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Effects of Developmental Activation of the Aryl Hydrocarbon Receptor by 2,3,7,8-Tetrachlorodibenzo-p-dioxin on Long-Term Self-Renewal of Murine Hematopoietic Stem Cells

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Running title: AHR activation in utero inhibits HSC self-renewal

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ABSTRACT

Background: Human epidemiological and animal studies suggest that developmental exposure to contaminants that activate the Aryl hydrocarbon receptor (AHR) lead to suppression of immune system function throughout life. The persistence of immune deficiency throughout life suggests the cellular target of AHR activation is a fetal hematopoietic progenitor or stem cell.

Objectives: The aim of this study was to identify the effect of transplacental exposure to an AHR agonist on long-term self-renewal of fetal hematopoietic stem cells.

Methods: Pregnant C57BL/6 or AHR+/- mice were exposed to the AHR agonist, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). On day 14 of gestation, hematopoietic progenitors from wild-type or AHR deficient fetuses were placed into in vitro T-lymphocyte differentiation cultures to identify the effects of transplacental TCDD on AHR activation in the fetus. We next analyzed the fetal hematopoietic progenitor cells for changes in reactive oxygen species (ROS). Finally, hematopoietic progenitors from fetuses exposed transplacentally to TCDD were mixed 1:1 with cells from congenic controls and used to reconstitute lethally irradiated recipients for analysis of long-term self-renewal potential.

Results: Our findings suggested that the effects of TCDD on the developing hematopoietic system were mediated by direct AHR activation in the fetus. Furthermore, developmental AHR activation by TCDD increased ROS in the fetal hematopoietic stem cells and the elevated ROS is associated with a reduced capacity of the TCDD-exposed fetal cells to compete with control cells in mixed competitive irradiation/reconstitution assay.

Conclusions: Our findings indicate that AHR activation by TCDD in the fetus during pregnancy leads to impairment of long-term self-renewal of hematopoietic stem cells.
INTRODUCTION

Since nutritional deficiency during pregnancy was first identified as a fundamental factor in the developmental origins of health and disease, there have been an explosion of factors identified that influence life-time health status for nearly every organ system in the body (Barker 2007; Haugen et al. 2015). Moreover, intrauterine environmental factors such as exogenous chemicals are clearly recognized as increasing risk for a spectrum of disorders that may appear during childhood and can persist throughout the life-course (Faulk and Dolinoy 2011; Haugen et al. 2015). Immune system function has been recognized as a particularly sensitive endpoint to changes in the intrauterine environment owing to its systemic distribution throughout the body and importance for both host defense and immunoregulatory function (Dietert 2011; Winans et al. 2011). Specifically, human epidemiological studies have found associations between developmental exposures and an array of later-life immune deficiencies including changes in cord blood lymphocyte composition, increased wheezing events, and increased autoinflammatory disorders (Choi et al. 2010; Herr et al. 2010; Jedrychowski et al. 2013). Additionally, animal studies have found that transplacental exposures to chemicals that bind to and activate the Aryl hydrocarbon receptor (AHR) adversely impact later-life immune effects by decreasing the immune response to influenza and also increasing autoimmune susceptibility in adults (Boule et al. 2014; Hogaboam et al. 2008; Mustafa et al. 2009). The diversity of adult diseases caused by related developmental exposures may be consistent with a two-hit mechanism whereby the prenatal exposure leads to epigenetic reprogramming of a progenitor cell that can have differential impacts on disease etiology depending on the genetic background, timing, and type of secondary environmental exposures. Indeed, this added complexity to the developmental origins of health and disease hypothesis has been proposed for other adult outcomes including
neurodevelopmental, reproductive, and obesogenic disorders (Bruner-Tran and Osteen 2011; Lahiri and Maloney 2010; Wadhwa et al. 2009).

The importance of the AHR for human health is demonstrated in part by epidemiological studies based on the population in Seveso, Italy accidentally exposed to the prototypical AHR agonist 2,3,7,8-tetrachlorodibenzo-\(p\)-dioxin (TCDD), where risk for lymphatic and hematological cancers were found to be slightly elevated in adults (Consonni et al. 2008; Pesatori et al. 2009). Furthermore, Vietnam Air Force Veterans exposed to TCDD-contaminated Agent Orange were reported to be at increased risk for melanoma and prostate cancer compared with unexposed veterans (Akhtar et al. 2004). In both the Seveso cohort and among Vietnam Veterans, small sample sizes for relatively rare diseases have made causal conclusions between TCDD exposure and disease difficult. Nevertheless, the International Agency for Research on Cancer (IARC) has classified TCDD as a group I human carcinogen based on sufficient epidemiological evidence for all cancers combined (Baan et al. 2009). Despite residual uncertainty on the carcinogenicity of TCDD in the general population, there is a need to identify and understand intergenerational impacts of dioxins given the unique vulnerability of children to developmental exposures (Landrigan and Goldman 2011). Notably, low-level background exposures to chemicals that activate the AHR occurs primarily through dietary intake (Kvalem et al. 2012; Liem et al. 2000; Schecter et al. 2001). Furthermore, human developmental evidence specific to the immune system comes from epidemiological studies that find an association between prenatal exposure to dioxins and dioxin-like PCBs and lower antibody titers to mumps and measles at preschool age (Weisglas-Kuperus et al. 2000).

