Aryl hydrocarbon receptor activation in hematopoietic stem/progenitor cells alters cell function and pathway-specific gene modulation reflecting changes in cellular trafficking and migration

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AhR activation alters migration of hematopoietic stem cells

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Non-standard abbreviations:
- 7-amino-actinomycin D, 7AAD;
- Allophycocyanin, APC;
- Aryl hydrocarbon Receptor, AhR;
- Bone Marrow, BM;
- Chemokine (C-X-C motif) Ligand 12, Cxcl12;
- Competitive Repopulation Units, CRU;
- Fluorescein, FITC;
- Hematopoietic Stem Cells, HSCs;
- Ingenuity Pathway Analysis, IPA;
- Lineage depleted, Sca-1+ and c-kit+ cells, LSKs;
- Multi-experiment Viewer, MeV;
- Multi-potent progenitor, MPP;
- Per-ARNT-Sim, PAS;
- Phycoerythrin, PE;
- Real-time Reverse Transcription Polymerase Chain Reaction, RT²-PCR;
- Signaling Lymphocyte Activation Molecules, SLAM;
- Stem cell factor, Scf;
- 2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD;
- University of Rochester Medical Center, URMC.
Abstract

The aryl hydrocarbon receptor (AhR) is a transcription factor belonging to the Per-ARNT-Sim family of proteins. These proteins sense molecules and stimuli from the cellular/tissue environment, and initiate signaling cascades to elicit appropriate cellular responses. Recent literature suggests an important function of AhR in hematopoietic stem cell (HSC) biology. However, the molecular mechanisms by which AhR signaling regulates HSC functions are unknown. In previous studies, we and others reported that treatment of mice with the AhR agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) compromises the competitive reconstitution of bone marrow (BM) cells into irradiated host animals. Additional studies indicated a requirement for AhR in hematopoietic cells and not marrow microenvironment cells. In this study, we tested the hypothesis that TCDD-mediated phenotypic and functional changes of HSCs are a result of changes in gene expression that disrupt stem cell numbers and/or their migration. TCDD treatment to mice increased the numbers of phenotypically-defined HSCs in BM. These cells showed compromised migration to the BM in vivo and to the chemokine CXCL12 in vitro, as well as increased expression of the leukemia-associated receptors CD184(CXCR4) and CD44. Gene expression profiles at six and twelve h after exposure were consistent with the phenotypic and functional changes observed. The expression of Scin, Nqo1, Flnb, Mmp8, Ilf9, and Slamf7 were consistently altered. TCDD also disrupted expression of other genes involved in hematological system development and function including Fos, JunB, Egr1, Ptgs2(Cox2) and Cxcl2. These data support a molecular mechanism for an AhR ligand to disrupt the homeostatic cell signaling of HSCs that may promote altered HSC function.
Introduction

The aryl hydrocarbon receptor (AhR) is a basic helix-loop-helix protein belonging to the Per-ARNT-Sim (PAS) superfamily of proteins. Physiologically, many of the PAS proteins sense molecules and stimuli from the cellular/tissue environment, and initiate signaling cascades to elicit appropriate cellular responses. Lipophilic compounds such as halogenated and polycyclic aromatic hydrocarbons, including the potent exogenous ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Poland and Glover 1973), bind to the cytoplasmic AhR leading to a sequence of conformational changes that ultimately transform the AhR into its high-affinity DNA binding form in the nucleus to function as a transcription factor (Soshilov and Denison 2008).

Epidemiological data has correlated accidental and occupational exposure to TCDD and related dioxins with increased risk for certain hematological diseases such as non-Hodgkin's lymphoma, chronic lymphocytic leukemia and multiple myeloma in humans (Frumkin, 2003). Also, there is much data indicating that persistent activation of AhR results in immunosuppression in different animal models (Stevens, et al., 2009). AhR-dependent thymic atrophy, a hallmark of exposure to varying doses of TCDD, may be elicited by different mechanisms including (1) reduced seeding of the thymus by bone marrow (BM)-derived progenitors (Fine, et al., 1990), (2) reduced stromal-mediated proliferation of thymocytes (Kremer, et al., 1994; Frericks, et al., 2007), and (3) a skewing of the thymic output through direct effects on developing thymocytes (Laiosa, et al., 2010). Additionally, AhR activation leads to (1) alterations of B lineage cells during gestation with possible consequences on autoimmunity (Mustafa, et al., 2008), (2) decreased numbers of immature B cells (Murante and Gasiewicz, 2000), and (3) altered functional activity of early hematopoietic progenitors (Murante and Gasiewicz, 2000; Sakai, et al., 2003; Singh, et al., 2009). A single dose of TCDD that results in BM TCDD concentrations of < 1 nM (Fine, et al., 1990) increases the relative numbers of phenotypically-defined HSCs/progenitors (Staples, et al., 1998) starting 2 days post-treatment and lasting until
day 31 (Murante and Gasiewicz, 2000). Furthermore, TCDD-treated phenotypically-defined long-term HSCs were unable to sustain peripheral blood repopulation (Sakai, et al., 2003), and had compromised ability to competitively repopulate the BM of irradiated mice (Singh, et al., 2009). These effects were dependent on the presence of the AhR in hematopoietic cells, but not supporting stroma (Staples, et al. 1998; Sakai, et al. 2003). Together, these data support a hypothesis that primitive progenitors of hematopoiesis in the BM are targeted by AhR-ligands resulting in AhR-mediated phenotypic and functional changes. Given recent interest in the possible use of AhR ligands for the expansion of HSCs and their use in BM transplants (Boitano, et al., 2010), as well as treatment of autoimmune disease (Quintana, et al., 2010), it is critical to further define mechanisms related to effects on hematopoietic cell function and the signaling pathways that may mediate these alterations.

