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In utero arsenic exposure and fetal immune repertoire in a US pregnancy cohort

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Abstract

Arsenic has wide-ranging effects on human health and there is evidence that it alters the immune response by influencing CD4+/CD8+ T cell ratios, IL-2 cytokine levels, and the expression of immune-response genes. We investigated the impact of *in utero* environmental arsenic exposure on immune development and function in newborns participating in a pregnancy cohort in New Hampshire, U.S., where arsenic levels have exceeded the current EPA maximum contaminant level of 10 μg/L. Our results showed that maternal urinary arsenic concentrations were inversely related to absolute total CD45RA+ CD4+ cord blood CD69+ T cell counts (N=116, p=0.04) and positively associated with CD45RA+ CD69− CD294+ cell counts (p=0.01). In placental samples (N=70), higher *in utero* urinary arsenic concentrations were positively associated with expression of IL1β (p=0.03). These data provide evidence that relatively low-level arsenic exposure *in utero* may alter the fetal immune system and lead to immune dysregulation.

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Keywords

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1. Introduction

Arsenic has wide ranging human health effects, including its established role as a human carcinogen and emerging data suggesting it enhances susceptibility to infection [1–4]. Arsenic has been shown to affect multiple aspects of immune function, such as T cell ratios [5], IL-2 cytokine levels, and the expression of immune-response genes, including those involved in T cell receptor signaling [6–8]. These arsenic-associated changes in lymphocyte subsets and cytokine profiles could impact not only susceptibility to infection, but also risk of atopic disorders.

T cells represent a subset of immune cells that play a key role in fighting infections, in vaccine responses, and in promoting inflammation [9–13]. Once a T cell becomes activated, T cell effector functions can initiate downstream events, such as the synthesis of antibodies by B cells. CD4+ T cells play a role in the etiology and maintenance of immune diseases such as allergies [14]. Different subsets of CD4+ T cells in the peripheral blood can be further identified phenotypically by surface markers as memory (CD45RO+) or naïve (CD45RA+), proliferating (Ki67+) or activated (CD69+), Th1 (IFN-γ containing) or Th2 (CD294+) by standard flow cytometry techniques [10, 12]. Another subset of T cells, regulatory T cells (Treg, defined by CD4+ CD25hi CD127lo cells), usually increases in response to inflammatory processes [9, 13]. T cells, especially Treg, have been shown to influence pregnancy outcomes [15, 16] and are affected by environmental pollutants [11, 17].

Gestation is a critical period for immune development [18] and there is evidence that Th2-skewed responses to common environmental allergens occur in newborns [19, 20]. Given their limited exposure to antigens, most T cells in cord blood are presumed to be naïve (i.e., CD45RA+) and can become activated during inflammation [15, 16]. However, circulating neonatal T lymphocytes are fundamentally different from naïve adult T cells and have characteristics of recent thymic emigrants that are impaired in their acquisition of Th1 function [21]. Additionally, a high proportion of circulating neonatal T cells are concurrently progressing through the cell cycle and have increased susceptibility to apoptosis, as compared to those of adults [22]. While strong experimental evidence demonstrates that arsenic exposure can alter immune function [8, 23], few human studies have examined the effects of arsenic on T cells, and in particular, the developing T cell repertoire of the neonate.

The prevalence of atopy and allergic diseases have increased dramatically in recent years [24, 25], affecting up to 30% of the population in industrialized countries [12] and causing substantial childhood morbidity, as well as social and medical costs. At the same time, infant infections remain prevalent in the first year of life, even in developed countries [26, 27]. We have established a pregnancy cohort comprised of individuals with exposures spanning the

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dose range of regulatory interest, (i.e., water arsenic 0–100 μg/L, with over 10% of the cohort's well water samples exceeding the US EPA standard of 10 μg/L arsenic in water). As part of this cohort study, we investigated whether neonatal T cells are potential targets of arsenic immunotoxicity by examining changes in immune cell profiles, T cell functionality and gene expression measures.

2. Methods

2.1. Ethics Statement

Study protocols and materials were approved by both the Dartmouth Committee for the Protection of Human Subjects and Stanford Administrative Panels on Human Subjects in Medical Research. All subjects provided written informed consent to participate.