Given the known risk for immune system impairment from developmental exposure to compounds that activate the AHR (Winans et al. 2011), it remains critical to identify the long-
term cellular and molecular targets on the hematopoietic system. In experimental systems the AHR has been identified as a negative regulator of hematopoietic stem cell proliferation (Singh et al. 2014). A study of mice exposed to TCDD as adults demonstrate AHR activation has adverse impacts on long-term self-renewal (Sakai et al. 2003). Furthermore, a transient inhibition of prothymocyte activity in fetal hematopoietic progenitors was reported following developmental TCDD exposure in mice (Fine et al. 1989). Despite a growing consensus that the AHR is an important regulator of hematopoiesis (Boitano et al. 2010; Carlin et al. 2013; Casado et al. 2011; Smith et al. 2013), it is entirely unknown what impact activation of the AHR in the fetus has on the long-term self-renewal function of the hematopoietic system.

To address this question, pregnant dams were exposed to a low oral dose of TCDD and we first tested the role of the AHR specifically in the fetus on the differentiation potential of fetal hematopoietic progenitors. Next, we found that developmental TCDD exposure increases fluorescence of an indicator dye that measures cellular reactive oxygen species (ROS) in fetal hematopoietic progenitor and stem cells. Using ROS as a marker of hematopoietic stem cell differentiation, we then sorted cells for gene expression analysis and found differential expression of Notch pathway genes in TCDD-exposed fetuses. Finally, we performed an irradiation / competitive reconstitution experiment using fetal liver progenitor cells from vehicle-exposed fetuses and fetuses exposed to TCDD transplacentally. We found that cells derived from the TCDD-exposed fetuses were significantly reduced in all immune tissues when competing against hematopoietic progenitors from the controls.
MATERIALS AND METHODS

Experimental animals

All animal procedures were conducted humanely and with regard for the alleviation of pain and suffering according to NIH’s Guide for the Care and Use of Laboratory Animals (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals 2011) and with the approval of the Institutional Animal Care and Use Committees (IACUC) at the University of Wisconsin-Milwaukee and the Blood Research Institute of Wisconsin. C57BL/6J mice, designated CD45.2, or B6.SJL-Ptprc<sup>a</sup> Pepc<sup>b</sup>/BoyJ mice, designated CD45.1, were offspring from original breeder pairs obtained from the Jackson Laboratory (Bar Harbor, ME). B6;129-Ahr<sup>tm1Bra</sup>/J mice, henceforth designated AHR<sup>+/−</sup> were obtained from Jackson Laboratory and bred as female heterozygote by male AHR<sup>−/−</sup>. After overnight pairings, the presence of a vaginal plug was designated gestational day (GD) 0.5. All mice were housed in Ancare 75 high temperature polycarbonate micro-isolator cages (11.75 x 7.25 x 5; 75 square inches) in a specified pathogen-free facility at the University of Wisconsin-Milwaukee using ¼ inch of Harlan Teklad corncob bedding. No more than five adult mice were housed per cage and reproductively active males were housed individually. Mice were provided Teklad Irradiated Global 19% Protein extruded rodent diet 2919 and autoclaved tap water ad libitum. Mice were maintained on a 12:12-h light:dark cycle and room temperature was maintained at \(22^{\circ}\pm1.5^{\circ}\text{C}\). Unless otherwise specified, four to six mice were used per group for each experiment.
**TCDD preparation and treatment protocol**

TCDD (Cambride Isotopes, Andover, MA) diluted in 1,4-dioxane (Sigma-Aldrich) at a working stock concentration of 0.2 mg/mL was suspended in olive oil (Filippo Berio) to a concentration of 0.3µg/ml as previously described (Ahrenhoerster et al. 2014). The Vehicle control was made with an equal volume of evaporated 1,4-dioxane added to olive oil. Timed pregnant mice were exposed to TCDD at a dose of 3µg/Kg body weight or olive oil vehicle control (0.1ml per 10g) by oral gavage on gestational days 0.5 and 7.5.

**Antibodies for lymphocyte staining**

Primary fluorochrome-conjugated monoclonal antibodies were used in flow cytometry analysis and cell sorting. Biotin-conjugated antibodies used for the lineage cocktail included CD3 (clone 145-2c11), LY-76 (clone TER119), CD45R/B220 (clone RA3-6B2), CD11b (clone M1/70), and LY6G/LY6C/GR-1 (clone RB6-8C5) coupled with Streptavidin-FITC. Lineage negative cells were further identified with Phycoerythrin (PE)-conjugated Sca-1 (clone E13-161.7) and Alexa647-conjugated cKit (clone 2B8; Life Technologies, Grand Island, NY). Thymocytes, and splenocytes were identified based on expression of Alexa647-conjugated CD8α (clone 53-6.7); PE-conjugated CD4 (clone L3T4); and APC-H7-conjugated B220 (clone RA3-6B2). FITC-conjugated CD45.1 (clone NDS58) and PE-Cy7-conjugated CD45.2 (clone 104) were used to identify chimerism within the bone marrow, thymus, and spleen. All antibodies were used at titrated concentrations and were purchased from BD Biosciences unless otherwise noted.

For analysis of relative intracellular ROS levels, cells were first stained for cell surface proteins, followed by incubation with 5µM 2’7’-dichloroflorescien diacetate (H2DCF-DA; Life
Technologies) in PBS containing 5% fetal bovine serum. H$_2$-DCF-DA is hydrolyzed by intracellular reactive oxygen species into the highly fluorescent DCF, detectable in the FITC channel of the FACS Aria III. After a 15 minute incubation in a 37°C water bath, cells were then acquired on the FACS Aria III.

For measurement of apoptosis, fetal progenitor cells from GD 14.5 were stained to identify LSKs, fixed, permeabilized and DNA breakage analyzed by the TdT-dependent nick-end labeling assay using the APO-BrdU kit from Phoenix Flow Systems according to the manufacturers instructions.