This work tests the hypothesis that TCDD- and AhR-mediated phenotypic and functional changes of murine HSCs are a consequence of disruption of stem cell numbers and/or their migration preceded by changes in gene expression. Understanding these changes in gene expression and their functional consequences in HSCs may provide a rationale to define a role of AhR in HSCs. Furthermore, this information may provide avenues to further explore the use of therapeutic AhR ligands.
Materials and Methods

Mice and Treatments

Five-week old C57BL/6J female mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and housed in the facilities of the University of Rochester Medical Center (URMC) (Singh, et al., 2009) for at least a week before treatment. CD45.1+ (B6.SJL-Ptprc<α>/BoAiTac) female mice used in repopulation experiments were purchased from Taconic Farms Inc (Germantown, NY).

TCDD was obtained from Cambridge Isotopes (Andover, MA) and 6 μg/mL aliquots were prepared as previously described (Laiosa, et al., 2010). Either 30 μg of TCDD in olive oil/kg body weight or olive oil alone in a volume of 0.1 mL/20 g body weight was given orally by gavage. Mice were euthanized after 7 days for functional and phenotypic experiments. This dose and time were chosen based on determined optimal effects of TCDD on HSCs (Singh, et al., 2009). To study gene expression, treated mice were euthanized 6 and 12 hours after treatment.

Cell Preparations

Bone marrow (BM) was harvested from femurs and tibias, red blood cells were lysed, and lineage positive leukocytes were depleted as previously described (Singh, et al., 2009). Only cell suspensions with viability > 95% as measured by exclusion of trypan blue were used for further experimentation. All flow cytometric analyses and separations were performed on viable populations only, as measured by exclusion of the fluorescent dye 7-amino-actinomycin D (7AAD). Lineage depleted Sca-1+ and c-kit+ cells (LSKs) used in microarray experiments were obtained by laser-assisted sorting of lineage depleted cells stained with fluorochrome-conjugated antibodies against Sca-1+ (D7, allophycocyanin (APC); ebioscience, San Diego, CA) and c-kit+ (2B8, fluorescein (FITC); BD Pharmingen, Santa Clara, CA) using a FACSARia cell
sorter (BD Biosciences, San Jose, CA) and a gating scheme recently published (Singh, et al., 2010). To examine differentiation of LSK cells under conditions in vitro, LSK cells were obtained from mice treated with TCDD (30 µg/kg, 7d) or vehicle. Thirty thousand cells (10,000 cells/mL) were cultured per well in 12-well plates. Each well contained StemSpan Serum-Free Expansion Media (Stem Cell Technologies) supplemented with IL-6 (50 ng/mL) or IL-6 plus stem cell factor (Scf) (100 ng/mL). Cells were harvested after 7 d or 14d and phenotypic characterization was performed by flow cytometry.

**Determination of Competitive Repopulation Units**

The engraftment and proportion of Competitive Repopulation Units (CRU), per million of donor’s BM cells injected into the irradiated recipients, were calculated for short-term and long-term reconstitutions (6 and 20 weeks post-transplantation, respectively). A limiting-dilution approach was used for quantification. BM cells were isolated from control or TCDD-treated mice. The donor’s (CD45.2+) cells were resuspended in 100 µL of PBS at three concentrations (0.1, 0.2, and 1 x 10^6) and mixed with 100 µL of PBS containing 2 x 10^5 competitive donor’s (CD45.1+) BM cells. Recipient (CD45.1+) mice were irradiated with two doses of 5.5 Gy from a Cesium source. Mixtures of donor and competitive donor cells were injected intravenously in eight recipients. After 6 (short-term) or 20 (long-term) weeks post-transplantation, recipients were sacrificed and BM cells were isolated. BM cells were stained using commercially fluorochrome-conjugated antibodies against CD45.1 (A20, APC; BD Pharmingen) and CD45.2 (104, FITC; BD Pharmingen), and analyzed using a FACSCanto (BD Biosciences) flow cytometer. Engraftment of the progeny was evaluated based on the percentages of donor-derived CD45.2+ BM cells. For the limiting-dilution analysis, the presence of more than 1% cells of donor origin (Cd45.2+) was considered as positive engraftment. The proportions of HSCs per million BM cells were calculated assuming a single-hit Poisson statistics (Purton and Scadden, 2007) and using the L-Calc software (StemCell Technologies, Vancouver, Canada).
Migration of Hematopoietic Precursors

The *in vivo* migration of hematopoietic precursors was examined 24 hours after i.v. injection. Two hundred and fifty μL of sterile PBS containing 5 million TCDD- or vehicle-treated donor BM cells were injected in the tail vein of non-irradiated recipients. The number of BM cells used was optimized experimentally, and represents about 6.3% of the total number of BM cells in WT mice (i.e. recipients). The migration of phenotypically-defined HSCs/progenitors was calculated as the ratio of the numbers of CD45.2+ LSK cells retrieved from transplanted recipients per the numbers of CD45.2+ donor LSK cells injected.

The *in vitro* migration was studied utilizing a transwell assay. One hundred and fifty μL of the chemo-attractant murine chemokine (C-X-C motif) ligand 12 (CXCL12), also known as stromal-derived factor-1α (Miltenyi Biotech, Bergisch Gladbach, Germany) was added to each well of a V-bottom 96-well plate (BD, Franklin Lakes, NJ) at concentrations of 0, 50, 100 or 300 ng/mL. The HTS Transwell 96 Permeable support with a 5 μm polycarbonate membrane (Corning Life Sciences, Lowell, MA) was placed on top of the plate containing chemo-attractant. Five hundred thousand of lineage depleted (Lin-) cells resuspended in 50 μL of PBS were placed on top of the permeable support and incubated at 37°C, for 3 hours at 5% CO₂. To determine migration, cells that were recovered from the bottom well were stained with 7AAD, and fluorochrome-conjugated antibodies against Sca-1 and c-kit.