2.2. Study Subjects

Pregnant women who obtained their prenatal care at clinics in the New Hampshire area, including regions with relatively high water arsenic levels identified by our earlier work [28], were enrolled at 24–28 weeks gestation beginning in 2009. T cell functions were analyzed for 16 cord blood samples from the extremes of exposure: high arsenic (> 5 μg/L arsenic in water, and >5 μg/L total urinary arsenic, n = 8) and low arsenic exposed women (< 0.5 μg/L arsenic in water, and < 5 μg/L total urinary arsenic, n = 8). Immune profiling was performed using cryopreserved lymphocytes from the first 116 of the 129 remaining deliveries with available cord blood samples. Our study included pregnant women screened for the following eligibility criteria at their first prenatal visit: (1) currently pregnant, (2) 18 to 45 years old, (3) receiving routine prenatal care at one of the study clinics, (4) living in a household served by a private well (defined as a well serving fewer than 15 households or 25 individuals), (5) residing in the same place since their last menstrual period and using the same water supply, and (6) not planning to move prior to delivery. As only a small fraction of our study population is non-English speaking, we limited our recruitment to English speaking women.

2.3. Data Collection

Women who consented were asked to complete a self-administered lifestyle and medical history questionnaire that covered socio-demographic factors such as age, race/ethnicity (of the mother and child's father), and level of education. Women were asked about their general health and medical history, reproductive history, as well as lifestyle and exposure questions, including tobacco and alcohol use. Trained study staff also performed a structured review of the prenatal and delivery medical records (maternal and infant) to document pregnancy and delivery information.

2.4. Assessment of Arsenic Exposure

We ascertained information about each participant's household water supply including type of system (e.g., drilled versus dug well, or spring), use of water filters and the average number of cups per day of water participants consumed (in food or drink) from their household tap and other sources. Participants completed a diary of water, fish and seafood, and rice intake for the three days prior to urine collection to aid the assessment of recent

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arsenic exposure measured in the urine samples. Upon enrollment, a maternal spot urine sample was obtained in a pre-labeled 120 ml urine specimen container with 30 μL of 10 mM diammonium diethyl dithiocarbamate (DDDC) to stabilize the arsenic species. Samples were stored upright and maintained at 4°C and sent via courier to the Dartmouth coordinating laboratory within 24 hours for pH testing, aliquoting and storage at −80°C. Samples of urine were analyzed for individual arsenic species (inorganic arsenic [inAs, including arsenic III and arsenic V], monomethylarsonic acid [MMA V], dimethylarsinic acid [DMA V]) concentrations by ion chromatography coupled to inductively coupled plasma mass spectrometry (ICP-MS) using standard approaches at the University of Arizona, as previously described [29]. All sample preparations and analyses were carried out in a trace-metal free HEPA-filtered-air environment with strict adherence to QA/QC procedures. Detection limits (LOD) were 0.15, 0.1, 0.14 and 0.11 for arsenic III, arsenic V, DMA V and MMA V, respectively. In the 116 samples, there were 39.66%, 91.38%, 0% and 9.48% below LOD, respectively. The values below LOD were imputed by LOD/sqrt(2) for statistical analyses. Additionally, 101 of the 116 individuals (87%) urine samples were analyzed for creatinine (Cayman's Creatinine Assay Kit).

2.5. Cord Blood Collection, Cell Isolation/Flow Cytometry, and Suppression/Proliferation Assays

At delivery, a physician or delivery room assistant at the hospital filled 8 ml CPT™ Cell Preparation Tubes containing sodium citrate and a lymphocyte gradient, allowing blood to drip into the collection tubes to maximize the collectable volume and minimize contamination with bacteria and maternal cells. After collection, the cord blood was sent to the hospital's main laboratory or to the laboratory at Dartmouth and stored at 4°C until processing. No later than 24 hours following collection, the lymphocytes were isolated according to the manufacturer's instructions using standard buoyant density centrifugation methods. Following centrifugation, plasma was harvested, aliquoted and frozen at −80°C. Peripheral blood mononuclear cells (PBMCs) were isolated by pipette, washed with PBS and cryopreserved (−120°C) using freezing media at a controlled rate of 1°C per minute. This procedure demonstrates approximately 90% viability after thawing [11] and has high reproducibility in our laboratory [30] and other laboratories [31].

Cord blood lymphocytes were shipped frozen (on dry ice) to the Stanford laboratory using a courier service or next morning overnight postal service where samples were processed and stored at −80°C according to published procedures [11, 32]. Flow cytometric staining was performed on cord blood samples using an LSRII digital flow cytometer (BD Biosciences) with 4 lasers (405,488, 535, 633 nm), 2 light scatter detectors (yielding forward and side scatter data), and 18 fluorescent detectors for analysis. A phenotyping method was used with colors of fluorescently conjugated antibodies (LSRII; BD Biosciences) for the following CD4+ T cell-surface markers: CD294+ (Th2 surface marker) [33], CD294−, CD45RO+ (surface memory T cell marker) and CD45RA+ (surface naïve T cell marker) (Stanford Human Immune Monitoring Center). Data was acquired with DiVa software (BD Biosciences) and analyzed with FlowJo software (Treestar, Ashland, Ore). A calibration procedure was performed with a standard set of multicolor fluorescence beads in order to

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standardize signal output by the cytometer prior to each session. Absolute cell numbers were determined by using the complete PBMC count per ml (Stanford Clinical Laboratories).