**Isolation of Fetal Liver hematopoietic progenitors for limiting dilution analysis**

Pregnant mice were euthanized by CO$_2$ asphyxiation followed by cervical dislocation to confirm death, according to the AVMA Guidelines on Euthanasia (Leary et al. 2013) on gestational day 11.5 or 14.5. For all experiments, mice were euthanized between 7:30 and 9:00 AM in our laboratory, which is inspected twice per year by the UW-Milwaukee IACUC. Fetal livers were dissected and cell suspensions made and used for limiting dilution analysis with exactly 1, 3, 10 or 30 LSK cells directly sorted into individual wells on a Costar tissue culture treated 96 well plate (Corning) containing mitomycin-C treated OP9-DL1 stromal cells grown at confluence as previously described (Ahrenhoerster et al. 2014). The limiting dilution assays testing the role of the AHR in TCDD responsiveness was accomplished by breeding AHR-/- male mice with AHR+/- females such that the dams and half the fetuses would be responsive to TCDD. In comparison, the AHR-/- fetuses were anticipated to be resistant to direct effects of *in utero* TCDD exposure. Cell sorting was conducted using a BD FACS Aria III, DIVA version 6.1.3, equipped with four laser (violet 405 nM, blue 488 nM, yellow/green 561 nM and red 633
nM), four way sorting capacity and an automated cell deposition unit capable of sorting a single cell into an individual well.

**Competitive irradiation chimeras**

Fetal liver hematopoietic progenitors on GD 14.5 were harvested as described for the limiting dilution experiment. After depletion of RBCs, cells from vehicle exposed CD45.1 fetuses were counted and mixed with an equivalent number of fetal liver cells from the vehicle- or TCDD-exposed CD45.2+ dams.

Four hours prior to reconstitution, host CD45.1+ mice were lethally irradiated with 11 Gy to eliminate all host hematopoietic cells. Subsequently, $1 \times 10^6$ fetal liver cells ($5 \times 10^5$ CD45.1 plus $5 \times 10^5$ CD45.2) were injected by IV injection into each host recipient mouse. Mice were then maintained for eight weeks to allow complete blood system reconstitution. At eight weeks, the primary competitive chimera recipients were euthanized by CO$_2$ asphyxiation followed by cervical dislocation as described above with the exception that the procedure was conducted at the Blood Research Institute between 9:00 and 10:00 AM. Bone marrow from one femur, along with the thymus and spleen was analyzed for the percent of chimerism. Bone marrow from the other femur was harvested under sterile conditions, depleted of RBCs, counted, and $1 \times 10^6$ cells were used in a secondary IV transfer into individual naive CD45.1+ irradiated host recipient mice. Transfers into the secondary recipients were performed by IV injection of cells from individual mice into each individual recipient to track any potential outliers throughout the full sixteen-week experiment. Eight weeks after the transfer, the secondary recipients were euthanized and lymphoid tissues analyzed for chimerism as was done in the primary recipients.
For analysis of competitive mixed chimeras, at least 100,000 viable lymphocytes from bone marrow, thymus, or spleen were acquired and chimerism was analyzed by comparing the frequency of CD45.1 or CD45.2. Additional analysis of cell subset distribution was determined by first gating on CD45.1 or CD45.2 followed by analysis of lineage markers, Sca-1, and ckit (bone marrow); or CD4, CD8, and B220 (thymus and spleen).

RNA preparation and Quantitative RT-PCR

Hematopoietic cells pooled from individual litters of vehicle or TCDD-exposed fetuses were isolated and sorted directly into Trizol (Life Technologies) on GD 11.5 using cKit and DCF fluorescence to discriminate between hematopoietic progenitor cells with long and short-term self-renewal potential. RNA was purified, quantified and processed as previously described (Ahrenhoerster et al. 2014). RNA was subjected to reverse transcription using Tetro cDNA Synthesis kit (Bioline) using both anchored-oligo(dT) 18 priming and random hexamer priming options and was stored at -20 until day of assay. Primers were selected using the Universal ProbeLibrary version 2.5 for Mouse (Roche) and checked for specificity using Primer-Blast (NCBI). Gene names, accession numbers, and primer sequences are provided in Supplemental Material, Table S1. cDNA was used as a template in 20µL reaction consisting of 10µM of forward and reverse primers and 10µL SensiFAST SYBR No-ROX (Bioline). Relative expression change was determined using the \([2^{\Delta\Delta Ct}}\) with standardization to housekeeping genes. Samples were run in triplicate wells with at least two independent litters per treatment analyzed. Cycling conditions were 95°C for 2 minutes, followed by 45 cycles of 95°C for 5s, 60°C for 10s, and 72°C for 10s.
Statistical analysis

The T-cell precursor frequency calculations and Chi-square statistical analysis for each genotype and exposure reported in figure 1 was accomplished using the “limdil” function contained in the “statmod” package in Rstudio as previously described (RStudio, 2012; Ahrenhoerster et al. 2014).

Graphpad Prism (Graphpad Software, LaJolla, CA) was utilized for the statistical analysis and graphical presentation of all other data. Specifically, Graphpad was utilized for the analysis of variance with Tukey’s t post-hoc tests used in the competitive reconstitution chimera experiments and analysis of DCF fluorescence (figures 2, 3, and 4). Statistical significance of changes in gene expression was determined by Student’s t test (figure 5). For all experiments, a p<0.05 was used to determine statistical significance.