Phenotyping of Cells

Flow cytometry was performed using a LSR-II flow cytometer (BD Biosciences) in the Flow Core Facility of the URMC, and was used to phenotypically define long-term and short-term HSCs (Kiel, et al., 2005) using fluorochrome-conjugated antibodies against CD48 (HM48-1, Phycoerythrin (PE)-Cy7, BD Pharmingen) and CD150 (9D1, APC; ebioscience). LSK cells (Sca1 (E13-161.7, APC; BD Pharmingen) and ckit (2B8, PE-Cy5; BD Pharmingen)) were
phenotyped for the expression of markers involved in the interactions between HSCs and their microenvironment such as: the integrin dimer $\alpha 4\beta 1$ (also, Very Late Antigen-4) composed of CD49d (also $\alpha 4$; R1-2, PE; BD Pharmingen) and CD29 (also $\beta 1$; Ha2/5, FITC; BD Pharmingen), CD184 (2B11/CXCR4, APC; BD Pharmingen), CD44 (IM7, PE-Cy5; BD Pharmingen), and CD162 (also P-selectin glycoprotein ligand-1; 2PH1, PE, BD Pharmingen). Flow cytometric analysis was performed in viable cells excluding 7AAD. For apoptosis analysis, Lin- cells were incubated with fluorochrome-conjugated antibodies against ckit (2B8, APC) and Sca-1 (D7, V450). Cells were washed and resuspended in 1X Annexin V Binding Buffer (BD Pharmingen), and then incubated with 7AAD and Annexin V-PE according to manufacturer's recommendations. 7AAD and Annexin V double-negative cells were gated as viable cells, and 7AAD negative and Annexin V positive cells were gated as apoptotic cells. Unstained, single fluorochrome and all-fluorochromes-except-one were used to compensate the fluorescent signals. Data were calculated and plotted using FlowJo software (Tree Star Inc., Ashland, OR).

**Microarray Experiments**

Total RNA was isolated from sorted LSK cells pooled from 20 mice using RNeasy Mini/Micro Kit (Qiagen, Valencia, CA), DNAse treated, and quantified using the ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Microarray analyses were done according to standard operating procedures by the Functional Genomics Center at the URMC. RNA quality was confirmed by the presence of two peaks on a 2:1 ratio of intensity of 28S:18S rRNA using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA). RNA (20 ng) was amplified using the NuGEN Ovation RNA Amplification System (San Carlos, CA) to yield single stranded cDNA, then hybridized with the GeneChip® Mouse Gene 1.0 ST Array (Affymetrix, Santa Clara, CA). Five microarrays from independent RNA preparations per treatment were used. The microarrays were processed as previously described (Henry, et al., 2010). The relative fold-changes of the signal intensity averages of TCDD with respect to control at the 6 h
and 12 h time-points were calculated for each of the 35,556 probes. Assuming a normal distribution of the data, the F-test was performed to evaluate variances between the treated and control group, and calculate the p-value obtained after performing the two-tailed Student’s T-test using Microsoft Excel.

**Gene-specific Primers and Real-time Reverse-transcription (RT²-PCR)**

Gene-specific primers and real-time reverse-transcription (RT²-PCR) analysis were used to validate microarray data using a set of genes selected because of their significant fold-change expression differences at 6h and/or 12h. First strand cDNA was prepared from 100 ng total RNA following the protocol for the SuperScript II First Strand cDNA Synthesis system (Invitrogen, Carlsbad, CA). After selecting a panel of genes of interest (Supplemental Table 1), RT²-PCR was performed using protocols provided by the commercial provider of primers and master mix (SA Biosciences, Frederick, MD) in an iCycler System (Biorad, Hercules, CA). Fold-changes in expression of genes from TCDD-treated LSK were calculated with respect to vehicle-treated LSK using the \(2^{-\Delta\Delta Ct}\) approximation method. Gapdh and Hprt1 were used as control endogenous genes to normalize gene expression. A linear regression coefficient close to one was used to assess validation of microarray data with RT²-PCR.

**Gene Expression Analysis**

Transcripts with a fold-change expression (TCDD normalized expression/Vehicle normalized expression) greater than 1.5 at each time-point were filtered. The most variable transcripts were visualized using the open-source software TIGR Multi-experiment Viewer (MeV) (version 4.6.1) (Saeed, et al., 2003). Relevant Functional Association Networks were generated by Ingenuity Pathway Analysis (IPA) Software (version 8.7) (Ingenuity Systems, Redwood City, CA). For this purpose, the data sets containing all gene identifiers and corresponding fold-change expression values were up-loaded into the application. Each identifier was mapped to its corresponding
object in Ingenuity's Knowledge Base. A 1.5 cut-off of fold-change was set to identify transcripts whose expression was regulated. Transcripts with altered expression were overlaid onto a global molecular network developed from information contained in Ingenuity’s Knowledge Base.

