14. Martin PA, Mayne GJ, Bursian FS, Tomy G, Palace V, Pekarik C, et al. Immunotoxicity of the commercial polybrominated diphenyl ether mixture DE-71 in ranch mink (Mustela vison). Environ Toxicol Chem. 2007; 26(5):988–97. PMID: 17521147

15. Suvorov A, Bissonnette C, Takser L, Langlois MF. Does 2,2',4,4'-tetrabromodiphenyl ether interact directly with thyroid receptor? Journal of applied toxicology: JAT. 2011; 31(2):179–84. doi: 10.1002/jat.1580 PMID: 20737425

16. Hales CN, Barker DJ, Clark PM, Cox LJ, Fall C, Osmond C, et al. Fetal and infant growth and impaired glucose tolerance at age 64. BMJ (Clinical research ed). 1991; 303(6809):1019–22.

17. Barker DJ, Osmond C, Simmonds SJ, Wield GA. The relation of small head circumference and thinness at birth to death from cardiovascular disease in adult life. BMJ (Clinical research ed). 1993; 306(6875):426–2.

18. Tang WY, Ho SM. Epigenetic reprogramming and imprinting in origins of disease. Reviews in endocrine & metabolic disorders. 2007; 8(2):173–82.

19. Jirtle RL, Skinner MK. Environmental epigenomics and disease susceptibility. Nature reviews Genetics. 2007; 8(4):253–62. PMID: 17363974

20. Ho SM, Tang WY, Belmonte de Frausto J, Prins GS. Developmental exposure to estradiol and bisphenol A increases susceptibility to prostate carcinogenesis and epigenetically regulates phosphodiesterase type 4 variant 4. Cancer research. 2006; 66(11):5624–32. PMID: 16740699

21. Tang WY, Levin L, Talaska G, Cheung YY, Herbstman J, Tang D, et al. Maternal exposure to polycyclic aromatic hydrocarbons and 5'-CpG methylation of interferon-gamma in cord white blood cells. Environmental health perspectives. 2012; 120(8):1195–200. doi: 10.1289/ehp.1103744 PMID: 22562770

22. Tang WY, Newbold R, Mardilovich K, Jefferson W, Cheng RY, Medvedovic M, et al. Persistent hypomethylation in the promoter of nucleosomal binding protein 1 (Nsbp1) correlates with overexpression of Nsbp1 in mouse uteri neonatally exposed to diethylstilbestrol or genistein. Endocrinology. 2008; 149(12):5922–31. doi: 10.1210/en.2008-0682 PMID: 18669593

23. Perera F, Tang WY, Herbstman J, Tang D, Levin L, Miller R, et al. Relation of DNA methylation of 5'- CpG island of ACSL3 to transplacental exposure to airborne polycyclic aromatic hydrocarbons and childhood asthma. PloS one. 2009; 4(2):e4488. doi: 10.1371/journal.pone.0004488 PMID: 19221603

24. Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat Rev Genet. 2012; 13(7):484–92. doi: 10.1038/nrg3230 PMID: 22641018

25. Rusiecki JA, Baccarelli A, Bollati V, Tarantini L, Moore LE, Bonefeld-Jorgensen EC. Global DNA hypomethylation is associated with high serum-persistent organic pollutants in Greenlandic Inuit. Environmental health perspectives. 2008; 116(11):1547–52. doi: 10.1289/ehp.11338 PMID: 19057709

26. Huen K, Yousefi P, Bradman A, Yan L, Harley KG, Kogut K, et al. Effects of age, sex, and persistent organic pollutants on DNA methylation in children. Environmental and molecular mutagenesis. 2014; 55(3):209–22. doi: 10.1002/em.21845 PMID: 24375655

27. Byun HM, Benachour N, Zalko D, Frisardi MC, Colicino E, Takser L, Baccarelli AA. Epigenetic effects of low perinatal doses of flame retardant BDE-47 on mitochondrial and nuclear genes in rat offspring. Toxicology. 2015; 328:152–159. doi: 10.1016/j.tox.2014.12.019 PMID: 25533936

28. Du P, Li Z, Zhang H, Zhou Y, Sun W, Xiao X, He Y, Sun B, Yu Y, Chen D. The effects of PBDE-209 exposure during pregnancy on placental ET-1 and eNOS expression and the birth weight of offspring. Int J Dev Neurosci. 2015 Mar 27.

