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Developmental Exposure to 2,3,7,8 Tetrachlorodibenzo-*p*-dioxin Attenuates Capacity of Hematopoietic Stem Cells to Undergo Lymphocyte Differentiation

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Abstract

The process of hematopoiesis, characterized by long-term self-renewal and multi-potent lineage differentiation, has been shown to be regulated in part by the ligand-activated transcription factor known as the aryl hydrocarbon receptor (AHR). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a ubiquitous contaminant and the most potent AHR agonist, also modulates regulation of adult hematopoietic stem and progenitor cell (HSC/HPC) homeostasis. However, the effect of developmental TCDD exposure on early life hematopoiesis has not been fully explored. Given the inhibitory effects of TCDD on hematopoiesis and lymphocyte development, we hypothesized that *in utero* exposure to TCDD would alter the functional capacity of fetal HSC/HPCs to complete lymphocyte differentiation. To test this hypothesis, we employed a co-culture system designed to facilitate the maturation of progenitor cells to either B or T lymphocytes. Furthermore, we utilized an innovative limiting dilution assay to precisely quantify differences in lymphocyte differentiation between HSC/HPCs obtained from fetuses of dams exposed to 3μg/kg TCDD or control. We found that the AHR is transcribed in yolk sac hematopoietic cells and is transcriptionally active as early as gestational day (GD) 7.5. Furthermore, the number of HSC/HPCs present in the fetal liver on GD 14.5 was significantly increased in fetuses whose mothers were exposed to TCDD throughout pregnancy. Despite this increase in HSC/HPC cell number, B and T lymphocyte differentiation is decreased by approximately 2.5 fold. These findings demonstrate that inappropriate developmental AHR activation in HSC/HPCs adversely impacts lymphocyte differentiation and may have consequences for lymphocyte development in the bone marrow and thymus later in life.

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Keywords

2,3,7,8-tetrachlorodibenzo-*p*-dioxin; Lymphocyte differentiation; Developmental Immunotoxicology; Limiting Dilution Analysis; Hematopoiesis; Aryl hydrocarbon receptor

INTRODUCTION

Hematological malignancies are the fifth most common cancer in the world and include lymphomas, acute lymphoblastic leukemias, and myeloproliferative neoplasms (Rodriguez-Abreu et al., 2007). These malignancies are the most common childhood cancer and the incidence rate for both children and adults has increased during the last 40 years (Howlader et al., 2013). One risk factor identified for leukemia comes from epidemiological analysis of Vietnam War military veterans exposed to the defoliant Agent Orange that was contaminated with 2,3,7,8 Tetrachlorodibenzo-*p*-dioxin (TCDD) (“Veterans and Agent Orange: Update 2010,” 2011). In present day China, children living near electronic waste recycling facilities experience daily exposure to a spectrum of dioxin congeners (Guangen et al., 2011; Leung et al., 2011; Shen et al., 2010). Moreover, humans in industrialized countries can be exposed to dioxins through daily intake of meat, dairy and seafood (Kvalem et al., 2012; Liem et al., 2010). Thus, dioxin exposure continues to represent a significant global public health issue with potentially greater adverse effects for populations exposed during development because the effects can be longer lasting and may occur at lower developmental doses (Dietert et al., 2010; Hogaboam et al., 2007).

TCDD is the most potent agonist of the Aryl hydrocarbon receptor (AHR) and is known to impact a spectrum of adverse health conditions including malignancy, neurological disorders, and immune suppression (“Veterans and Agent Orange: Update 2010,” 2011). We and others have shown that exposure to TCDD leads to thymic atrophy and impairs T lymphocyte development (Laiosa et al., 2002; 2003). Recently, the AHR has been implicated in mechanisms required for maintenance and differentiation of adult hematopoietic stem cells (Boitano et al., 2010; Casado et al., 2010; Gasiewicz et al., 2010; Lindsey and Papoutsakis, 2012; Smith et al., 2013). However, AHR-dependent effects on fetal hematopoiesis has received little recent attention despite the possibility that exposure to AHR agonists early in life could lead to long-term defects of hematopoietic stem cell self-renewal or differentiation.

Hematopoiesis is the process by which immature hematopoietic stem and progenitor cells (HSC/HPCs) develop into mature blood and immune cells, and during development this process is initiated in the yolk sac (Orkin and Zon, 2008; Samokhvalov et al., 2007). In fetal mice, the anatomical location and timing of hematopoiesis proceeds such that the site of hematopoiesis migrates from the yolk sac to the aorta-gonads-mesonephros (gestational day (GD) 8-9), followed by the fetal liver and spleen (GD11-GD16). Ultimately HSC/HPCs localize to the bone marrow, which remains the site of hematopoiesis throughout adulthood (Orkin and Zon, 2008; Robin et al., 2003). In response to micro-environmental signals in the stem cell niche, a proportion of the long-term HSCs begin differentiation along multiple blood cell lineages, and become committed to more specific effector cell fates (Luc et al.,

2008). For example, progenitors referred to as common lymphoid precursors that receive a signal through the trans-membrane protein Notch1, further differentiate into T lymphocytes whereas B lymphocytes are generated from common lymphoid precursors that fail to engage Notch1 (Deftos et al., 2000; Zúñiga-Pflücker and Schmitt, 2005).