Statistical Analyses

Unless otherwise specified, results were analyzed and plotted using GraphPad Prism (version 5.03) (GraphPad Software, Inc, La Jolla, CA). The p-value of Pearson’s Chi-square test for goodness of fit to a binomial distribution (p=1) was used to analyze the CRU data. When appropriate, two-tailed Student’s T-test and two-way ANOVA were used to analyze statistical significance. P-values smaller than 0.05 were considered to be statistically significant *p<0.05, **p<0.001, and ***p<0.0001. Right-tailed Fisher’s exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that data set by IPA is due to chance alone.
Results

AhR activation by TCDD alters phenotype and functions of HSCs

Our laboratory and others have consistently observed alterations of phenotypically-defined HSCs/progenitors after TCDD treatment (Murante and Gasiewicz, 2000; Sakai, et al., 2003; Singh, et al., 2009). In this study, we asked questions about the relative expression of newly recognized phenotypes of HSCs (Kiel, et al., 2005) as well as qualitative and quantitative functional characteristics of BM cells. Seven days after in vivo treatment with TCDD, populations of HSCs were analyzed using flow cytometry based on their relative expression of the Signaling Lymphocyte Activation Molecule (SLAM) receptors (Wilson, et al., 2008) as phenotypic markers of BM stem cells. Figure 1A shows representative gating applied to all samples used to determine the relative percentages of LSK, LSKCD48^-CD150^- and LSKCD48^-CD150^+ populations. As observed previously (Murante and Gasiewicz, 2000; Singh, et al., 2009), TCDD treatment resulted in a significant increase in the relative percentage of LSK cells (Fig. 1B). However, only a small proportion of the LSK population is made up of those cells defined as HSCs, with the largest sub-population being composed of predominantly more mature multi-potent progenitor (MPP) cells. As such, it is notable that TCDD also increased the relative percentages of the sub-populations LSKCD48^-CD150^- and LSKCD48^-CD150^+ which have been correlated with short-term HSCs and MPPs, and long-term HSCs, respectively (Fig. 1C).

To characterize the functional quality of HSCs, the progeny of short-term and long-term HSC donor cells was analyzed after 6 or 20 weeks of competitive repopulation, respectively. Hematopoietic cells from a donor or competitive donor origin were distinguished using flow cytometry (Figs. 2A-C). Under the experimental conditions used, no differences between TCDD and vehicle were observed 6 (Fig. 2D) or 20 (Fig. 2E) weeks post-transplantation. The proportions of short- and long-term HSCs per million BM cells (Table 1) were calculated from
limiting dilution analysis to characterize quantitatively the functional integrity of these cells. By this analysis, the numbers of competitive functional HSCs from TCDD-treated animals were not significantly different from vehicle controls. To complement this analysis, we evaluated the ability of LSK cells from TCDD-treated mice to undergo expansion and differentiation under ex vivo conditions. Supplemental Figure 1 shows that, in the presence of IL-6 ± stem cell factor (Scf), TCDD-treated LSKs are as able to grow and generate different Lin⁻ and Lin⁺ populations in vitro similarly as vehicle-treated LSKs. Also, LSKs from TCDD-treated mice didn’t show any difference in the level of apoptosis as measured by Annexin and 7AAD staining (Supplemental Figure 2). These results suggested that the TCDD dosing paradigm used to activate the AhR elicited phenotypic changes, but did not alter the absolute proportions of functional HSCs when measured independently from their correlated phenotype. Also, there were no alterations of their ability to survive, expand and differentiate under ex vivo conditions.

**TCDD affects migration of LSKs**

TCDD-treated LSK cells were reported to have a decreased engraftment (Sakai, et al. 2003; Singh, et al. 2009). However, TCDD treatment elicited no difference in the overall proportions of functional HSCs independently from their phenotype (Table 1). Therefore, we hypothesized that critical events during engraftment such as trafficking behavior of LSKs may be affected. Figure 3A shows that migration of LSK cells from TCDD-treated animals to BM of recipients is decreased when measured as in vivo accumulation of cells to the BM after 24 hours of transplantation. As a complement to this study, we also observed that the directional migration of TCDD-treated LSK cells through a semi-permeable membrane to a chamber containing the chemokine CXCL12 was decreased (Fig. 3B).

The best studied receptor of CXCL12 is CD184 (CXCR4) (Sasaki, et al., 2009). As such, we analyzed LSK cells for the expression of CD184/CXCR4 as well as other molecules known to
participate in the interactions between HSCs and their microenvironment such as CD44 which is a receptor for osteopontin present in the extra-cellular matrix of the endosteum. Within the LSK population from TCDD-treated animals, there was increased Mean Fluorescent Intensity for CD184/CXCR4 and CD44 (Figs. 3C and 3D). Together these results support that AhR activation leads to cell signaling-mediated disruptions in the ability of LSKs to interact with molecules present in their microenvironment.