RESULTS

Effects of developmental TCDD exposure on T lymphocyte differentiation capacity of hematopoietic progenitor cells from AHR deficient or AHR+-/ fetuses

Adverse effects of developmental exposures may be either directly mediated by the toxicant in the developing fetus, or via a toxic response occurring in the mother adversely affecting the endocrine and nutrient exchange systems required for growth of the fetus. In order to identify the fetal or maternal environment as the dominant target of TCDD that impacts the developing hematopoietic system we tested the T lymphocyte differentiation capacity of hematopoietic progenitor cells obtained from AHR+/ or AHR-/- fetuses from pregnant dams exposed to vehicle or 3µg/Kg TCDD. Assessment of T lymphocyte differentiation capacity is done using the OP9-DL1 ex vivo assay previously employed to demonstrate that developmental
exposure to TCDD from GD 0.5 through 14.5 attenuates the capacity of fetal hematopoietic progenitor cells to complete lymphocyte differentiation (Ahrenhoerster et al. 2014). Indeed, as shown in Figure 1, only hematopoietic progenitors obtained from TCDD-exposed AHR+/- fetuses had an attenuated T lymphocyte differentiation potential. Specifically, T cell progenitor frequency of hematopoietic progenitors was nearly identical in all but the TCDD-exposed AHR+/- cells with a progenitor frequency of 1 in 15.6 (95% CI: 13.6, 18.0) for vehicle-exposed AHR+/-, 1 in 14.2 (95% CI: 12.4, 16.3) for vehicle-exposed AHR-/- cells and 1 in 14.3 (95% CI: 12.7, 16.1) for TCDD-exposed AHR-/- cells. In comparison, the T cell progenitor frequency of LSK cells from TCDD-exposed AHR+/- hematopoietic progenitor cells was 1 in 30.4 (95% CI: 27.7, 33.5). These data indicate that the pool of hematopoietic progenitor cells from TCDD-exposed AHR+/- fetuses have a two-fold decrease in T lymphocyte differentiation potential compared with vehicle and TCDD-exposed AHR-/- fetuses.

Effect of developmental TCDD exposure on reactive oxygen species in fetal hematopoietic progenitor cells

Given the deleterious effects of developmental AHR activation on T cell differentiation, it was necessary to more specifically probe the function of the hematopoietic stem and progenitor cell pools in the fetus. As an initial approach to testing this function, the levels of intracellular reactive oxygen species (ROS) following developmental exposure to TCDD was assessed. ROS was analyzed because hematopoietic stem and progenitor cells maintain a lower basal metabolic rate thus preventing accumulation of DNA-mutagenic reactive oxygen species that could impact multi-lineage differentiation and long-term self-renewal (Ito et al. 2006; Jang and Sharkis 2007). Intracellular ROS was detected in hematopoietic stem and progenitor cells from GD 11.5 and 14.5 fetuses using the esterified vital dye H₂DCF-DA that is oxidized into the more highly
fluorescent DCF in the presence of oxidative stress. The GD 14.5 time point was chosen to identify potential cellular changes occurring in the cells that coincide with the time point used in the differentiation experiment shown in Figure 1. In comparison, GD 11.5 cells were initially analyzed to characterize the hematopoietic stem cells prior to when differentiation potential was interrogated. Furthermore, GD 11.5 represents an important transition in fetal hematopoiesis where hematopoietic progenitors are starting to be defined as true stem cells because of their acquisition of long-term self-renewal (Ciriza et al. 2013; North et al. 2002). As shown in Supplementary Material, Figure S1, the DCF fluorescence changes dependent on the specific cell population analyzed. Thus it was necessary to analyze DCF specifically within the putative hematopoietic progenitor and stem cell populations. Furthermore, we analyzed DCF in progenitor cells from age matched AHR-/- fetuses given previous reports that ROS is elevated in bone marrow hematopoietic stem cells from AHR-/- mice (Singh et al. 2014). As shown in Figure 2A, there is a bimodal distribution of DCF within the cKit+ hematopoietic progenitor cells on GD 11.5. Thus, an electronic gate surrounding each of the two DCF populations was made using the vehicle sample and the populations are designated cKit+DCF<sub>hi</sub> (top gate in Figure 2A) and cKit+DCF<sub>int</sub> (lower gate in Figure 2A). Notably, total DCF fluorescence is clearly elevated in the cells from TCDD-exposed fetuses indicated by the population shift toward the upper gate in the right hand plot of Figure 2A. In AHR-/- fetuses, the basal level of DCF fluorescence in cKit+ progenitors is nearly the same as observed in the TCDD-exposed fetuses. Specifically, developmental AHR activation by TCDD in C57Bl/6 and absence of the AHR increases the overall fluorescence intensity of DCF by nearly 75% indicating higher levels of intracellular ROS in fetal hematopoietic stem and progenitor cells (Figure 2B). Moreover, by taking a ratio of the percentage of cKit+DCF<sub>hi</sub> cells to the cKit+DCF<sub>int</sub>, it is evident that
developmental AHR activation increases the frequency of hematopoietic progenitors with high intracellular ROS levels by nearly 2-fold with $p \leq 0.05$ (Figure 2C). The elevated ROS was not associated with an increase in apoptosis as measured by TdT dependent nick end labeling assay in hematopoietic progenitors (Supplemental Material, Figure S2). Similar effects of developmental AHR activation on ROS were observed on GD 14.5 (Figure 2D), however, the magnitude of change in DCF fluorescence was less pronounced (Figure 2E). Despite the slightly lower basal ROS at GD 14.5 than 11.5 there still exists an slight, but not statistically significant increase in the ratio of cKit+DCF$^{hi}$ cells to cKit+DCF$^{int}$ cells in progenitors from TCDD-exposed fetuses on day 14.5. In comparison, there was more than a 2-fold increase in this ratio in AHR-/- fetuses ($p \leq 0.05$; Figure 2F).