There is a growing recognition that factors that disrupt the processes of HSC self-renewal or multipotent lineage differentiation into immune effector cells can have profound effects on long-term immunity (Seita and Weissman, 2010). For example, mice developmentally exposed to TCDD display persistent immune defects indicative of later-life autoimmune disorder (Mustafa et al., 2008) and an inability to mount an effective immune response to viral insult (Head and Lawrence, 2009; Vorderstrasse et al., 2004). Moreover, mice exposed during the perinatal period to TCDD exhibit longer-lasting effects than adult-exposed mice (Hogaboam et al., 2007).

While there is considerable evidence connecting adult TCDD exposure to immune suppression, the target hematopoietic cell population and the mechanism by which immune suppression after developmental exposure is occurring have not yet been fully elucidated. Therefore, we set out to test the hypothesis that developmental aryl hydrocarbon receptor activation by TCDD modulates the capacity of hematopoietic stem cells to undergo lymphocyte differentiation. In order to test this hypothesis, we co-cultured fetal liver HSC/HPCs in an *in vitro* co-culture system designed to drive differentiation along either the B or T lymphocyte lineage. Our method of determining B or T lymphocyte differentiation capacity is particularly innovative because we have created an assay that is a hybrid of stem cell limiting dilution analysis and traditional toxicological assay. Importantly, we can accomplish this while approaching environmentally-relevant levels of TCDD exposure *in utero*. Using this quantitative *in vitro* co-culture system, we found a significant decrease in the lymphocyte differentiation potential of HSC/HPCs developmentally exposed to TCDD. This decrease in the ability of fetal HSC/HPC to complete normal hematopoietic differentiation may increase risk for later-life hematological diseases such as malignancies or stem cell exhaustion (Lento et al., 2013; Stein and Baldwin, 2013).

Materials and Methods

Antibodies used for HSC/HPC and 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). Immature thymocytes and B cells were identified based on expression of PECy7-conjugated CD8 α (clone 53-6.7); APC-H7-conjugated CD4 (clone L3T4); Biotin-conjugated CD25 (clone 7D4) coupled with Streptavidin PE-Texas Red; PECy5-conjugated CD44 (clone IM7); PE-conjugated B220 (clone RA3-6B2); and FITC-conjugated CD19 (clone ID3). All antibodies were used at titrated concentrations and were purchased from BD Biosciences (San Jose, CA) unless otherwise noted.

Experimental animals

All animal procedures were conducted 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 Committee (IACUC) at the University of Wisconsin-Milwaukee. C57BL/6J mice used were offspring from original pups obtained from the Jackson Laboratory (Bar Harbor, ME). After overnight pairings, the presence of a vaginal plug was designated gestational day (GD) 0.5. All mice were housed in micro-isolator cages in a specified pathogen-free facility at the University of Wisconsin-Milwaukee, and were provided food and water *ad libitum* and maintained on a 12:12-h light cycle.

TCDD preparation and treatment protocol

TCDD (Cambridge Isotopes, Andover, MA) was diluted in 1,4-dioxane (Sigma-Aldrich, St. Louis, MO) to a working stock concentration of 0.2 mg/mL. Appropriate volume of TCDD in 1,4-dioxane was subsequently transferred to a sterile 15 ml conical tube and the liquid was evaporated in a chemical fume hood. The TCDD residue was then suspended in olive oil (Filippo Berio, Hackensack, NJ) to a concentration of 0.3µg/ml and was mixed by rotation at room temperature. Control olive oil was added to a tube with an equal volume of evaporated 1,4-dioxane. Mice were exposed to either 3µg TCDD/Kg body weight by oral gavage on gestational days 0.5 and 7.5, or olive oil vehicle control (0.1ml per 10g). Doses were given 7 days apart to insure a relatively constant level of TCDD throughout because the half life of TCDD in a C57BL/6 mouse is approximately one week (Miniero et al., 2001; Weber and Birnbaum, 1985).

Fetal Liver HSC isolation and cell sorting

Pregnant C57BL/6J mice were euthanized by CO₂ asphyxiation according to the AVMA Guidelines on Euthanasia (Leary et al., 2013) on gestational day 14.5. Dissections and tissue harvest were carried out within a 2 hour window (7:00 am-9:00am CST) each day to minimize time-of-day fluctuations in HSC numbers (Singh et al., 2011). Fetuses were removed to culture dishes containing cold Dulbecco's Modified Eagle Medium (DMEM; Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Grand Island, NY), 1% L-glutamine (Invitrogen, Grand Island, NY), 1% 1 M HEPES (Invitrogen, Grand Island, NY), 0.01% 0.5 M 2-Mercaptoethanol (EMD, Gibbstown, NJ), and 0.001 mg/mL gentamycin (Life Technologies, Carlsbad, CA). For all HSC experiments, fetal livers were removed from individual fetuses, crushed in DMEM, and passed through sterile Nitex nylon mesh (80µm; Wildco, Yulee, FL) to form single cell suspensions. Mature fetal liver cells were eliminated by CD24-mediated (clone J11d.2) complement dependent lysis with low tox Rabbit Complement (Cedarlane, Burlington, NC) at 37°C for 30 minutes. The pellet was then re-suspended in DMEM without serum and depleted of dead cells by centrifugation over Lympholyte Mammal (Cedarlane, Burlington, NC) at 800g for 20 minutes. Remaining cells were washed in DMEM and labeled with fluorochrome-conjugated antibodies directed against hematopoietic progenitor cell markers Sca-1 and cKit, and a cocktail of lineage antibodies (CD3, TER119, CD45R, CD11b, and LY6G/LY6C/GR-1).