**TCDD alters pathways involved in cell signaling, cellular movement and cancer.**

The above data suggest that the previously observed TCDD-elicited changes in HSC/progenitor engraftment (Sakai, et al., 2003; Singh, et al., 2009) were due predominantly, if not exclusively, to altered homing of HSCs from TCDD-treated animals. Alterations in the expression and/or function of CD184/CXCR4 and CD44 on these cells are likely concomitant with functional changes. Being that the AhR is a transcription factor, we hypothesized that the phenotypic and cellular changes observed above were the consequence of AhR-mediated changes in gene expression. Differential gene expression in LSKs from mice treated with TCDD (30 μg/kg) or vehicle for 6h and 12h was analyzed using microarray technology. Hierarchical clustering of the expression profiles showed that the most significant changes in regulation occurred for 105 transcripts (Fig. 4A). Transcripts consistently up-regulated at 6h and 12h included Scinderin (Scin), Mmp8, Interleukin 1 family member 9 (Il1f9), NAD(P)H dehydrogenase quinone 1 (Nqo1), Filamin beta (Flnb), mouse predicted gene Gm5662, and SLAM family member 7 (Slamf7) (Table 2). The lists of up- or down-regulated transcripts at 6h and 12h can be found in Supplemental Tables 2, 3, and 4. Results from RT²-PCR analyses, (Figs. 4B and 4C) validate the changes observed at 6h and 12h. There were some notable differences between the responses at 6 and 12 h. At 6 h, several genes (Olfr1095, Mmp9, Ceacam10, Retnig, Msr1, and Lcn2) were up-regulated > two-fold, but demonstrated no significant change at 12 h (Fig. 4A, Table S2). In contrast, other genes including Fosb, Cxcl2, Egr1, Atf3, Nr4a1, Fos, Jun,
Junb, Dusp1, Plk2, Ptgs2 (Cox2), Cd69, Ighg, Slc10a1, and Myd116, were down-regulated at 6 h, but were significantly up-regulated (Cxcl2, Nr4a1, Plk2, Ptgs2, Cd69) or not altered at 12 h (Fig. 4A, Tables S3 and S4).

In order to understand the biological relevance of the observed changes, IPA was used to generate Functional Association Networks with the following settings: direct relationships from the Ingenuity Knowledge Base Reference Set reported in all human, mouse and rat immune primary cells; and immune, leukemia, lymphoma, macrophage and myeloma cell lines; cut-off fold-changes values 1.5; up to 25 networks per analysis; up to 35 molecules per network. Table 3 shows that the common top biological functions at 6h and 12h were cell-to-cell signaling and interaction, and cellular movement. Also, at these time-points, hematological system development and function was significantly affected by treatment. Supplemental Tables 5 and 6 show the detailed lists of transcripts assigned by IPA to the most relevant molecular and cellular functions, and physiological systems and functions. Notably, changes in genes involved in the biological functions of antigen presentation, cell-to-cell signaling and interaction, cellular movement, hematological system development and function, and immune cell trafficking appeared to be most significant (defined by p-values < 10⁻⁷) in the 12h response to TCDD (Table 3). Figure 5 shows functional association networks of transcripts assigned to hematological system development and function, generated by IPA at 6h and 12h. An analysis was also run using information reported from hematopoietic and non-hematopoietic cells. According to this analysis, the top biological functions and diseases associated with the network generated by the molecular interactions common to both time-points were gene expression (RNA polymerase II, histone h3, histone h4), inflammation (Ptgs2 also known as Cox2, NFκB complex), and cancer (Ptgs2, Cxcl2, Jun, Fos, Att3, Mmp9, Nqo1, Nr4a1, Cd69, Btg2, Egr1, Dusp1).
Discussion

There is increasing evidence supporting a role of the AhR in hematopoiesis, and HSC biology in particular. In part, this evidence comes from reports of increased incidence of leukemia and non-Hodgkin's lymphoma in TCDD-exposed populations (Frumkin 2003), as well as recent studies suggesting a potential therapeutic application of AhR antagonists to expand human HSCs for BM transplants (Boitano, et al., 2010). As such, there is a need to define cellular and molecular mechanisms whereby the AhR mediates these effects.

It has been shown that TCDD exposure to mice increases the percentages of LSKs, and some LSK sub-populations in BM (Sakai, et al., 2003; Singh, et al., 2009). Here we expand on these reports by showing that the percentage of LSKCD48^-CD150^- and LSKCD48^-CD150^+ stem cells, identified by the recently described SLAM markers (Kiel, et al. 2005), are also increased following TCDD treatment (Fig. 1). These results present the apparent paradox that higher percentages of phenotypically-defined HSCs (LSKs) occurred with a decreased functional ability of these cells to generate progeny (Sakai, et al. 2003; Singh, et al. 2009). This may not necessarily be contradictory since it has been reported that when homeostasis is disrupted, cells with the phenotype of HSCs may have an altered functional capacity (Purton and Scadden, 2007). To avoid potential biases inherent with phenotypic-based sorting and defined relative ratios of donor to competitor cell numbers, we used a limiting dilution approach to quantify the functional integrity of these cells. These studies indicated no significant differences in the absolute proportions of LT- and ST-HSCs from TCDD- and vehicle-treated mice (Table 1). Together, these data suggested that the higher percentages of phenotypically-defined HSCs reflected changes in HSC biology and cell signaling that may affect different steps of the engraftment process.

Successful engraftment of HSCs involves a number of different events such as leaving the
circulation to home to the BM, taking residence in the vascular and endosteal niches, and finally lineage differentiation. Our studies further demonstrated that TCDD-mediated activation of the AhR alters the migration and trafficking of phenotypically-defined HSCs under in vivo and in vitro conditions (Figs. 3A and 3B). Thus, previously observed decreased engraftment (Sakai, et al. 2003; Singh, et al. 2009) is likely due to decreased migration (Figure 3) rather than decreased numbers of functional HSCs (Fig. 2 and Table 1) or altered viability and differentiation (Supplemental Figs. 1 and 2). A modified ability of HSCs and progenitors to move within the marrow niche may also explain the effects of AhR activation by TCDD on more differentiated cells i.e. decreased thymic seeding (Fine, et al., 1990; Laiosa, et al., 2010), and altered B cell numbers (Thurmond, et al., 2000).