To explore the relationship between arsenic exposure and T cell function, we analyzed 16 cord blood samples selected from the extremes of exposure: high arsenic (> 5 μg/L arsenic in water, and >5 μg/L total urinary arsenic, n = 8) and low arsenic exposed women (< 0.5 μg/L arsenic in water, and < 5 μg/L total urinary arsenic, n = 8). Treg and Teff cells were separated (CD4+CD25hiCD127lo=Treg and CD4+CD25lo=Teff) by FACS Aria (BD Biosciences, San Jose, CA). Treg suppression assays were conducted in round-bottom 96-well microtiter plates using Miltenyi kits for cell isolation, according to previously published methods [32]. A fixed number of each type of live cells were used per well: Teff (20,000 cells), Treg (20,000 cells), and irradiated, CD3+ depleted antigen-presenting cells (APCs) (200,000 cells). We added autologous CD4+CD25− conventional T cells, and autologous-irradiated CD3-depleted peripheral blood mononuclear cells with or without autologous Treg to test three parameters for each sample: 1) Treg-cell anergy (Treg cell alone with APC) as an assay control; (2) Treg-cell suppression (Treg cell: Treg and Teff cells at 1:1 ratios with APC); and (3) T cell proliferation (Teff cell with APC). StemSep human CD3+ T cell depletion (StemCell Technologies) was used to deplete APCs of T cells, followed by irradiation with 40 Gy, and anti-CD3 mAb (clone HIT3a at 5.0 mg/ml) was then added to all wells. T cells were cultured for 7 days at 37°C in a 5% CO_2-humidified atmosphere. 1.0 mCi ^{3}H-thymidine was added to each well 16 hours before the end of incubation, followed by plate harvesting using a Tomtec cell harvester. A Perkin Elmer scintillation counter was used to determine proliferation by ^{3}H-thymidine incorporation. The percentage of Teff cell proliferation was measured by using standard published thymidine incorporation methods, and the percentage of Treg cell function was determined as follows: (Teff cell proliferation – Teff cell:Treg cell proliferation)/Teff cell proliferation.

2.6. Placenta collection, RNA extraction and gene expression analysis

Following delivery, the placenta was biopsied adjacent to the cord insertion (to minimize heterogeneity), removing any maternal decidua, placed immediately in RNAlater (Life Technologies) and frozen at −80°C within 24 hours. RNA extraction and subsequent gene profiling were performed in two similarly sized batches (44 or 45 samples per batch). For placental samples, an approximately 200 μg wet weight representative sample was homogenized in Tri Reagent (Molecular Research Center) by an electronic homogenizer. RNA was extracted according to the vendor's instructions and purified using an RNeasy column (Qiagen). Quality of RNA was evaluated using the Agilent Bioanalyzer. Gene expression analysis of approximately 100 ng RNA was performed for 72 of the 116 newborns on whom we collected placental biopsies using the Nanostring system (Nanostring Technologies) at the Oncogenomics Core Facility (University of Miami) custom designed to include interleukin (IL)1β and other arsenic-related genes including Aquaporin-9 (AQP9). Data were compiled and normalized in the nSolver software and normalized to the geometric mean of the expression of 5 housekeeping genes (*ATCB*, *GAPDH*, *HPRT1*, *RPL19*, and *RPLP0*) and presented as normalized counts per gene per sample. A complete descriptive list of the genes measured can be found in Supplemental Table S2.

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2.7. Statistical Analysis

To estimate *in utero* exposure to arsenic we summed the individual arsenic species (inAs, MMA, DMA) in maternal urinary arsenic taken at 24–28 weeks gestation, with the exclusion of arsenobetaine an unmetabolized form of arsenic found in fish and shellfish. Absolute counts of T cells have right skewed distributions, and hence were log10 transformed for normalization before statistical testing. Multivariable linear regression models to determine the association between *in utero* arsenic exposure and profiles of T cells in infant cord blood were controlled for multiple potential confounders (e.g., maternal age, parity, pre-pregnancy body mass index [BMI], smoking, and infant sex, gestational age, and birth weight). Level of maternal education was unrelated to urinary arsenic concentrations and therefore not included in our models. We further assessed whether results differed by infant sex. Locally smoothed curves [34] were generated and compared with estimated linear regression lines. The spans of local intervals for smoothed curves were determined by minimizing the mean squared error. Linear regression assumptions were evaluated for each of the outcomes. For models with small sample sizes ($N \leq 10$), nonparametric Spearman correlations were also used to test the associations. To compare the Teff proliferation and Treg function assays between the high and low arsenic groups ($n = 16$), we used the nonparametric Wilcoxon rank-sum test [35] and examined their Spearman correlations with the individual arsenic exposure levels. For the gene expression data, we first adjusted for potential batch effects using the COMBAT method [36]. Using the batch-adjusted expression data, we used multivariable linear regression to model the natural log-transformed gene expression as a function of urinary arsenic, controlling for potential confounders (as described above). We similarly evaluated the association between AQP9 and IL1β expression. In sensitivity analyses, we included urinary creatinine concentrations in our models, and we examined the robustness of our results with exclusion of potential outliers. A two-sided significance threshold of $p < 0.05$ was used for all statistical tests.