**Effects of developmental TCDD exposure on long-term self-renewal capacity of hematopoietic stem cells**

Self-renewal is a fundamental property of hematopoietic stem cells and refers to the principle that each time a stem cell divides, one of the daughter cells will replace the functions of the original parent cell while the other cell undergoes multi-lineage differentiation. Testing the functional capacity of a hematopoietic stem cell pool to maintain long-term self-renewal potential is accomplished by mixing wild-type control progenitor cells with a test population and following the ability of each population to sustain hematopoiesis in a lethally irradiated host (Richie Ehrlich et al. 2011; Weissman 2000). As illustrated in Figure 3A, we performed this competitive reconstitution experiment by competing GD 14.5 fetal liver cells from vehicle exposed CD45.1 fetuses with CD45.2 fetal liver cells from either vehicle exposed or TCDD-exposed dams. Eight weeks after initial reconstitution, the contribution of each donor pool was analyzed in the bone marrow, and thymus of the initial recipients. Furthermore, bone marrow
from each recipient was transferred into a secondary recipient to confirm long-term self-renewal potential of each donor pool. We found that the hematopoietic stem cell pool obtained from the CD45.2+ TCDD-exposed fetuses failed to compete effectively over the course of the primary and secondary irradiation/reconstitution (Figure 3B). Notably, the diminished competitive capacity of the hematopoietic stem cells from the TCDD-exposed fetuses was observable after the initial reconstitution where the cells from the control exceeded the TCDD by nearly 2.5-fold (Figure 3C, p<0.01). This difference was further exacerbated following the secondary transfer with the control cells outcompeting the TCDD-exposed hematopoietic progenitors by approximately five-fold (Figure 3D, p<0.01).

In order to determine if the diminished competitive capacity of TCDD-exposed fetal progenitors is reflected by a reduction in hematopoietic stem and progenitor cells in the reconstituted primary and secondary bone marrow, we analyzed the frequency of CD45.1 or CD45.2 derived hematopoietic stem and progenitor cells defined by absence of lineage markers and expression of Sca-1+ and cKit+ (LSKs). In the chimeras where vehicle-exposed CD45.1 cells competed with vehicle-exposed CD45.2 cells there was no significant difference in the frequency of cells identified as LSK (Figure 4A). However, in primary chimeras where vehicle-exposed CD45.1 fetal liver progenitors competed with TCDD-exposed CD45.2 fetal liver chimeras there was a nearly 50% reduction in the frequency of LSK cells recovered in the bone marrow eight weeks after initial transfer (Figure 4B). Furthermore, following the secondary bone marrow transfer, LSK cells from TCDD-exposed CD45.2 cells were severely depleted compared to the vehicle-exposed CD45.1 competitor cells (Figure 4D). Specifically, in the secondary bone marrow chimeras, LSK cells from TCDD-exposed fetuses were reduced by nearly 80% compared to the control competitors (Figure 4E, p<0.01). In comparison to the hematopoietic
stem and progenitor cell population in the bone marrow, mature effector cells in the peripheral organs were able to complete their normal maturation program, however, cells from the TCDD-exposed fetuses were still at a competitive disadvantage. For example in the thymus, while both CD45.1 and CD45.2 derived progenitor cells produced thymocytes according to their expected proportion, the CD4+CD8+ thymocytes derived from the TCDD-exposed CD45.2 cells were reduced by more than 50% compared with vehicle-exposed CD45.1 cells (Supplemental Material, Figure S3).

**Effects of in utero TCDD exposure on cellular differentiation-induced gene expression changes**

Given the adverse impacts of developmental TCDD exposure on fetal hematopoietic progenitor cells and long-term self-renewal we analyzed the gene expression changes that occur between two distinct hematopoietic progenitor cell populations to determine how normal differentiation-induced gene expression changes were impacted by developmental TCDD exposure. Specifically, we sorted GD 11.5 lineage-negative cKit+ fetal hematopoietic progenitor cells based on DCF fluorescence as illustrated by the gating strategy used in figure 2 with the sorting strategy further delineated in Supplemental Material, figure S4. We chose to compare gene expression between these two cell populations because of the hypothesized difference in self-renewal potential between the DCF^hi and DCF^int populations (Ito et al. 2006; Jang and Sharkis 2007). Given the earlier reports that higher ROS signals a transition to short-term self-renewal and multi-lineage differentiation, the approach of comparing gene expression changes between cKit+DCF^int and cKit+DCF^hi was considered to be an innovative approach to determine the effect of in utero TCDD exposure on this putative developmental transition. Within these cell
populations, we focused our analysis on transcripts known to be involved in Notch and Notch-dependent signal transduction, cellular metabolism, and oxidative stress.