Trafficking events are dependent on the ability of HSCs to “sense” their microenvironment by cell-cell interactions and the recognition of soluble factors through cell surface proteins. Markers used for phenotypic discrimination are functional molecules themselves involved in cell signaling through recognition of chemical cues from the microenvironment to migrate, differentiate, proliferate, or self-renew. For example, c-kit is a tyrosine kinase serving as a receptor of the stem cell factor, which provides cues for quiescence and survival of the most primitive progenitor cells in the BM (Askmyr, et al., 2009). CD184/CXCR4 is critical during development for seeding of the BM with HSCs, and mediates migration and quiescence throughout adulthood (Nie, et al., 2008). CD44 is highly expressed in pro-thymocytes (CD4^low^CD25^-^c-kit^+) of the BM serving as a homing receptor for the thymus (Wu, et al., 1993), and CD44 expression was found to be up-regulated in thymic emigrants exposed to TCDD (Esser, et al. 2004). As such, it is reasonable, if not expected, that functional alterations in HSCs following TCDD treatment would be a reflection of altered expression and/or function of cell surface proteins. However, we observed an increase, rather than an expected decreased, phenotypic expression of both CD184/CXCR4 and CD44 at 7 d following TCDD treatment (Fig. 3). No differences were
observed in CD184/CXCR4 or CD44 mRNA expression at 6 or 12h post TCDD exposure as determined by the microarray data. Also, CD184/CXCR4 mRNA expression at 24h or 7d following TCDD exposure was not changed as determined by RT²-PCR analysis (not shown). Together these data suggest that TCDD may cause altered CD184/CXCR4 and CD44 protein expression in HSCs by a post-transcriptional mechanism, and that other molecules are being affected by AhR activation that either alter the function of these proteins or that are involved the migration and trafficking of these cells.

Given that the AhR is a ligand-activated transcription factor, we hypothesize that TCDD-mediated changes in phenotype and signaling of HSCs were preceded by changes in gene expression. The results shown in Figures 4 and 5 support this hypothesis. Multiple transcriptional changes occurred 6h after exposure, some of which were transient and other were also observed 6h later. The genes modulated by AhR activation in LSKs that have been reported to have putative AhREs (Sun, et al., 2004) in the promoters of their murine homologs include Scin, Nqo1, Egr1, Ptgs2, Cxcl2, Nr4a1, Dusp1, Btg2, Junb, Fosb, Jun, Ceacam10, Scrg1, C1qb, Bst1, Slc10a1, Spp1, Snord14a, ler2, Ccl4, Skil, and Klrb1c, although it is not yet clear whether all of these sites represent AhREs that are functional.

Scinderin (Scin, also known as adseverin) was highly up-regulated both after 6h and 12 h of TCDD-treatment (Table 2). In hematopoietic stem and progenitor cells, Scin is one of the key regulators accounting for chemotactic responses to CXCL12 (Evans, et al., 2004). This calcium/proton-regulated protein binds actin monomers, severs and caps actin filaments, and has been reported to respond to TCDD treatment in other immune cells (Svensson, et al., 2002). Sequential coordinated polymerization and depolymerization of the actin cytoskeleton is necessary to maintain the functional expression of cell surface proteins and for directed migration (Friederich, et al., 1992). CD184/CXCR4 function and cell surface expression is known to depend on endocytosis, intracellular trafficking and recycling (Kumar, Kremer et al.
2011), all of which are dependent on cytoskeleton regulation. Lin-Sca-1^ckit^ and LSKs move with different efficiencies towards CXCL12 due at least in part to different expression of Scin protein (Evans, et al., 2004). Given that TCDD can regulate Scin transcripts and responses to CXCL12 in LSKs, our results support a role for the AhR in cytoskeleton regulation that may have consequences for cell motility.

*Mmp8* was also up-regulated at both time points. Even though there is no evidence of AhR transcriptionally regulating metalloproteinases, there is literature linking AhR activation with their expression, probably via *Jun* or *Fos* (Hillegass, et al., 2006). Together with CD184, metalloproteinases have been proposed to be involved in cell migration and invasion of leukemic cells (Shao, et al., 2011). Different transcripts involved in NFκB signaling such as *Il1f9*, *Cxcl2*, *Ptgs2*, *Junb*, and *Fos* (*Table 2 and Supplemental Table 3*) were also altered at both time-points. Whether these changes in gene expression are directly related to AhR-DNA binding in LSKs is uncertain since there is evidence that AhR and NFκB proteins can interact directly (Tian, 2009). Further kinetic studies to clarify these interactions are of therapeutic interest considering that NFκB complexes regulate CD184 (Chua, et al., 2010) and CD44 (Damm, et al., 2010) protein expression. It is also of interest that *Spp1* was down-regulated 6h after TCDD-exposure. *Spp1* encodes osteopontin, one of the ligands of CD44. The reduced expression of a CD44 ligand (*Supplemental Table 4*) at early time-points may be related with increased CD44 expression (*Fig. 3C*) several days later. Given that some of the transcripts changed in our data sets are themselves transcriptional regulators and are regulated by the AhR (e.g. *Fos*, *JunB*, *Ptgs2*, *Egr1*), it seems likely that some of the functional changes are secondary to initial AhR signaling. While supporting the functional changes that we observed in hematopoietic stem/progenitor cells after TCDD treatment, the changes in gene expression also suggest a complex crosstalk of the AhR with pathways associated with the ability of HSCs to sense their microenvironment, alter their trafficking behavior, and promote hematological diseases, which is
a common sequence of events in leukemogenesis.