3. Results

3.1. Study population

Women in the study were on average 31.6 years of age at pregnancy (SD = 4.3), with one prior pregnancy and a body mass index of 24.4 (SD = 4.4) (Table 1). Less than 10% reported smoking during pregnancy and 38% reported having less than a college degree (Table 1). Infants were 53% male, on average 39.5 weeks gestation (SD = 1.3) and weighed 3481 grams at birth (median = 3458.6, SD = 466.3) (Table 1). Maternal urinary arsenic concentrations were skewed, with a median value of 4.23 (IQR = 4.1; mean = 7.8; SD = 26.8 μg/L) (Table 1).

3.2. T cell phenotype

To determine the effects of *in utero* arsenic exposure on neonatal immune function, cord blood T cell lymphocytes were phenotyped on the 116 subjects and identified by surface marker staining and flow cytometry. The majority of proliferating T cells in umbilical cord blood are CD45RA+ and can be primed by antigen or cytokines in the immune environment to express early activation markers like CD69 prior to effector function and differentiation, therefore we have separated CD45RA+ T cells into CD69+ and CD69− T cell subsets [22,

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37–40]. CD294 (CRTH2) can be used as a surrogate surface marker for characterizing Th2 cells, therefore we further characterized T cells based on their CD294 and CD69 expression [33, 41, 42]. As not all subtypes of T cells were detected for each subject, sample sizes varied for the various models (Table 2, Supplemental Table S1). Using multiple linear regression models (adjusted for maternal age, parity, body mass index [BMI], smoking, and infant sex, gestational age, and birth weight) maternal urinary arsenic concentrations were inversely related to absolute counts of CD45RA+ CD69+ T cells in cord blood (Table 2; Figure 1A) with each unit (μg/L) increase in urinary arsenic associated with a 15% (95% CI: −0.7%, −28%) decrease in CD45RA+ CD69+ T cell counts. Urinary arsenic was positively associated with absolute counts of CD45RA+ CD69− CD294+ cells (Table 2; Figure 1B) whereby each urinary arsenic unit (μg/L) increase associated with a 17% (95% CI: 2%, 35%) increase in CD4+ CD45RA+ CD69− CD294+ cell counts (Table 2). We did not detect any appreciable difference by infant sex in these results (data not shown). Further, adjustment for urinary creatinine strengthened the associations (for CD45RA+ CD69+ T cells: −17.89%; 95% CI: −33.34%, 1.13%; n = 35, and for CD45RA+ CD69− CD294+ cells: 30.50%; 95% CI: 10.99%, 53.39%; n = 30), although the former association lost statistical precision. In contrast to findings for CD45RA+ CD4+ cells, there was no association of urinary arsenic with phenotypic changes in memory CD45RO+ CD4+ T cells (Table 2). As mentioned, Spearman correlations with arsenic also were calculated when the sample size was small, e.g., for CD45RO+ CD69+ CD294+ cells (Spearman r=0.33, p=0.35) and CD45RO+ CD69− CD294+ cells (Spearman r=0.12, p=0.77).

3.3. T cell function

To assess the functional capacity of T cells from neonates exposed to high levels of arsenic *in utero*, we measured the ability of cord blood effector T cells to proliferate following *in vitro* TCR stimulation, as well as the ability of cord blood Tregs to suppress this effector T cell function. Effector T cells from neonates exposed to high levels of arsenic *in utero* had increased proliferation as compared to T cells from neonates exposed to lower levels of arsenic *in utero* ($p = 0.03$) (Figure 2A) and, on a continuous scale, maternal urinary arsenic concentrations were positively correlated with increased cord blood T cell proliferation (Spearman r = 0.55, $p = 0.03$). In addition, cord blood Treg suppressor function was diminished at higher urinary arsenic concentrations (Figure 2B), although the difference ($p = 0.60$) and correlation coefficient were not statistically significant in this small initial sample (Spearman r= −0.24, $p = 0.37$). Characteristics of the 16 subjects with extreme arsenic exposure levels on whom we performed T-cell function assays are provided in Supplemental Table S1.