As shown in figure 5A, the importance of Notch dependent signal transduction in the transition from long-term (cKit+DCF<sup>int</sup>) to short-term self-renewal (cKit+DCF<sup>hi</sup>) is illustrated by decreases in the relative expression of Notch1 and the Notch-dependent transcription factor Hes-1 in control fetal cells, and an increase in the relative gene expression of the structurally distinct Notch family member protein, Notch4. In comparison, in utero TCDD exposure attenuates this differentiation-dependent reduction in Notch message (p<0.05) and furthermore significantly increases expression of Hes-1 between these two cell populations (p<0.05). The differentiation-induced increase of Notch4 message in the cKit+DCF<sup>int</sup> to cKit+DCF<sup>hi</sup> control transition is abrogated with a significant reduction occurring in TCDD-exposed cells (p<0.05). In addition, we show that among a panel of metabolic and oxidative stress regulatory genes, TCDD significantly increases pyruvate kinase expression (p<0.05) in cKit+DCF<sup>hi</sup> cells relative to cKit+DCF<sup>int</sup> cells (figure 5B) in contrast to the reduced relative expression observed in controls. Finally, expression of the known TCDD-target gene and eNOS regulator GTP-cyclohydrolase (Andreasen et al. 2006; Carney et al. 2006), is decreased by TCDD within this cellular developmental transition (p<0.05).

**DISCUSSION**

Given the dual function that hematopoietic stem cells possess to undergo self-renewal and multi-lineage differentiation for production of the effector cells responsible for immune system function, identification of the impact of developmental environmental exposure on these cells is urgently needed. Moreover, the sensitivity of the immune system to developmental insults that persist throughout life is consistent with the conclusion that a progenitor population
present in the fetus is a target of intrauterine environmental exposures. Thus, we believe that we are the first to report experimental evidence that is consistent with a direct effect of prenatal TCDD exposure on impairment of hematopoietic stem cell long-term self-renewal.

Our conclusion that transplacental TCDD has a direct effect on hematopoiesis is based on our studies with the AHR deficient hematopoietic stem cells placed into T lymphocyte differentiation cultures. Specifically, this experiment examined the contribution of AHR activation directly in the fetus on T lymphocyte differentiation potential. We report that fetal hematopoietic stem cells from AHR deficient fetuses were resistant to the inhibitory effects of TCDD. These data suggest that AHR activation directly in the fetus is the primary mechanism by which TCDD mediates its developmental immunotoxicity.

Given this data supporting the hypothesis that the developmental immunotoxic effects of TCDD was through direct AHR activation in the fetus, the next question is to identify the target cell population affected. We found that AHR activation in the fetus increases oxidative stress in hematopoietic progenitor cells. While activation of the AHR by TCDD is known to impact ROS (Kennedy et al. 2013; Kopf and Walker 2010; Wan et al. 2014), it is unclear from the present study whether the observation is due to increased induction of P450 enzymes or due to potential changes in the cell’s metabolic activity. However, induction of the phase I enzymes Cyp1a1 and Cyp1b1 is not nearly as robust in hematopoietic-derived cells as in other tissues (Ahrenhoerster et al. 2014; Lawrence et al. 1996), suggesting this change could be attributable to energetic changes occurring in the cell. It is known that hematopoietic stem cells in the adult bone marrow maintain low cytoplasmic ROS by producing energy via glycolytic anaerobic respiration (Jang and Sharkis 2007; Simsek et al. 2010). In comparison, the fetal hematopoietic stem cell energetic balance is less well understood and changes throughout development (Imanirad et al. 2014;
Regardless, it is well established that an elevated oxidative state is detrimental for long-term self-renewal (Iiyama et al. 2006; Ito et al. 2006; Takubo et al. 2013).

To further evaluate the implications of the elevated oxidative state, we sorted fetal hematopoietic progenitors based on DCF and thus ROS levels and conducted a focused analysis of gene expression changes dependent on the developmental stage of the cells and TCDD exposure. Genes were selected based on their role in Notch signaling, cellular metabolism, and oxidative stress. Notch and Hes-1 were selected based on the previously described function during self-renewal whereby decreased Notch-activity leads to differentiation (Duncan et al. 2005), and Hes-1 has been identified as a target gene of TCDD (Thomsen et al. 2004). Thus, the decrease in Notch1 and Hes-1 expression (figure 5A) in control cKit+DCF^{hi} cells compared with control cKit+DCF^{int} cells is consistent with a loss of self-renewal potential during the normal process of maturation. In contrast, Hes-1 expression was increased in TCDD-exposed cKit+DCF^{hi} cells compared with TCDD-exposed cKit+DCF^{int} cells, while the decrease in Notch1 was significantly less in the TCDD-exposed cells than the corresponding decrease in control cells. Notably, Hes-1 is a transcriptional repressor involved in a Notch feedback loop that is under strict regulation in stem cells (Guiu et al. 2013), and loss of that strict regulation via TCDD-exposure could impact hematopoietic stem cell renewal capacity. Furthermore Notch4, which is reported to be involved in hematopoietic stem cell maintenance (Vercauteren and Sutherland 2004), has elevated expression in control cKit+DCF^{hi} cells compared with control cKit+DCF^{int} cells and is significantly reduced following in utero TCDD exposure. Taken together, the altered expression patterns of Notch1, Hes-1 and Notch4 in the TCDD-exposed cKit+DCF^{hi} cells relative to their cKit+DCF^{int} precursors combined with the pronounced shift toward elevated ROS in these fetal cells could be indicative of premature differentiation of the
hematopoietic stem cell pool. A potential consequence of this premature differentiation is that it effectively depletes the pool of long-term self-renewing cells at a critical time during development that the organism cannot recover.

In addition to the effects of TCDD on the Notch pathway, we also measured potential changes in cellular metabolism and oxidative stress regulatory genes due to the elevated ROS. Notably, TCDD-induced a significant increase in expression of pyruvate kinase (PKM2) in the maturation from cKit+DCF\textsuperscript{int} to cKit+DCF\textsuperscript{hi} compared with control cells undergoing the same developmental maturation, suggestive of a transition to a more metabolically active cell with potentially elevated ROS. However, while other metabolic and oxidative stress genes were largely unchanged, these studies do not rule out potential effects of developmental AHR activation on the activity level of the enzymes encoded by the genes analyzed. Thus, a more comprehensive metabolomic and proteomic approach designed to test the normal energy regulation of these hematopoietic cells and determine the impact of TCDD may offer novel mechanistic insight into AHR regulation of cellular metabolism in hematopoietic stem cells during critical developmental transitions.