Together, these results suggest a complex role of AhR signaling in HSC function. Additional studies in \textit{AhR} null mice suggest that AhR may regulate HSC quiescence/proliferation (Singh, et al., 2010) that, consistent with the present study, depend on the ability of HSCs to sense cues within their microenvironment. It is also important to consider that the exact role of the AhR and its physiological relevance in other tissue stem cell populations may be context-specific (Latchney, et al., 2010; Panteleyev and Bickers, 2006). Clinical use of the AhR to treat hematological diseases (Boitano, et al., 2010; Quintana, et al., 2010) will also require answering daunting questions regarding the biological implications of different, and possibly endogenous, ligands in AhR activity and the relevance of non-genomic pathways in AhR signaling. Nevertheless, further investigations in this area will provide important information needed for our understanding of processes that regulate stem cells and their role in human disease.
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**Authorship contributions**

Participated in research design: Casado, Singh and Gasiewicz

Conducted experiments: Casado and Singh

Performed data analysis: Casado, Singh, Gasiewicz,

Wrote or contributed to the writing of this manuscript: Casado, Gasiewicz and Singh
References


Footnotes

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Legends for figures

Figure 1. TCDD treatment altered phenotypically-defined HSC populations.

Lin- cells were harvested from mice treated with TCDD (30 μg/kg, 7d) or vehicle. Flow cytometry was used to phenotypically characterize populations of HSCs. (A) Dead cells were excluded and the LSK population was gated from the viable cells. From the LSK population, further gating was applied based on CD48 and CD150. Cumulative data for the percentages of the HSC-enriched population are indicated. (B) LSK (p-value = 0.0059), and sub-populations (C) LSKCD48-CD150- (p-value = 0.0075) and LSKCD48-CD150+ (p-value = 0.0007) in Lin- cells. Error bars are S.E.M. for n=3 independent experiments using data from 5-6 individual mice.

Figure 2. Qualitative analysis of engrafted TCDD–treated BM cells shows no difference in the engraftment potential of HSCs.

Donor CD45.2+ BM cells from TCDD-treated (30 μg/kg, 7d) mice were simultaneously injected with CD45.1+ competitor BM cells at different ratios into CD45.1+ irradiated recipient mice. Representative gating applied for (A) competitive donor BM cells and (B) donor BM cells. After reconstitution, the long-term (20 weeks) and short-term (6 weeks) ability to reconstitute the BM was qualitatively analyzed. (C) Flow cytometry was used to discriminate CD45.2+ donor cells from CD45.1+ competitive donor cells in repopulated recipients. Cumulative data for the percentage of donor's progeny (CD45.2+) show no differences (D) 6 or (E) 20 weeks post-transplantation. Data shown represent one independent experiment of two with eight recipients per group per experiment. Squares represent values for individual mice. Average values for vehicle and TCDD groups are represented by dashed and solid lines, respectively.

Figure 3. TCDD exposure altered the migration of HSCs in vivo and in vitro.
BM cells were harvested from mice treated with TCDD (30 μg/kg, 7d) or vehicle. (A) Five million CD45.2+ BM cells from TCDD-treated or untreated mice were injected intravenously into CD45.1+ recipients. After 24 h, flow cytometry was used to determine the numbers of donor LSKs that migrated to BM in recipients with respect to the LSK numbers injected. (p-value = 0.0242). Error bars are S.E.M of n=3 independent experiments using data from 5-6 individual mice per experiment. (B) Half a million Lin- cells were placed on top of a transwell migration chamber containing 50-300 ng/mL of CXCL12 in the bottom chamber. Three hours later, LSK cells that migrated to the bottom chamber were counted by flow cytometry. Two-way ANOVA analysis indicated significant differences at 100 and 300 ng/mL (p-values < 0.0001). Error bars are S.E.M. of n=3 independent experiments using data from 12 wells with cells from three pooled mice. (C) LSK cells were analyzed for expression of surface molecules by flow cytometry. Two-way ANOVA analysis indicated significant differences between groups for CD184/CXCR4 and CD44 (p-values = 0.0037 and <0.0001, respectively). Error bars are S.E.M. of n=3 independent experiments using data from 5-6 individual mice. (D) Representative histograms obtained using flow cytometry to determine the Mean Fluorescent Intensity of CD184/CXCR4 and CD44 in viable LSK cells. The filled histograms are representative of TCDD– (black) and vehicle-treated cells (grey). Unfilled histograms are representative of the negative controls used to compensate fluorescent signals, that were stained with all of the fluorochromes used in the respective panels except with the one conjugated to the protein being analyzed (CD184 or CD44). The horizontal bars represent the gating used to determine the positive population of cells where the Mean Fluorescent Intensity was calculated.

**Figure 4. TCDD exposure induced changes in the expression of genes in LSK cells.**

Total RNA was obtained from sorted LSK cells. Fold-changes in normalized values of gene expression for TCDD or vehicle control groups were calculated using microarray technology. Microarray data represent averages of five experiments using RNA from LSKs pooled from 20
mice per treatment. (A) The most variable transcripts from the 6h and 12h datasets (Fold-change > 1.5) were visualized using MeV. The intensity of the colors represents the changes in fold-change for the up- (yellow) or down- (blue) regulation of the transcripts. Genes with significant fold-changes in expression were used to validate microarray results using RT²-PCR after (B) 6h and (C) 12h of treatment. RT²-PCR data are averages of three experiments from LSK cells pooled from 20 mice.

Figure 5. TCDD treatment changes the expression of transcripts involved in hematopoietic system development and function.

Data sets containing 35,556 microarray probes were analyzed at 6h and 12h. Transcripts with regulated expression were overlaid onto a global molecular network developed from information contained in the IPA’s knowledge base. All relationships are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the IPA’s knowledge base. Hematopoietic system development and function was regulated after TCDD-treatment. Association networks of the transcripts regulated in this category after 6h and 12h were generated using IPA. In the graphical representation of the molecular relationships, transcripts are represented as nodes with shapes indicating the function of the expressed protein. The color of the node indicates the up- (red) or down- (green) regulation (fold-changes > 1.5) of the transcripts. The non-color transcripts were present in our dataset, and have a direct relationship with the regulated transcripts. Also, according to IPA’s knowledge base, these are categorized within hematopoietic system development and function.
Table 1. Quantitative analysis of engrafted TCDD–treated BM cells shows no difference in the engraftment potential of HSCs.