3.4. Placental IL1β expression is associated with maternal urinary arsenic concentration

The placenta plays a critical role in fetal development and arsenic has been shown to easily cross the placental barrier [43]. IL1β is a marker of general inflammation and plays direct and indirect roles in T cell activation and differentiation. AQP9 is an arsenic transporter, which we [44] and others [45–47] found to play a central role in mediating cellular response to arsenic exposure. Thus, we determined the association between the expression level of IL1β in the placenta and maternal urinary arsenic concentrations and the association between the expression of IL1β and AQP9. Placental expression of IL1β was positively associated

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with urinary arsenic concentrations during pregnancy (beta = 0.04, $p = 0.04$) in the multiple regression analysis of the normalized gene expression data, and with adjustment for batch effects and maternal age, parity, body mass index [BMI], smoking, and infant sex, gestational age, and birth weight. There was a slight difference in the coefficient estimates by infant sex (for males: beta = −0.03; $p = 0.56$, and for females: beta = 0.06; $p = 0.01$; p for interaction = 0.11). Moreover, a stronger correlation was observed between the expression of AQP9 and IL1β (beta = 0.93, $p < 0.0001$) (Figure 3) without evidence of differences by infant sex (data not shown). This finding was robust to creatinine adjustment and exclusion of a subject with an extreme urinary arsenic value (data not shown).

4. Discussion

We performed a preliminary study using flow cytometry approaches to profile immune cell populations from the cord blood from newborns in a US pregnancy cohort. We found *in utero* arsenic exposure to be related to the number of specific CD4+ T cell populations present in cord blood, increased cord blood T cell proliferation, and greater IL1β expression in the placenta. Previously, studies of more highly exposed populations of adults [7] and children 6 to 10 years of age [48] found reduced T cell proliferation in arsenic-exposed versus unexposed individuals. While methods for T cell stimulation differed between our study and the previously published work, differences also may be explained by the age of our cohort and the fact that our assessment is based on newborn cord blood [49]. The fetal T cell compartment consists of cells with high thymopoietic capacity and high proliferative turnover of post-thymic T cells [50]. Thus, our initial findings may help our understanding of differences in immune response between cord blood and peripheral blood of older children after toxicant exposures. Additionally, unlike the adult, the majority of proliferating T cells in umbilical cord blood are also CD45RA+ [22], which can be maintained through the first years of life. Consistent with our findings, in experimental systems, low exposure levels of arsenic enhance cell proliferation in a variety of cell lines [51–53]. Overall, our preliminary data suggest that arsenic exposure may have an effect on fetal/neonatal T cells.

The short and long-term consequence of these arsenic-related changes in cord blood and placenta are uncertain. Several studies, including recent work based on our own cohort [54], have reported maternal urinary arsenic concentrations during pregnancy to be associated with increased infectious disease morbidity during infancy [2, 3]. During pregnancy, fetal T cells tend to be tolerogenic in order to prevent a fetal immune response against maternal alloantigens [55], characterized by immature antigen-presenting cell function, a delayed antibody response, and reduced T cell function [56, 57]. In particular, neonatal T cell responses are characterized by minimal Th1 function and increased Th2 activity/cytokine production. Although the Th2 bias of the human newborn immune response is not as evident as that observed in newborn mice, human neonatal Th1 cell responses are often diminished and produce less IFNγ than their adult counterparts [58, 59]. Biasing fetal immunity toward Th2 polarization appears to be an evolutionary adaptation to maintain maternal-fetal tolerance during pregnancy and this dominant anti-inflammatory environment is maintained through early infancy [60, 61]. As the fetus grows and approaches birth, the immune system shifts to a more immunogenic phenotype, capable of defending the newborn from the microbes that it will encounter outside of the womb [55], which continues to mature in an

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age-dependent manner postpartum. Although the neonatal immune system affords some level of protection to a variety of pathogens, infants are clearly more susceptible to infectious disease and respond with less efficiency to vaccinations [62]. Alterations to this layered, precisely regulated process of immune development, as may occur with *in utero* arsenic exposure, could increase risks of infection if the tolerogenic phase is extended [56]. Thus, our findings suggest that neonates exposed to even low amounts of arsenic may experience a Th2 bias, and in turn, enhanced vulnerability to bacterial infections. However, further studies are ongoing to confirm our findings in a larger cohort with longitudinal follow-up.

Additionally, the heightened Th2 responses among *in utero* arsenic exposed newborns may contribute to exacerbated allergic and atopic conditions later in life [49]. Early maturation to an immunogenic state may result in abnormal antigen responses, potentially leading to autoimmune disease or allergy [55]. To our knowledge, the relation between arsenic and allergy and atopy has not yet been investigated. Thus, the clinical consequences of *in utero* arsenic exposure's association with neonatal CD4+ CD45RA+ CD69− CD294+ T cells, a surrogate Th2 cell surface phenotype, will be determined through continued follow-up for health outcomes.