Cells were sorted on 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 on a 96 well tissue culture plate. Prior to cell sorting, cells were stained with 10µl of the vital dye Sytox Blue (Life Technologies, Carlsbad, CA), 1mM, diluted 1:80, to label nuclei from dead or dying cells with permeable membranes. Sytox Blue negative cells were electronically gated on the 405 violet B detector. Subsequently, doublets were excluded using FSC Height X Width and SSC Height × Width discrimination. Lineage-Sca-1+cKit+ (LSK) cells were sorted into a single tube and then divided into aliquots of 4000 cells per well in a 24 well plate or directly sorted into individual wells on a Costar tissue culture treated 96 well plate (Corning, Corning, NY) at concentrations ranging from 1-30 cells per well.

In vitro LSK short-term self-renewal assay

LSK cells were sorted as described above and cells were placed in StemSpan SFEM serum free media (Stem Cell Technology, Vancouver, BC, Canada) supplemented with 100 ng/ml SCF (PeproTech). Five hundred cells per well in multiple wells on a 96 well plate were seeded on day 0 with feeding of fresh media and SCF occurring every two days. On days 3, 6, 9, 12, and 15, cells were harvested in quadruplicate, stained for lineage and hematopoietic stem cell surface markers as described above and cells were acquired on the FACS Aria III. Cell counts were obtained flow cytometrically using counting beads (Life Technologies) according to the manufacturer's instructions.

OP9 co-culture system

OP9 bone marrow-derived stromal cells transduced with either GFP (OP9-GFP) or Notch ligand delta-like 1-GFP-IRES (OP9-DL1), were a generous gift from Dr. Juan Carlos Zúñiga-Pflücker (Department of Immunology, University of Toronto, Sunnybrook, Ontario, Canada). Approximately one week prior to experiments, OP9-DL1 or OP9-GFP cells were thawed and grown in OP9 complete media consisting of Minimum Essential Medium alpha (α-MEM; Life Technologies, Carlsbad, CA) containing 20% FBS (lot tested for supporting OP9 co-cultures; Life Technologies, Carlsbad, CA) and 0.05mg/mL gentamycin (Life Technologies Carlsbad, CA). Two days prior, OP9-DL1 or OP9-GFP cells were plated into either 24-well or 96-well tissue culture treated plates (Corning, Corning, NY) and allowed to grow to approximately 80% confluency, in a humidified 37°C incubator maintained with 5% CO₂. Prior to introduction of LSK cells, OP9 cells were treated for 30 minutes in the incubator with 5µg/mL Mitomycin C (Acros Organics, Thermo-Fisher Scientific, Waltham, MA) followed by five washes with sterile Phosphate Buffered Solution containing 2.5% FBS. After the final wash, complete OP9 medium was added to the wells, supplemented with 5ng/mL Flt3L and 5ng/ml Interleukin-7 (PeproTech, Rocky Hill, NJ). Sorted LSK cells were maintained in co-culture system for up to 15 days with media replacement (complete OP9 medium, supplemented with 5ng/mL Flt3L and 5ng/ml Interleukin-7) occurring every three days of co-culture.

Flow cytometry analysis of lymphocytes derived from LSK co-cultures

At varying times after initiation of co-culture, lymphocytes were harvested, stained and analyzed for surface expression of CD4, CD8, B220, CD19, CD44 and/or CD25. For cell cycle experiments, co-cultures were incubated in the presence of 10 mM 5-bromo-2'-deoxyuridine (BrdU) for one hour prior to harvest. Cells were then stained with appropriate surface markers followed by fixation and permeabilization using the BD FITC BrdU flow cytometry kit according to manufacturers instructions (BD). All flow cytometry data were analyzed using FlowJo software (Version 9.5, Treestar, Ashland, OR). Thymocyte precursors were identified by presence of both CD4 and CD8 α surface markers. B cell precursors were identified by presence of both B220 and CD19 surface markers.

RNA preparation

Hematopoietic cells from vehicle or TCDD-treated fetuses were isolated and sorted in Trizol (Life Technologies) from GD 7.5 yolk sac, GD 11.5 AGM, and GD 14.5 fetal liver. 1-bromo-3-chloropropane (Sigma) was used to separate RNA into the aqueous phase after centrifugation. Resulting pellet was resuspended in molecular-biology grade water. Yield and purity were quantified spectrophotometrically on a NanoquantTM plate using OD260/OD280 ratio (Tecan) and was either made into cDNA or stored at -80. 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 and Quantitative RT-PCR

Primers were selected using the Universal ProbeLibrary version 2.5 for Mouse (Roche) and checked for specificity using Primer-Blast (NCBI). cDNA was used as a template in 20 μ L reaction consisting of 10 μ M of forward and reverse primers and 10 μ L SensiFAST SYBR No-ROX (Bioline). Gene names, accession numbers, and sequences are provided in table 1. Relative expression change was determined using the $[2^{\Delta\Delta C_t}]$ with standardization to housekeeping genes. Samples were run in triplicate for each result. 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