Donor BM cells were harvested from mice seven days after treatment with TCDD (30 μg/kg) or vehicle (olive oil) by gavage as described in Fig. 2 legend. Repopulated recipients contained >1% of CD45.2+ cells in total BM cells. The proportions of HSCs per million of BM cells were calculated using the L-Calc Software entering the numbers of total surviving and repopulated mice. Representative results from one of two independent experiments (n=8 recipients per dilution of donor BM cells per group). Pearson’s Chi Square p-value = 1 was consistent with a Poisson’s distribution of data.

<table>
<thead>
<tr>
<th></th>
<th>Short-term (6 weeks)</th>
<th>Long-term (20 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>TCDD</td>
</tr>
<tr>
<td>Proportion (HSCs per million BM cells)</td>
<td>13.4</td>
<td>11.0</td>
</tr>
<tr>
<td>95% Confidence Interval</td>
<td>6.0-30</td>
<td>5.3-22</td>
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<tr>
<td>p-value of Pearson’s Chi-Square</td>
<td>0.7966</td>
<td>0.4722</td>
</tr>
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</table>
Table 2. Transcripts consistently up- or down-regulated at 6h and 12h in LSKs after TCDD treatment.

Microarray experiments were performed from LSKs isolated using immuno-magnetic sorting. Hierarchical clustering of microarray results was used to determine groups of genes co-regulated. (n=5 microarrays from pooled samples of 20 mice per group).

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>mRNA Accession Number</th>
<th>6h</th>
<th>12h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scin</td>
<td>NM_001146196</td>
<td>6.4</td>
<td>7.9</td>
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<tr>
<td>Mmp8</td>
<td>NM_008611</td>
<td>4.4</td>
<td>1.8</td>
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<td>Il1f9</td>
<td>NM_153511</td>
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<td>NM_008706</td>
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<td>Gm5662</td>
<td>NM_001013824</td>
<td>2.1</td>
<td>3.9</td>
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<tr>
<td>Slamf7</td>
<td>NM_144539</td>
<td>-1.6</td>
<td>-3.2</td>
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</table>
Table 3. Important biological functions were significantly altered in LSKs 6 and 12 h after TCDD treatment.

A 1.5 cut-off of fold-change (TCDD/vehicle) was set to identify transcripts whose expression was regulated. The top biological functions based on their Fisher’s exact test p-value (in brackets) are shown. The numbers in parentheses after the name of the functions are the number of transcripts in our data sets common to the Ingenuity’s Knowledge Base reference set.

<table>
<thead>
<tr>
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<th>6h</th>
<th>12h</th>
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<td><strong>Cellular Functions</strong></td>
<td><strong>Cellular Functions</strong></td>
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<td>Cell-To-Cell</td>
<td>[9.52x10^-3 - 3.92x10^-2]</td>
<td>[2.11x10^-8 - 4.44x10^-2]</td>
</tr>
<tr>
<td>Signaling and</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interaction</td>
<td>23</td>
<td>17</td>
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<tr>
<td>Cellular</td>
<td>[2.20x10^-3 - 4.48x10^-2]</td>
<td>[2.11x10^-8 - 4.89x10^-2]</td>
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<tr>
<td>Movement</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td>Antigen</td>
<td>[2.51x10^-3 - 3.92x10^-2]</td>
<td>[1.54x10^-1 - 4.44x10^-2]</td>
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<td>14</td>
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<tr>
<td>Cellular</td>
<td>[4.54x10^-3 - 4.31x10^-2]</td>
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<tr>
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<td>[9.90x10^-3 - 2.67x10^-2]</td>
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<td><strong>System Development and Function</strong></td>
<td><strong>System Development and Function</strong></td>
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<tr>
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<tr>
<td>System</td>
<td>Function</td>
<td>Function</td>
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<tr>
<td>Function</td>
<td>(44)</td>
<td>(26)</td>
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<tr>
<td>Tissue</td>
<td>[2.79 x10^-5 - 2.67x10^-2]</td>
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<tr>
<td>Morphology</td>
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<td>Immune Cell Trafficking (17)</td>
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<td></td>
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<td>[2.11x10^-8 - 4.89x10^-2]</td>
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<tr>
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<td>Hematopoiesis (20)</td>
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<td>Immune Cell Trafficking (23)</td>
<td>[2.20x10^{-3} - 4.96x10^{-2}]</td>
<td>Humoral Immune Response (3)</td>
</tr>
</tbody>
</table>
Figure 1

A. % of Max. Events

B. LSK

C. % of Lin- cells

D. CD48-CD150+

E. CD48-CD150−

F. CD48−CD150−
Figure 2

A. CD45.2  0.031%
    CD45.1  83%

B. CD45.2  90%
    CD45.1  0%

C. CD45.2  59%
    CD45.1  29%

D. Short-term BM transplantation
   (6 weeks)

E. Long-term BM transplantation
   (20 weeks)
Figure 3

A. % Migration of LSKs (Total BM cells)

B. % Migration of LSKs (Lin+ cells)

C. Mean Fluorescent Intensity

D. % of Max Events

Legend:
- Control
- No CD184
- Vehicle
- TCDD
Figure 4.

A.

B. LSK - 6h after TCDD

C. LSK - 12h after TCDD