Taken together, by analyzing the gene expression changes that are associated with normal developmental transitions occurring during hematopoiesis, we have identified two potential mechanisms accounting for the loss of self-renewal and increase in ROS of fetal hematopoietic stem cells. The data with TCDD-induced alterations of Notch and Hes-1 expression combined with the shift in the proportion of cells in cKit+DCF\textsuperscript{int} to cKit+DCF\textsuperscript{hi} are suggestive of premature maturation of the hematopoietic stem cell pool, effectively depleting the number of long-term hematopoietic stem cells. Alternatively, the increased PKM2 expression could be indicative of conversion of the cells from glycolysis to oxidative phosphorylation and if
this occurred prematurely during development it could have an adverse effect on establishment of long-term self-renewal in the fetus.

In comparison to fetal development where we are just starting to understand the role for the AHR, it is well established that the AHR acts as an important regulator of hematopoiesis in adult model systems. Specifically, new classes of non-toxic AHR agonists and antagonists implicate a role for the AHR in hematopoietic stem cell expansion and maturation while maintaining multi-potential differentiation capacity (Boitano et al. 2010; Carlin et al. 2013; Smith et al. 2013). Others have reported that the AHR maintains hematopoietic stem cells in a quiescent state by acting as a negative regulator of cell proliferation (Gasiewicz et al. 2014; Singh et al. 2009), while maintaining a low intracellular oxidative state (Singh et al. 2014). In support of this model, a possible interpretation of our data is that the cytoplasmic-localized AHR complex has a normal function maintaining low ROS in hematopoietic stem cells. Thus, in the presence of a potent agonist such as TCDD, dissociation of the AHR from its cytoplasmic complex and concomitant nuclear translocation of the AHR removes a ROS regulator from the quiescent hematopoietic stem cell cytoplasm. In possible support of this model, our data and that of others demonstrates that baseline levels of ROS are elevated in the absence of the AHR. Taken together, our ROS data in both AHR-/- and TCDD-exposed cells along with our gene expression findings suggests a potential mechanism whereby TCDD-activation of the AHR in fetal hematopoietic stem and progenitor cells increases Notch-dependent signal transduction in the cell and accelerated maturation. Elevated Notch signaling combined with potentially elevated metabolic activity and the associated ROS adversely impacts long-term self-renewal.

Beyond the mechanistic implications for developmental AHR activation on hematopoiesis, the environmental health impact of AHR activation by TCDD in the fetus on
hematopoiesis is more sensitive than comparable studies in adult mice. Specifically, the dose of 3µg/Kg TCDD administered to the pregnant dam in the current study is ≥ 10 fold lower than the dose used in experiments testing self-renewal potential of adult bone marrow hematopoietic stem cells (Casado et al. 2011; Sakai et al. 2003). Moreover, the dose used for these studies is well within the 1 to 10 µg/Kg TCDD exposures utilized in recent developmental basis of health and disease rodent studies testing cardiomyocyte, epidermal and immunological end points (Muenyi et al. 2014; Wang et al. 2013; Winans et al. 2015). Collectively, these and our studies all result in an exposure to the fetus that is in the ppt range based on measurements that show only ~0.5% of the TCDD administered to a pregnant C57BL/6 dam is transferred to the fetus (Weber and Birnbaum 1985). While comparisons between rodent studies and human exposures are inherently complicated by differences in pharmacokinetics, gestation, reproductive biology, life-span and complex mixtures exposures, these in utero exposures are within an order of magnitude of the TEQ estimates for several populations across the globe based on food consumption (Hochstenbach et al. 2012; Schecter et al. 2001), or GC/MS exposure assessment of maternal blood, cord blood and placenta (Suzuki et al. 2005).

Identification of the hematopoietic system as a sensitive target impacted by in utero exposures has broad implications for later life health and disease given the essential role for hematopoiesis in the formation of the blood and immune system. Any perturbation of the developing hematopoietic system has the potential to adversely impact a spectrum of later-life blood diseases including anemia, cancer, and immune suppression. It should be noted however, that developmental TCDD exposure may be more complex with adverse effects occurring in both the hematopoietic and stromal compartments (Boule et al. 2014). It is also unknown from the current study if exposure to other AHR agonists during development will produce a similar
outcome. Nevertheless, these findings should initiate further research to determine the effects of different classes of AHR active compounds on long-term self-renewal along with identification of other environmental factors present in the intrauterine environment that have impacts on development of the hematopoietic system.

CONCLUSIONS

To our knowledge, we have shown for the first time that developmental exposure to a low dose of TCDD impairs long-term self-renewal of hematopoietic stem cells. The potential mechanism of this self-renewal impairment is consistent with AHR activation occurring directly in the fetus and is associated with increased ROS levels in fetal hematopoietic stem cells.
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FIGURE LEGENDS

Figure 1: Effects of developmental TCDD exposure and fetal AHR expression on the capacity of hematopoietic progenitor cells to undergo T lymphocyte differentiation. Fetal liver hematopoietic stem cells from GD 14.5 fetuses were placed into OP9-DL1 limiting dilution in vitro co-culture experiments. Twelve days later, hematopoietic stem cell differentiation into thymocytes as a measure of T cell differentiation potential was analyzed. Data are reported as the average number of hematopoietic stem cells placed into culture needed to produce a single thymocyte colony. Error bars represent the upper bound of the 95% confidence interval for each group. Data from AHR+/- fetuses are represented by solid bars and AHR-/- fetuses are represented by bars containing diagonal slashes. The white bars represent fetuses from dams exposed to vehicle, whereas the blue bars are from TCDD-exposed fetuses. The *** reflects a p≤0.01 by Chi Square analysis comparing the TCDD-exposed AHR+/- to the vehicle exposed AHR+/- fetuses. Data are pooled from three independent experiments.