For the precursor frequency calculations, we first determined an acceptable threshold value for a positive response. The percentage of CD4+CD8+ cells for each well (Supp. Fig. 1A), was used to discriminate responsive from non-responsive wells. Using the extreme limiting dilution analysis (ELDA) program (Hu and Smyth, 2009), T cell precursor frequencies were calculated for CD4+CD8+ percentages at every point from 0.1% to 5.0% of the total cell population. The ELDA program returns a Chi Square value for each vehicle/TCDD frequency comparison. Chi Square values were plotted at each percentage point (0.1-5.0%) against the matching cell population percentage (Supp. Fig. 1B). The peak of this graph, where the Chi Square value indicated the greatest difference between control and exposed wells, was determined to be a range including the three highest peaks (2.0-2.8). The highest peak value was chosen (2.8%) and used to determine progenitor frequency for both B and T

lymphocyte precursors. Data for the limiting dilution was analyzed and graphed according to the “limdil” function contained in the “statmod” package in Rstudio (RStudio, 2012). Our calculations are based on a compilation of all data from three replications of the OP9-DL1 co-culture system and three of the OP9-GFP co-culture system. Graphpad Prism (Graphpad Software, LaJolla, CA) was utilized for analysis of variance with Dunnett's t post-hoc tests and graphical presentation of all other data. Statistical significance was determined and is denoted on graphs and tables with an asterisk with a p 0.05 or three asterisks for p 0.01.

RESULTS

Developmental exposure to TCDD increases the number of hematopoietic progenitor cells in the fetal liver

Hematopoietic progenitor cells cultured in the presence of both agonist and antagonistic AHR modulators lead to progenitor cell expansion *in vitro* and these new selective AHR modulators hold promise for improved clinical stem cell therapies and approaches (Boitano et al., 2010; Smith et al., 2013). However, *in vivo* comparisons with the *in vitro* data are complicated by differences in dose of the modulators, metabolism of the modulator, and presence of additional intrinsic and extrinsic factors present in the hematopoietic stem cell niche that could modulate progenitor cell differentiation and self-renewal. Thus, as a first step to determine the effect of persistent developmental AHR activation on hematopoiesis, we tested the effects of transplacental exposure to the non-metabolizable TCDD on fetal liver HSC homeostasis. Specifically, to determine if developmental TCDD exposure affects fetal HSCs, we used flow cytometry to examine GD14.5 fetal liver cells from mice developmentally exposed to either 3µg/kg TCDD or vehicle control administered to the pregnant dam by gavage on GD 0.5 and 7.5. The percentage of HSC/HPC cells defined by absence of lineage markers and expression of Sca-1 and cKit (referred to as LSK) is more than two-fold higher in the fetal livers of mice developmentally exposed to TCDD (Fig. 1). Furthermore, the absolute number of LSK cells per fetus and the mean fluorescence intensity of Sca-1 containing cells is also significantly increased in fetal livers of mice exposed to TCDD transplacentally (Fig. 1C & 1D; p 0.01).

The AHR is expressed and is transcriptionally active in fetal hematopoietic cells

Alterations in the proportion of fetal HSC/HPC cells following TCDD exposure is predicted to be due to AHR expression and transcriptional activation in hematopoietic cells. Thus, gene expression of the AHR and its heterodimerization partner ARNT were analyzed in hematopoietic cells obtained from yolk sac on GD 7.5, the aorta-gonads-mesonephros (AGM) on GD 11.5 or GD 14.5 fetal liver during early fetal development. The AHR was expressed at all three time points and its gene expression was significantly increased in TCDD-exposed fetuses relative to control fetuses on GD 11.5 (Fig. 2A; p<0.01). In comparison, ARNT was relatively unchanged except in the maternal liver where its expression was down modulated following 14.5 days of exposure (Fig. 2B). Maternal liver was used as a control to demonstrate the dose and method of TCDD-exposure (gavage) was sufficient to activate the adult AHR throughout the 14.5 day exposure window. Furthermore, induction of Cyp1A1 by TCDD exposure in GD 11.5 AGM HSC/HPC cells suggests the AHR-ARNT heterodimer is transcriptionally active in the fetus (Fig. 2C). Taken together,

these data demonstrate the potential of the ligand-activated AHR to impact hematopoiesis in the fetus through transcriptional changes in gene expression.

LSK cells obtained from GD 14.5 fetal livers exposed to TCDD have normal growth potential *in vitro*

In order to determine if the increase in the LSK cell population in TCDD-exposed mice was attributed to differences in their proliferative potential, LSK cells were sorted and cultured *in vitro* using serum free media optimized for expansion of short-term self-renewing HSCs and HPCs. To obtain a pure population of LSK cells, fetal livers from GD 14.5 mice were dissected and mature CD24+ cells were depleted by J11d mediated complement dependent lysis as previously described (Schmitt and Zúñiga-Pflücker, 2002). Subsequently, cells were prepared for fluorescence activated cell sorting and isolated with greater than 95% purity (Fig. 3A & 3B).