29. Liu X, Zhan H, Zeng X, Zhang C, Chen D. The effects of PBDE-209 exposure during pregnancy and lactation on glucose tolerance at age 64. BMJ (Clinical research ed). 1991; 303(6809):1019–22.

30. Viviani B, Boraso M, Marchetti N, Marinovich M. Perspectives on neuroinflammation and excitotoxicity: a neurotoxic conspiracy? Neurotoxicology. 2014; 43:10–20. doi: 10.1016/j.neuro.2014.03.004 PMID: 24662010

31. Evans HL. Markers of neurotoxicity: from behavior to autoantibodies against brain proteins. Clin Chem. 1995; 41(12 Pt 2):1874–81. PMID: 7497648

32. Galic MA, Riazl K, Pittman OJ. Cytokines and brain excitability. Front Neuroendocrinol. 2012; 33(1):116–25. doi: 10.1016/j.yfne.2011.12.002 PMID: 22214786

33. Hong X, Wang X. Early life precursors, epigenetics, and the development of food allergy. Seminars in immunopathology. 2012; 34(5):655–69. doi: 10.1007/s00281-012-0323-y PMID: 22777545

34. Wang D, Liu X, Zhou Y, Xie H, Hong X, Tsai HJ, et al. Individual variation and longitudinal pattern of genome-wide DNA methylation from birth to the first two years of life. Epigenetics: official journal of the DNA Methylation Society. 2012; 7(6):594–605.

35. Wang X, Zuckerman B, Pearson C, Kaufman G, Chen C, Wang G, et al. Maternal cigarette smoking, metabolic gene polymorphism, and infant birth weight. Jama. 2002; 287(2):195–202. PMID: 11779261
36. Liu X, Wang G, Hong X, Tsai HJ, Liu R, Zhang S, et al. Associations between gene polymorphisms in fatty acid metabolism pathway and preterm delivery in a US urban black population. Human genetics. 2012; 131(3):341–51. doi: 10.1007/s00439-011-1079-5 PMID: 21847588

37. Sjodin A, McGaaee EE 3rd, Focant JF, Jones RS, Lapeza CR, Zhang Y, et al. Semiautomated high-throughput extraction and cleanup method for the measurement of polynuclear diphenyl ethers and polynuclear chlorobiphenyls in breast milk. Anal Chem. 2004; 76(15):4508–14. PMID: 15283595

38. Matoba N, Yu Y, Mestan K, Pearson C, Ortiz K, Porta N, et al. Differential patterns of 27 cord blood immune biomarkers across gestational age. Pediatrics. 2009; 123(5):1320–8. doi: 10.1542/peds.2008-1222 PMID: 19403498

39. Sullivan KE, Reddy AB, Dietzmann K, Suriano VP, Kocieda VP, Stewart M, et al. Epigenetic regulation of tumor necrosis factor alpha. Molecular and cellular biology. 2007; 27(14):5147–60. PMID: 17515611

40. Liu X, Chen Q, Tsai HJ, Wang G, Hong X, Zhou Y, et al. Maternal preconception body mass index and offspring cord blood DNA methylation: exploration of early life origins of disease. Environmental and molecular mutagenesis. 2014; 55(3):223–30. doi: 10.1002/em.21827 PMID: 24243566

41. Golikova EA, Lopatnikova JA, Kovalskaya-Kucheryavenko TV, Nepomnyashih VM, Sennikov SV. Levels of TNF, TNF autoantibodies and soluble TNF receptors in patients with bronchial asthma. The Journal of asthma: official journal of the Association for the Care of the Asthma. 2013; 50(7):705–11.

42. Blandizzi C, Gionchetti P, Armuzzi A, Caporal R, Chimenti S, Cimaz R, et al. The role of tumour necrosis factor alpha in the pathogenesis of immune-mediated diseases. International journal of immunopathology and pharmacology. 2014; 27(1 Suppl):1–10. PMID: 24774503

43. Nasiri R, Amirzargar AA, Movahedi M, Hirbod-Mobarakeh A, Farhadi E, Behniafard N, et al. Single-nucleotide polymorphisms of TNFA and IL1 in allergic rhinitis. Journal of investigational allergology & clinical immunology. 2013; 23(7):455–61.