Although we were only able to quantify one indicator of general inflammation in human placenta, IL1β, we found an inverse relation with *in utero* arsenic exposure and expression of AQP-9, an arsenic transporter that is expressed in trophoblasts of human placenta [63, 64]. IL1β is a marker of general inflammation and can directly and indirectly influence T cell activity and differentiation. The placenta plays a critical role in fetal development and arsenic has been shown to easily cross the placental barrier [43]. Our findings indicate increased IL1β expression with increasing arsenic exposure, findings that mirror results from a pregnancy cohort from Bangladesh with high levels of arsenic exposure [65, 66]. Overall, our results support the possibility that the effects of *in utero* arsenic exposure observed in more highly exposed populations may be occurring at the lower arsenic exposure levels common to the US. Further, the striking association between AQP9 and IL1β expression could reflect a unique regulation by arsenic on these genes in human placenta by which increased expression of *AQP9* leads to increased uptake of arsenic, and in turn the expression of IL1β ultimately resulting in increased general inflammation and T cell activation and responses. Admittedly, this is speculative as our study did not directly measure arsenic concentrations in the placenta.

Our study has a number of limitations. For example, despite the physical separation of fetal and maternal circulation by the placenta, transfer of maternal cells to the fetus, and vice versa, has been observed, and should be carefully considered when interpreting results of cord blood studies. Numerous studies have demonstrated that maternal DNA is present in cord blood at varying levels [66–68]. It has recently been hypothesized that mothers can harbor fetal cells from previous pregnancies (microchimerism), leading to potential immune responses in younger siblings through transmaternal cells [69]. In aggregate, however, these studies suggest that the extent of maternal cell contamination is not great enough to account for the altered cell populations we observed in our cord blood samples. Further, mothers in our study had on average one prior pregnancy, and adjustment for parity in our analyses did

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not diminish the significant associations with the detected immune changes. For efficiency and ease of collection, we chose to collect a spot urine sample at GW 24–28, a time point that coincides with a standard prenatal appointment during which women undergo a routine blood draw and glucose challenge test. While immune development and programming occurs throughout pregnancy, this time period during the second trimester also represents a critical exposure window for the immune system, as hematopoiesis shifts from the liver to the bone marrow and the thymus undergoes a period of rapid growth, and B cells start to develop [67]. We acknowledge that it is possible that assessment at one time point may not account for variations in arsenic exposure over time. However, there is evidence that intra-individual total urinary arsenic measurements are relatively stable in adults [68, 69] and repeated measures of total maternal urinary arsenic during pregnancy are consistent [65, 70], although the profile of arsenic metabolites may change over pregnancy [71, 72]. For our study, we collected samples within a narrow gestational window, which has shown little variation in urinary arsenic [29] and adjusted for gestational age in our analyses. Further, our study also did not comprehensively test all types of PBMCs in cord blood. For example, using DNA methylation profiles to infer white blood cell mixture in cord blood, we noted a positive association between utero arsenic exposure and proportion of CD8+ cells [73]. Thus, T, B, natural killer, dendritic cells, neutrophils, basophils, eosinophils and monocyte subsets will be investigated in the future. Additionally, while we considered a number of potentially confounding factors, residual confounding still is possible. As mentioned earlier, we also lacked clinical outcome data in this study, and additionally had limited statistical power to detect associations. Another limitation is that in some individuals, certain subtypes of T cells were not detected, resulting in limited statistical power for subtype analyses. This may have led to null associations that will require investigations in larger studies. However, the characteristics of those included in the subtype models did not appreciably differ (Supplemental Table S1). Despite these limitations, to our knowledge, this is the first study to evaluate cord blood immune profiles and function, as well as placenta gene expression of IL1β, in relation to levels of arsenic common to populations throughout the world.

In summary, our data suggest that specific immunophenotypes of cord blood cells and impaired function of T cell subsets including CD45RA+ CD69+ T cells and CD45RA+ CD69− CD294+ cells are associated with the extent of arsenic exposure prenatally. Moreover, our initial study represents an example of high throughput immune monitoring in a well-characterized birth cohort - a capacity that is critical to the assessment of possible mechanisms by which early life exposure to toxicants could impair the immune system and potentially lead to human disease. Available biomarker data on *in utero* arsenic exposure in our cohort open up the opportunity for determining whether arsenic exposure and other measures of immune response and function (e.g., vaccine response and cytokine production) are linked to clinical outcomes (e.g., infection, allergy and atopy) in the future.