Once placed in culture, LSK cells were supplemented with the stem cell growth factor (SCF), where there is an approximately 1000 fold expansion of LSK cells over the course of a two-week culture period (data not shown). During the initial phase of the culture period, there appeared to be a slight increase in the proportion of LSK cells in the culture but this was not statistically significant (Fig. 3C-3D). Moreover, by the end of the culture, there were no differences between the frequency or number of LSK cells obtained from vehicle or TCDD exposed fetuses (Fig. 3C-3D). These data indicate that the process of short-term self-renewal that is facilitated by this *in vitro* culture system is not a primary target of *in utero* TCDD exposure.

TCDD induces cell cycle arrest in *in vitro* generated thymocytes

We have previously demonstrated that thymocytes obtained from adult mice exposed to TCDD undergo cell cycle arrest within hours of exposure (Laios et al., 2003), and that this effect is due to activation of the AHR in the hematopoietic cell population directly (Staples et al., 1998). Given the importance of AHR signaling in HSCs (Boitano et al., 2010; Gasiewicz et al., 2010), and thymic atrophy, a critical question that remains is what effect TCDD exposure has on HSC differentiation into cells of the lymphoid lineage. Up until now, answering this question has proven difficult because of the different anatomical locations where hematopoiesis and thymopoiesis occur. However, by using the OP9-DL1 *in vitro* system of thymocyte differentiation (Schmitt and Zúñiga-Pflücker, 2002), we can track the transition of hematopoietic stem and progenitor cells into thymocytes and determine the effect of AHR activation on this process. The first step in this process was to determine if OP9-DL1 *in vitro* generated thymocytes have TCDD sensitivity similar to that of thymocytes obtained *in vivo*. This was accomplished by sorting unexposed LSK cells harvested from murine fetal livers into OP9-DL1 co-cultures and maintaining cells for 12 days. On day 11, co-cultures were exposed to various concentrations of TCDD or DMSO vehicle control. Twelve hours after exposure, BrdU incorporation within each stage of thymocyte development was analyzed. We observed an equivalent percentage and number of cells expressing CD44 and CD25 twelve hours after *in vitro* TCDD exposure indicating the distribution of early thymocyte progenitor populations within the co-cultures are unaffected by this short exposure (Fig. 4A). However, BrdU incorporation is significantly

attenuated in the CD44-CD25⁺ (DN3) population of thymocyte progenitors (Fig. 4B; $p < 0.05$). Moreover, significant reduction in BrdU incorporation can be detected in cultures exposed to as low as 0.001nM TCDD (Fig. 4C; $p < 0.05$). Importantly, the nearly 50% reduction in BrdU within the CD44-CD25⁺ and CD44-CD25⁻ populations is highly correlated with the TCDD-induced cell cycle inhibition observed in these specific thymocyte progenitor populations *in vivo* (Laiosa et al., 2003).

Kinetics of DL1-mediated thymocyte differentiation are equivalent in control and developmental TCDD-exposed HSCs

The demonstration that *in vitro* TCDD exposure in the OP9-DL1 – LSK co-culture system models the *in vivo* response to TCDD suggested that this system could be employed to follow the lymphocyte differentiation potential of LSK cells exposed to TCDD *in utero*. Thus, pregnant dams were exposed to 3µg/Kg TCDD on GD 0.5 and 7.5 followed by cell sorting of LSK hematopoietic stem and progenitor cells on GD 14.5. LSKs were then placed into OP9-DL1 or OP9-GFP control co-cultures and thymocyte or B lymphocyte differentiation was tracked every two days in cultures seeded with either vehicle or TCDD-exposed LSK cells. Following developmental exposure to TCDD, no statistically significant difference was found in the rate of LSK maturation into thymocytes in OP9-DL1 co-culture when 4000 cells were plated per well (Fig. 5). Similar results were found with B cell progenitors grown in co-culture with OP9-GFP cells (supplemental Fig. 2). Notably, a slight but not statistically significant difference in the frequency of CD4⁺CD8⁺ thymocytes produced by OP9-DL1 co-cultures was detectable between the vehicle- and TCDD-exposed wells at the end of the culture period. This slight difference led us to conclude that any subtle variation may have been obscured by the high number (4000) of starting LSK cells per well.

Developmental TCDD exposure decreases lymphocyte differentiation potential of LSK cells

In order to precisely quantify the lymphocyte differentiation potential on a per cell basis following developmental vehicle- or TCDD-exposure, a limiting dilution approach was employed to the OP9 – LSK co-culture system. Following prenatal TCDD exposure, exactly 1, 3, 6, 10, or 30 LSKs from GD 14.5 fetuses were deposited by FACS into individual wells in 96 well plates containing OP9-DL1 or OP9-GFP stromal cells. On day 12 for the OP9-DL1 co-cultures, cells from individual wells were harvested and stained with the thymocyte surface markers CD4 and CD8 and the percentage of wells containing CD4⁺CD8⁺ cells was recorded. We found 1 in 16.9 (95% CI: 14.2-20.2) LSK cells have T cell potential compared with nearly 1 in 39.6 LSK (95% CI: 31.5-49.9) cells possessing T cell potential from the developmentally TCDD-exposed fetuses (Fig. 6A; Table 2; $p < 0.01$). Developmental TCDD exposure was observed to also reduce by nearly two-fold the B lymphocyte precursor frequency for LSK cells as determined by day 14 OP9-GFP limiting dilution co-culture analysis (Fig. 6B; Table 3). Taken together, these data demonstrate that prenatal TCDD exposure significantly decreases the number of fetal liver LSK cells with potential to produce T and B lymphocyte progeny.