44. Braunsmuller H, Wieder T, Brenner E, Assmann S, Hahn M, Alkhaled M, et al. T-helper-1 cytokines drive cancer into senescence. Nature. 2013; 494(7437):361–5. doi: 10.1038/nature11824 PMID: 23376950

45. Goldfeld AE, Tsai E, Kincaid R, Belshaw PJ, Schrieber SL, Strominger JL, et al. Calcineurin mediates human tumor necrosis factor alpha gene induction in stimulated T and B cells. The Journal of experimental medicine. 1994; 180(2):763–8. PMID: 8046352

46. El Gazzar M, Yoza BK, Chen X, Hu J, Hawkins GA, McCall CE. G9a and HP1 couple histone and DNA methylation to TNFalpha transcription silencing during endotoxin tolerance. The Journal of biological chemistry. 2008; 283(47):32198–208. doi: 10.1074/jbc.M803446200 PMID: 18809684

47. Barthel R, Tsytyskova AV, Barczak AK, Tsai EY, Dascher CC, Brenner MB, et al. Regulation of tumor necrosis factor alpha gene expression by mycobacteria involves the assembly of a unique enhancerosome dependent on the coactivator proteins CBP/p300. Molecular and cellular biology. 2003; 23 (2):526–33. PMID: 12509451

48. Li Y, Reddy MA, Miao F, Shanmugam N, Yee JK, Hawkins D, et al. Role of the histone H3 lysine 4 methyltransferase, SET7/9, in the regulation of NF-kappaB-dependent inflammatory genes. Relevance to diabetes and inflammation. The Journal of biological chemistry. 2008; 283(39):26771–81. doi: 10.1074/jbc.M802800200 PMID: 18650421

49. Ranjarb S, Rajbaum R, Goldfeld AE. Transactivator of transcription from HIV type 1 subtype E selectively inhibits TNF gene expression via interference with chromatin remodeling of the TNF locus. Journal of immunology (Baltimore, Md: 1950). 2006; 176(7):4182–90.

50. Tsytyskova AV, Falvo JV, Schmidt-Supprian M, Courtois G, Thanos D, Goldfeld AE. Post-induction, stimulus-specific regulation of tumor necrosis factor mRNA expression. The Journal of biological chemistry. 2007; 282(16):11629–38. PMID: 17303559

51. Li Z, Chao TC, Chang KY, Lin N, Patil VS, Shimizu C, et al. The long noncoding RNA THRIL regulates TNFalpha expression through its interaction with hnRNPL. Proceedings of the National Academy of Sciences of the United States of America. 2014; 111(9):1002–7. doi: 10.1073/pnas.1313768111 PMID: 24371310

52. Tsytyskova AV, Rajbaum R, Falvo JV, Ligeiro F, Neely SR, Goldfeld AE. Activation-dependent intrachromosomal interactions formed by the TNF gene promoter and two distal enhancers. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104(43):16850–5. PMID: 17940009

53. Sullivan KE, Suriano A, Dietzmann K, Lin J, Goldman D, Petri MA. The TNFalpha locus is altered in monocytes from patients with systemic lupus erythematosus. The Journal of immunology. 2007; 123(1):74–81. PMID: 17276734

54. Hermesdorff HH, Mansego ML, Campion J, Milagro FI, Zulet MA, Martinez JA. TNF-alpha promoter methylation in peripheral white blood cells: relationship with circulating TNFalpha, truncal fat and n-6 polyunsaturated fatty acids. The American journal of clinical nutrition. 2008; 88(6):1391–401. doi: 10.3945/ajcn.2008.26884 PMID: 18951860

55. Nishizuka Y. Tumor necrosis factor and related cytokines as activators of protein kinase C. Cell. 1992; 70(6):817–25. doi: 10.1016/0092-8674(92)90454-K PMID: 1381837

56. Liu X, Wang G, Hong X, Tsai HJ, Liu R, Zhang S, et al. Associations between gene polymorphisms in fatty acid metabolism pathway and preterm delivery in a US urban black population. Human genetics. 2012; 131(3):341–51. doi: 10.1007/s00439-011-1079-5 PMID: 21847588

57. Sjodin A, McGaaee EE 3rd, Focant JF, Jones RS, Lapeza CR, Zhang Y, et al. Semiautomated high-throughput extraction and cleanup method for the measurement of polynuclear diphenyl ethers and polynuclear chlorobiphenyls in breast milk. Anal Chem. 2004; 76(15):4508–14. PMID: 15283595