Figure 2: Effects of developmental TCDD exposure and fetal AHR expression on reactive oxygen species in fetal hematopoietic progenitors. For the analysis shown in figure 2, viable lymphocyte-sized cells were electronically gated based on lineage-cKit+ as illustrated in supplementary material figure S1. (A) Representative cKit vs DCF flow cytometry plots on GD 11.5 for vehicle, TCDD and AHR-/- fetuses. The number to the right of each cKit+DCF gate indicates the percentage of cells within each population. (B) Mean fluorescence intensity of total DCF in cKit+ cells is plotted. (C) The percentage of GD 11.5 cKit+ cells in the DCFhi is compared to the percentage of DCFint in order to illustrate that TCDD increases both the overall DCF profile and also changes the distribution of cells within the bimodal distribution. (D) On GD 14.5 lin-cKit+Sca-1+ cells were further analyzed for cKit vs DCF as shown in fig 2A. (E) Mean fluorescence intensity of total DCF in LSK cells on GD 14.5. (F) Ratio of DCFhi to DCFint in the GD 14.5 LSK population. Data in the bar graphs are the Mean ± SEM with n=8 individual fetuses per C57BL/6 group from two separate litters and 4 AHR-/- fetuses. Experiment was repeated twice. Statistical significance determined by Tukey’s t test after ANOVA is denoted with an * for p≤0.5 or *** for p≤0.01 compared to the vehicle.

Figure 3: Effects of transplacental TCDD-exposure on long-term self-renewal potential of fetal liver hematopoietic stem cells. (A) Schematic model of experimental design for the
primary reconstitution experiment. (B) Representative flow cytometry plot of bone marrow from the primary chimera. Numbers in each quadrant of the flow cytometry plots represent the percentage of bone marrow cells identified by the antibody specific for CD45.1 or CD45.2 congenic surface proteins. (C) Percent of bone marrow cells from each donor in the primary and secondary recipients. White bars represent control competitive chimeras, and blue bars are from the chimeras where vehicle cells are competed with cells obtained from TCDD-exposed fetuses. Solid bars represent CD45.1+ cells, and CD45.2+ cells are denoted with diagonal slash-filled bars. Data are the mean+SEM with 5 mice per group. Experiment was repeated twice. Three *** denotes statistically significant by ANOVA followed by Tukey’ with p<0.01 compared with the congenic cells from the same chimera. (D) Percent of bone marrow cells from each donor after the secondary bone marrow transfer.

Figure 4: Effects of developmental TCDD-exposure on bone marrow hematopoietic progenitor cells after long-term competitive reconstitution. (A) cKit vs Sca-1 flow cytometry plots from the primary vehicle-to-vehicle competitive chimeras with CD45.1+lin- cells in the left panel and CD45.2+lin- cells in the right panel. The percentage of lin- cells is specified by the number in each quadrant. (B) Flow cytometry plots from the primary vehicle to TCDD competitive chimeras. (C) Representative cKit versus Sca-1 flow cytometry plots from the vehicle-to-vehicle secondary bone marrow chimera. (D) Flow cytometry plots from the secondary vehicle to TCDD competitive chimeras. (E) Relative frequency of LSK hematopoietic progenitor cells in the CD45.2 population of cells compared to the CD45.1 competitor cells from the primary and secondary chimeras. White bars compare vehicle-to-vehicle chimeras and blue bars compare the vehicle to TCDD chimeras. Solid bars are from the primary chimeras and secondary chimeras are represented by bars containing slashes. The percent of control was calculated by dividing the frequency of ckit+Sca-1+ cells from the CD45.2+lin- population by the same population of CD45.1+lin- cells. Data are the mean+SEM from n=5 chimeras and the experiment was repeated once. Three *** denotes statistically significant by ANOVA followed by Tukey’ with p<0.01 compared with the congenic cells from the same chimera.

Figure 5: Impact of developmental TCDD exposure on differentiation-induced changes in gene expression in hematopoietic progenitor cells. Hematopoietic stem and progenitor cells were purified by FACS sorting as illustrated in figure 2A and with the sorting strategy shown in
Supplemental Material, figure S4. (A) Gene expression changes associated with Notch signal transduction between cKit+DCF\textsuperscript{int} compared to cKit+DCF\textsuperscript{hi} cells from control fetuses (white bars) and TCDD-exposed fetuses (blue bars). (B) Gene expression changes associated with cellular metabolism (CD36, TSC1, CPT1, CPT2, FASN, PKM2), and oxidative stress (SOD2, GTPCH1) in cKit+DCF\textsuperscript{int} compared to cKit+DCF\textsuperscript{hi} cells from control fetuses (white bars) and TCDD-exposed fetuses (blue bars). Data are presented as the mean±standard deviation of replicate wells from a single qPCR reaction with the experiment performed on two independent litters of mice. Statistical significance by Student’s t test between the two treatments is denoted by an asterisk next to the gene name with p<0.05.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.