DISCUSSION

In the present study, we demonstrate that prenatal exposure to TCDD causes alterations in fetal HSC/HPC homeostasis and lymphocyte differentiation. As shown in a novel co-culture limiting dilution assay, the ability of HSC/HPCs to differentiate into T lymphocytes is decreased nearly two and a half fold following developmental exposure to environmentally-relevant levels of the AHR agonist TCDD. This decrease in T lymphocyte differentiation ability occurs despite an increase in the overall number of fetal liver HSCs on GD14.5. The consequences of *in utero* activation of the AHR on HSC/HPC cell differentiation may provide insight into a spectrum of later life blood disorders ranging from stem cell exhaustion to leukemia (Seita and Weissman, 2010).

The observed increase in fetal LSK cells is consistent with studies showing similar alterations in adult bone marrow, however, the *in vitro* co-culture functional assay offers advantages compared with more traditional immunotoxicological hematopoiesis assays. Previous research has used stem cell transfer to determine the eventual differentiation ability of TCDD-exposed cells (Casado et al., 2011; Fine et al., 1989). However, these methods use an abundance of stem cells isolated from fetal livers and/or bone marrow. For example, when seeding *in vitro* OP9 cultures with 4000 LSK cells a sufficient number of LSK cells possessing T or B lymphocyte potential were present impacting the sensitivity necessary for determining if *in utero* exposure to TCDD impairs differentiation. It is possible that earlier LSK transfer experiments may have the same effect. To overcome the obstacle an excess of cells in an assay may present, limiting dilution is used to determine frequencies of cells in a mixed population that have a particular function and therefore is commonly used for progenitor cell applications. The benefit of the *in vitro* limiting dilution co-culture differentiation system that we use is the ability to track the progression of a single HSC to its eventual progeny. Furthermore, numerous single cells from one sample of sorted HSCs can be tested to determine a numerical differentiation potential. This method allows focus on not only individual effects, but also on the earliest steps in differentiation. While the frequency of lymphocyte differentiation using the OP9 co-culture system has been determined using limiting dilution analysis from hematopoietic precursors in mice (Balcunaite et al., 2005) and in humans (De Smedt et al., 2011; Kyoizumi et al., 2013), this is the first time it has been employed to test the effect of a developmental toxicological exposure on a progenitor cell population's ability to differentiate.

A limitation of the limiting dilution analysis is that cells identified by an absence of lineage markers, and the presence of Sca-1 and cKit (LSK cells) comprise a heterogeneous population of progenitor cells with a range of long- and short-term renewal capacity. Thus one interpretation of this research is that developmental exposure to TCDD causes a shift in the composition of long- to more short-term self-renewing progenitor cells identifiable by the LSK surface proteins. For example, LSK cells with long-term self-renewal potential have full multi-lineage differentiation potential. In comparison, cells transitioning from long-term to short-term self-renewal still maintain multi-lineage potential. However, their capacity to differentiate into any lineage becomes more restricted as they mature and can be biased away from production of the common lymphoid precursors that directly produce lymphoid restricted cells. In support of this interpretation, we performed a test of

heterogeneity for each limiting dilution experiment known as a log-dose slope test. We found that all treatment groups produced a log-dose slope of less than 1 (data not shown). A slope less than 1 demonstrates that the fetal liver LSK populations tested are a heterogeneous cell population (Hu and Smyth, 2009), with the TCDD-exposed fetuses producing a LSK cell population with significantly more heterogeneity than controls.

In support of the conclusion that developmental TCDD exposure changes the composition of the self-renewal potential of fetal LSKs, we observe higher Sca-1 expression on GD 14.5 (Fig. 1). Others have correlated high levels of Sca-1 expression with decreased differentiation in the erythroid lineage (Azalea-Romero et al., 2012) and decreased ability to form myeloid colonies (Bradfute et al., 2005) as well as patterns of decreased T cell lymphopoiesis in constitutively-expressing Sca-1 transgenic mice (Bamezai et al., 1995). Furthermore, in a mouse model for the autoimmune disease lupus, up-regulation of Sca-1 correlated with a more severe disease state (Kumar et al., 2005). Taken together, this supports Sca-1 as a potential target identifying loss of multi-lineage differentiation due to developmental exposure to TCDD. The higher mean fluorescence, and thus protein expression of Sca-1 could predict an increased risk of hematopoiesis defects over the life-course.