58. Matoba N, Yu Y, Mestan K, Pearson C, Ortiz K, Porta N, et al. Differential patterns of 27 cord blood immune biomarkers across gestational age. Pediatrics. 2009; 123(5):1320–8. doi: 10.1542/peds.2008-1222 PMID: 19403498

59. Sullivan KE, Reddy AB, Dietzmann K, Suriano VP, Kocieda VP, Stewart M, et al. Epigenetic regulation of tumor necrosis factor alpha. Molecular and cellular biology. 2007; 27(14):5147–60. PMID: 17515611

60. Liu X, Chen Q, Tsai HJ, Wang G, Hong X, Zhou Y, et al. Maternal preconception body mass index and offspring cord blood DNA methylation: exploration of early life origins of disease. Environmental and molecular mutagenesis. 2014; 55(3):223–30. doi: 10.1002/em.21827 PMID: 24243566

61. Golikova EA, Lopatnikova JA, Kovalskaya-Kucheryavenko TV, Nepomnyashih VM, Sennikov SV. Levels of TNF, TNF autoantibodies and soluble TNF receptors in patients with bronchial asthma. The Journal of asthma: official journal of the Association for the Care of the Asthma. 2013; 50(7):705–11.
55. Falvo JV, Jasenosky LD, Kruidenier L, Goldfeld AE. Epigenetic control of cytokine gene expression: regulation of the TNF/LT locus and T helper cell differentiation. Advances in immunology. 2013; 118:37–128. doi:10.1016/B978-0-12-407708-9.00002-9 PMID: 23683942

56. Almasry SM, Eldomiaty MA, Elfayomy AK, Habib FA. Expression pattern of tumor necrosis factor alpha in placenta of idiopathic fetal growth restriction. Journal of molecular histology. 2012; 43(3):253–61. doi:10.1007/s10735-012-9410-6 PMID: 22461198

57. Houseman EA, Accomando WP, Koestler DC, Christensen BC, Marsit CJ, Nelson HH et al. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC bioinformatics. 2012; 13:86 doi: 10.1186/1471-2105-13-86 PMID: 22568884

58. Toperoff G, Aran D, Kark JD, Rosenberg M, Dubnikov T, Nissan B et al. Genome-wide survey reveals predisposing diabetes type 2 related DNA methylation variations in human peripheral blood. Hum Mol Genet. 2012; 21(2):371–83. doi: 10.1093/hmg/ddr472 PMID: 21994764

59. Reinius LE, Acevedo N, Joerink M, Pershagen G, Dahlén SE, Greco D et al. Differential DNA methylation in purified human blood cells: implications for cell lineage and studies on disease susceptibility. PLoS One. 2012; 7(7):e41361. doi: 10.1371/journal.pone.0041361 PMID: 22848472

60. Singh AK, Kashyap MP. An Overview on Human Umbilical Cord Blood Stem Cell-Based Alternative In Vitro Models for Developmental Neurotoxicity Assessment. Mol Neurobiol. 2015. [Epub ahead of print].

61. Stock W, Hoffman R. White blood cells 1: non-malignant disorders. Lancet. 2000. 355: 1351–57. PMID: 10776761

62. Sanders JM, Lebetkin EH, Chen LJ, Burka LT. Disposition of 2,2′,4,4′,5,5′-hexabromodiphenyl ether (BDE153) and its interaction with other polybrominated diphenyl ethers (PBDEs) in rodents. Xenobiotica. 2006; 36(9):824–37. PMID: 16971346

63. Staskal DF, Diliberto JJ, DeVito MJ, Birnbaum LS. Toxicokinetics of BDE 47 in female mice: effect of dose, route of exposure, and time. Toxicological sciences: an official journal of the Society of Toxicology. 2005; 83(2):215–23.

64. Staskal DF, Hakk H, Bauer D, Diliberto JJ, Birnbaum LS. Toxicokinetics of polybrominated diphenyl ether congeners 47, 99, 100, and 153 in mice. Toxicological sciences: an official journal of the Society of Toxicology. 2006; 94(1):28–37.

65. Emond C, Sanders JM, Wikoff D, Birnbaum LS. Proposed mechanistic description of dose-dependent BDE-47 urinary elimination in mice using a physiologically based pharmacokinetic model. Toxicology and applied pharmacology. 2013; 273(2):335–44. doi: 10.1016/j.taap.2013.09.007 PMID: 24055880