Supplementary Material

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Abbreviations

Treg	regulatory T cell
APCs	antigen-presenting cells
AQP9	Aquaporin-9
IL1 β	Interleukin 1 beta

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Research Highlights

- *In utero* arsenic exposure related to altered lymphocyte profiles in newborns.
- Higher arsenic exposure associated with a decrease in naïve activated T cells.
- Higher arsenic exposure also related to an increase in activated Th2 cells.
- Further, placental AQP9 expression correlated with IL1b-expression.

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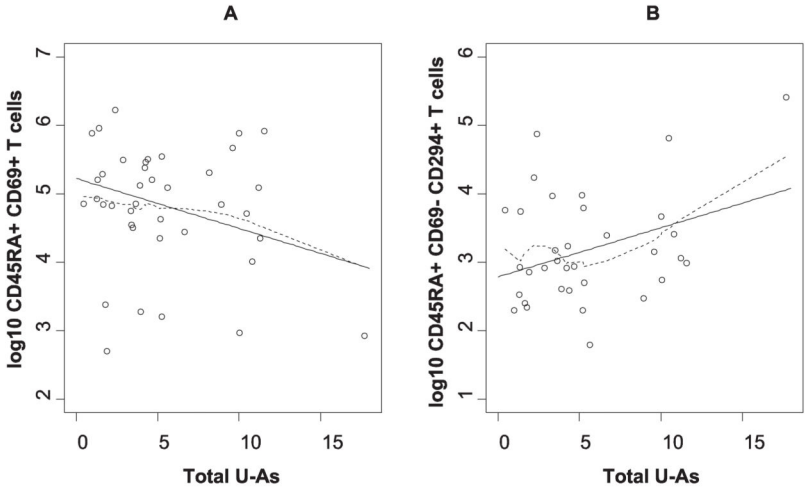

Figure 1.

Locally smoothed curves (dashed curves) for CD45RA+ CD69+ T cells (A) and CD45RA+ CD69− CD294+ T cells (B) compared with estimated linear regression lines (solid lines). Dots denote the observed data points.

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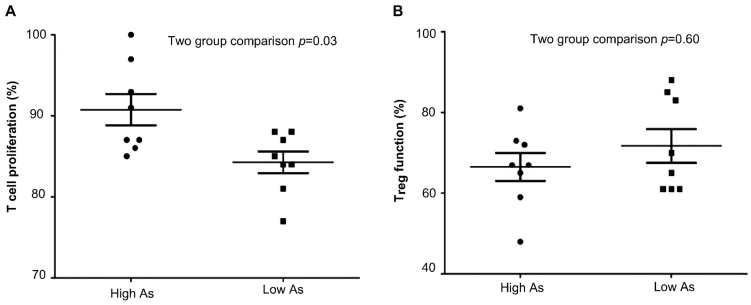

Figure 2.

(A) Percentage of effector T cell proliferation measured by cord blood effector T cells from neonates exposed to high levels of arsenic *in utero* (circles) or low levels of arsenic *in utero* (squares). (B) Treg cell suppressive activity on effector T cell proliferation by Tregs from neonates exposed to high levels of arsenic *in utero* (circles) or low levels of arsenic *in utero* (squares). % Treg function = (Teff proliferation – Teff:Treg proliferation)/Teff proliferation.

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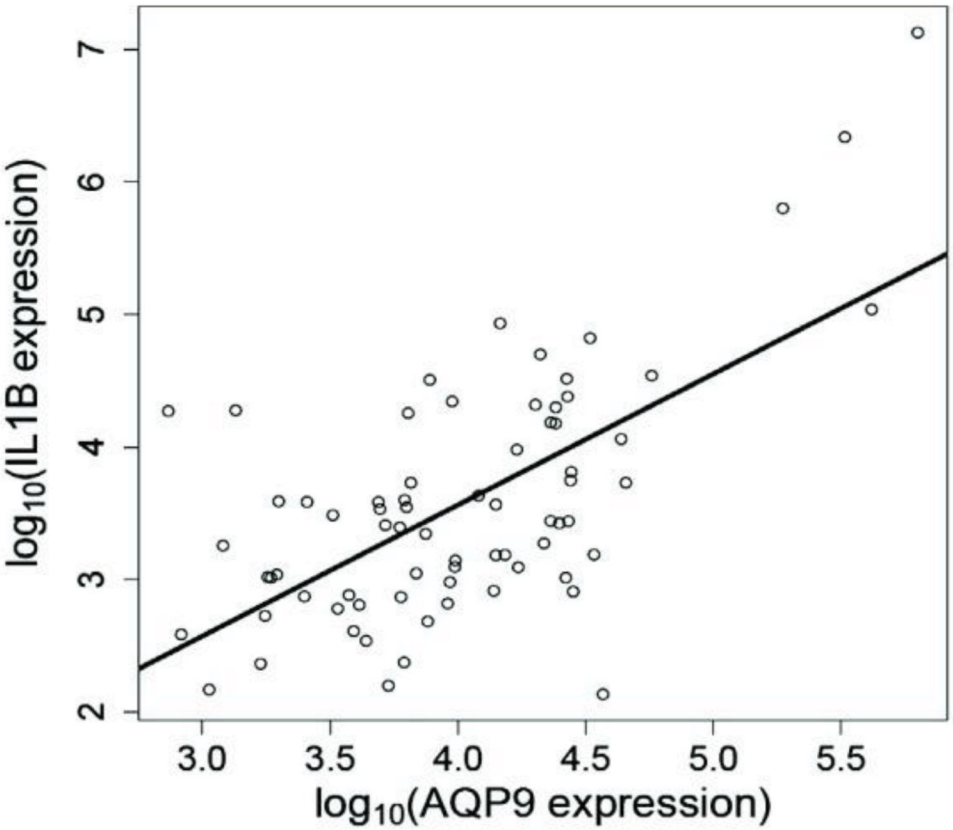