In summary, we have for the first time demonstrated that prenatal exposure to the environmental toxicant and AHR agonist TCDD significantly increases the number of HSCs, but on a per-cell-basis decreases the ability of fetal liver LSK cells to differentiate into B and T lymphocytes. As with *in vivo* experiments, the cell cycle effects of TCDD exposure were shown to be the most dramatic in the DN3/DN4 stage of thymocyte differentiation *in vitro*. We used a lymphocyte differentiation assay combined with extreme limiting dilution analysis (Hu and Smyth, 2009) to follow prenatally-exposed LSK cells from the progenitor stage through to lymphocyte commitment, arriving at a numerical differentiation potential. The *in vitro* co-culture assay allowed us to focus on TCDD's effect on only the LSK cells, without any potential cross-signaling from TCDD-exposed supporting tissues. Additionally, the single cell sorting procedure enabled us to examine one LSK cell at a time to determine its ability to differentiate into a lymphocyte. Utilization of an *in vitro* co-culture system to test the effects of developmental toxicological exposures on hematopoietic stem cells is not only novel, but preferable to some types of *in vivo* studies due to the fast concise measures of immunological impact. Our approach to determining lymphocyte differentiation potential could be modified to test the developmental immunotoxicological effects of other compounds. Finally, this approach could facilitate analysis of the sensitivity of other tissue-specific stem and progenitor cells to developmental exposures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Developmental TCDD exposure increases fetal liver hematopoietic progenitor cell frequency *in vivo*
- AHR is transcriptionally active in fetal hematopoietic cells
- TCDD reduces cell proliferation in *in vitro* generated thymocytes
- Developmental TCDD exposure decreases lymphocyte differentiation potential of hematopoietic stem and progenitor cells.

Figure 1.

Hematopoietic progenitor cell frequency and number increase following developmental TCDD exposure *in vivo*. Pregnant C57Bl/6 dams were exposed to 3µg/Kg TCDD or vehicle control throughout gestation and fetal liver cells were isolated on GD 14.5. A) representative fetal liver c-Kit versus Sca-1 flow cytometry plots are shown from vehicle- and TCDD-exposed dams. The numbers in each quadrant represent the percentage of lineage negative cells expressing cKit, Sca-1 or both. B) The percentage of fetal liver LSK cells from vehicle- and TCDD-exposed dams are shown. C) The percentage of LSK cells was multiplied by total fetal liver cell number to determine absolute number of LSK cells. D) The mean fluorescence intensity of Sca-1+ cells. Open histograms represent vehicle and shaded histograms represent TCDD. Data are presented as the mean + SEM (vehicle n=9; TCDD n=10). Statistical differences were determined by ANOVA and Dunnet's t post-hoc test. An *** is used to denote statistical significance with p 0.001.

Figure 2.

AHR and ARNT are expressed in fetal HSC/HPC cells and are transcriptionally active. Pregnant C57Bl/6 dams were exposed to 3 μ g/Kg TCDD or vehicle control throughout gestation and HSC/HPC cells were sorted and RNA obtained from the maternal liver or fetal yolk sac on GD 7.5, aorta-gonadal-mesonephros on GD 11.5 and fetal liver on GD 14.5. RNA was reverse-transcribed and cDNA PCR amplified by quantitative real-time PCR. A) The TCDD-induced fold change of AHR gene expression is shown for the three gestational time points and in maternal liver. The housekeeping genes used for each developmental time point are indicated in Table 1. B) TCDD-induced fold change for ARNT. C and D) The TCDD-induced fold change for AHRARNT target genes Cyp1A1 and Cyp1B1. The black dot represents the average fold change from two independent experiments with the error bars indicating the fold-change from each individual experiment. RNA was pooled from HSC/HPCs sorted from an entire litter.

Figure 3.

LSK cells obtained from GD 14.5 fetal livers exposed to TCDD have normal growth potential *in vitro*: LSK cells were sorted on the FACS Aria III following J11d mediated complement-dependent lysis. A) The gating strategy for sorting is shown and results from a pre- and post-sort representative sample are displayed. Briefly, viable cells based on Sytox blue dead cell exclusion were first gated, followed by doublet discrimination using Forward and Side scatter (FSC, SSC) height by width gating. Fluorescence gating strategy is shown with top panels showing lineage staining versus SSC-area. B) c-Kit+Sca-1+ events were

sorted using the FACS Diva 6.0 software purity mask. This sorting strategy was employed and purity was determined (>95%; data not shown) for all subsequent experiments. After cell sorting, five hundred LSK cells were grown in StemSpan SFEM media + 100 ng/ml SCF and cultured for 15 days. C) Representative histograms of lineage stained cells (CD11b, Ter-119, Gr-1, B220, CD3) are shown for days 3, 6 and 9. LSK cells from Vehicle-exposed dams are shown with a black line, and LSK cells from TCDD-exposed dams are illustrated in grey. D) cKit versus Sca-1 expression in LSK growth cultures is shown for Vehicle (left hand contour plots) or TCDD (right hand countour plots). The numbers in each quadrant represent the percentage of viable lineage negative cells. Experiment was repeated three times.

Figure 4.

BrdU incorporation is decreased in TCDD-exposed thymocyte progenitors derived from LSK cells grown in OP9-DL1 co-cultures. A) Representative CD25 versus CD44 flow cytometry plots for day 12 OP9-DL1 co-cultures exposed *in vitro* to vehicle or 1nM TCDD for 24 hours. Numbers inside each quadrant represent the percentage of CD4-CD8- (DN) cells. B) Intracellular BrdU incorporation was measured in each thymocyte progenitor population and representative BrdU histograms are shown for the CD44-CD25⁺ population from one vehicle and one 1nM TCDD exposed culture. C) The percent of BrdU⁺ cells for

each population of thymocyte progenitors 12 hours after *in vitro* exposure to TCDD or vehicle. Cultures were dosed on day 11, and surface staining and intracellular BrdU staining was conducted on day 12. Experiment was repeated two times. Data are presented as the mean + SEM with 6 wells analyzed per group. Statistical differences are determined by ANOVA and Dunnet's t post-hoc test. An * is used to denote statistical significance with $p < 0.05$.

Figure 5.

Fetal liver LSK cells co-cultured with OP9-DL1 bone marrow stromal cells undergo thymocyte differentiation with similar kinetics following developmental vehicle or TCDD exposure. Fetal liver LSK cells were isolated on GD14.5 as described and placed into OP9-DL1 co-cultures. Beginning on day 6 of co-culture, and continuing every other day, cells were harvested, counted, and stained with fluorochrome-labeled antibodies directed against CD4, CD8, CD44, and CD25. A) Representative CD4-CD8- gated cells were analyzed for the percentage of CD44 versus CD25 from vehicle- and TCDD-exposed dams. The numbers in each quadrant represent the percentage of viable cells within the CD4-CD8- gate. B) CD8 versus CD4 expression on each day of the differentiation assay is shown. The numbers in each quadrant represent the percentage of viable cells. Experiment was repeated five times.

Figure 6.

Developmental TCDD exposure decreases lymphocyte differentiation potential of murine fetal liver HSCs. Fetal liver LSK cells were isolated on GD14.5 as described. Exactly 1 to 30 LSK cells were directly sorted into individual wells of a 96 well culture plate containing a confluent layer of mitomycin C-treated OP9-DL1 or OP9-GFP cells and maintained for 12 (DL1) or 14 (GFP) days. Individual wells were analyzed for the generation of thymocytes ($CD4^+CD8^+$) or B cells ($CD19^+B220^+$). The number of positive responses for each cell concentration was entered into the *limdil* package in R Studio (RStudio, 2012) and plotted using ELDA software. A) Thymocyte wells from OP9-DL1 co-cultures were scored as a positive response if 2.8% of total cells in the well were $CD4^+CD8^+$. B) B lymphocyte wells from OP9-GFP co-cultures were scored as a positive response if 2.8% of total cells in

the well were CD19+B220+. Precursor frequency is reported in Tables 2 and 3. Vehicle samples are represented by red circles (3 replicates) and TCDD-exposed samples are represented by blue filled circles (3 replicates). 95% CI is shaded around each treatment (vehicle-pink; TCDD-light blue; p 0.001).

Table 1Gene name, accession number and primer sequence used for qRT²PCR

Name	Accession	5'→3' Forward	5'→3' Reverse
Cyp1A1	NM_009992.4	ccaagagggaaggcatagg	gagcagctctgttgctgac
Cyp1B1	NM_009994.1	agccaggacaccctttcc	gcctgaacatccgggtatc
AHR	NM_013464.4	cgggttcctgctcagta	ccagaagttcaagctccaaa
ARNT	NM_001037737.2	ccagatttcacgtcactcca	ttgggaagaacgagtcttgg
TBP ^{1,2}	NM_013684.3	ggcggtttgctaggttt	gggttatcttcacacacatga
SDHA ^{1,3}	NM_023281.1	gggaatgggttggaacacag	tgcgacagcatcagatt
HPRT ^{1,4}	NM_013556.2	gtcaacggggacataaaag	caacaatcaagacattcttcca

¹ At least two housekeeping genes were used for each time point. Housekeeping genes were selected for each day of development based on an initial analysis demonstrating no effect of TCDD.

² TBP housekeeping gene used on GD 7.5

³ SDHA housekeeping gene used on GD 11.5 and 14.5

⁴ HPRT housekeeping gene used on GD 7.5 and maternal liver

Table 2Thymocyte progenitor frequencies after developmental exposure^a

	Progenitor frequency (95% CI)	p-value
Thymocyte vehicle	1 in 16.9 (14.2-20.2) ^{b,c}	
Thymocyte TCDD	1 in 39.6 (31.5-49.9) ^{b,c}	0.001

^aPregnant C57Bl/6J mice were exposed on gestational days (GD) 0.5 and 7.5 to either 3µg/Kg of TCDD or vehicle; fetal liver LSK cells were isolated on GD 14.5 as described.

^bProgenitor frequencies were calculated using the *limdil* package in R-Studio after 12 days of co-culture with OP9-DL1 cells and as described in Fig. 5 legend.

^cEach limiting dilution was repeated three times and the total number of wells for each cell concentration from all three experiments was pooled for the *limdil* analysis.

Table 3B lymphocyte progenitor frequencies after developmental exposure^a

	Progenitor frequency (95% CI)	p-value
B lymphocyte vehicle	1 in 28.0 (21.6-36.2) ^{b,c}	
B lymphocyte TCDD	1 in 62.3 (44.0-88.2) ^{b,c}	0.001

^aPregnant C57Bl/6J mice were exposed on gestational days (GD) 0.5 and 7.5 to either 3µg/Kg of TCDD or vehicle; fetal liver LSK cells were isolated on GD 14.5 as described.

^bProgenitor frequencies were calculated using the *limdil* package in R-Studio after 14 days of co-culture with OP9-GFP cells and as described in Fig. 5 legend.

^cEach limiting dilution was repeated three times and the total number of wells for each cell concentration from all three experiments was pooled for the *limdil* analysis.