Figure 3.

Relationship between placental expression of arsenite transporter Aquaporin-9 (AQP9) and Interleukin 1 beta (IL1 β).

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Table 1

Selected characteristics of New Hampshire Birth Cohort Study participants included in the phenotyping studies (n=116).

Selected Characteristics	Mean, Median or N (%)
Maternal age (years)	
Mean (SD)	31.6 (4.3)
Median (IQR)	31.3 (6.2)
Parity	
Mean (SD)	1.1 (1.1)
Median (IQR)	1.0 (2.0)
Pre-pregnancy maternal body mass index	
Mean (SD)	24.4 (4.4)
Median (IQR)	23.4 (5.3)
Missing	17
Maternal smoking	
Yes	7 (7%)
No	94 (93%)
Missing	15
Level of Education	
Less than college graduate	38 (38%)
College graduate	42 (42%)
Post-graduate school	19 (19%)
Missing	17
Infant sex	
Male	61 (53%)
Female	55 (47%)
Gestational age (weeks)	
Mean	39.5 (1.3)
Median	39.6 (2.0)
Birth weight (grams)	
Mean (SD)	3480.1 (466.3)
Median (IQR)	3458.6 (652.0)
Total Urinary As (μg/L)*	
Mean (SD)	7.8 (26.8)
Median (IQR)	4.2 (4.1)
Water As (μg/L)	
Mean (SD)	5.7 (10.8)

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Selected Characteristics	Mean, Median or N (%)
Median (IQR)	1.2 (5.7)
missing	10

*The total urinary As includes As III, As V, DMA V and MMA V.

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Table 2

Log10-transformed absolute counts of CD4+ T cell populations per ml of PBMCs in newborn cord blood and associations with maternal pregnancy urinary arsenic concentrations.

	N	Mean (SD)	Adjusted percent change (95% CI) per 1 μg/L increase in urinary arsenic*	P value
Overall T cells				
Total T cells	116	5.01 (0.67)	−0.46 (−5.81, 5.44)	0.88
CD69+ T cells	39	4.02 (0.38)	4.71 (−2.28, 12.20)	0.27
CD25hi CD127lo Treg	37	4.25 (0.39)	4.71 (−2.28, 12.20)	0.29
CD45RA+ T cells				
Total CD45RA+ T cells	114	4.88 (0.76)	−0.23 (−6.46, 6.66)	0.99
CD69+ T cells	39	4.80 (0.90)	−14.89 (−28.39, −0.69)	0.04
CD69+ CD294+ cells	27	2.55 (1.28)	−6.67 (−29.37, 20.50)	0.46
CD69− CD294+ cells	34	3.21 (0.81)	17.49 (2.33, 34.90)	0.01
CD45RO+ T cells				
Total CD45RO+ T cells	110	3.68 (0.53)	−0.23 (−4.72, 4.23)	0.95
CD69+ CD294− cells	33	3.61 (0.54)	2.33 (−10.87, 17.49)	0.65
CD69+ CD294+ cells	10	1.63 (1.03)	2.33 (−39.74, 73.78)	0.99
CD69− CD294+ T cells	9	2.52 (0.39)	−6.67 (−27.56, 20.23)	0.42

*N represents the number of individual cord blood samples in which these cell types were detected.

**Regression models adjusted for maternal age, parity, body mass index [BMI], smoking, and infant sex, gestational age, and birth weight.

**Spearman correlations between arsenic and CD45RO+ CD69+ CD294+ T cells and CD45RO+ CD69− CD294+ T cells are 0.33 (p=0.35) and 0.12 (p=0.77) respectively.