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The Aryl Hydrocarbon Receptor Agonist 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Alters the Circadian Rhythms, Quiescence, and Expression of Clock Genes in Murine Hematopoietic Stem and Progenitor Cells

Russell W. Garrett[1] and Thomas A. Gasiewicz

Department of Environmental Medicine, University of Rochester, School of Medicine and Dentistry, Rochester, New York

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ABSTRACT

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), an aryl hydrocarbon receptor (AhR) agonist, has been identified as a potent immunohematopoietic toxicant with the ability to alter the number of Lin^- $Sca\text{-}1^+$ $cKit^+$ (LSK) bone marrow cells, a population enriched for murine hematopoietic stem cells. The biology of these cells is governed by circadian rhythms and TCDD has been shown to disrupt circadian rhythms of other biological endpoints. We investigated the effect of TCDD on the circadian rhythms of hematopoietic precursors. Female C57BL/6 mice were treated with a single oral dose of 10 μg/kg TCDD. Five days later, bone marrow was harvested every 4 h for 24 h and stained for specific hematopoietic populations using fluorescently labeled antibodies. In addition, cells were placed into semisolid culture to measure different functionally defined populations. Activation of the AhR by TCDD elicited disruptions in the rhythms of LSK cell numbers and phenotypically defined myeloid and erythroid precursors. Simultaneous DNA and RNA staining revealed an abnormal in vivo rhythm of percentage of total number of LSK cells in G_0 phase of the cell cycle, suggesting disruption of stem cell quiescence. Finally, quantitative reverse transcription-polymerase chain reaction revealed that expression of AhR and Arnt mRNA within enriched hematopoietic precursors oscillates with a circadian period. Modest changes in the 24-h expression of mPer1 and mPer2 mRNA and increased AhR repressor mRNA after TCDD exposure suggest a direct effect on the molecular machinery responsible for these rhythms. Together, these data demonstrate that activation of the AhR by TCDD disrupts the circadian rhythms associated with murine hematopoietic precursors.

The aryl hydrocarbon receptor (AhR) is a basic helix-loop-helix protein and a member of a relatively homologous, but functionally diverse, family of proteins containing one or more PAS (Per-Arnt-Sim) domains. This family contains the AhR's heterodimerization partner, the aryl hydrocarbon receptor nuclear translocator (Arnt), as well as hypoxia inducible factor-1α and principal components of circadian rhythm regulators, Clock and BMAL1. In addition to being a highly conserved, developmentally regulated, and ligand-activated transcription factor (Hahn, 2002; Williamson et al., 2005), the AhR is also the protein mediator for the action of several organic toxicants, typified by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Altered immunohematopoietic parameters, including both humoral and cellular, are among the most consistently observed toxicities after activation of the AhR by xenobiotic agonists such as TCDD. A hallmark of AhR-mediated toxicity is a persistent atrophy/aplasia of the thymus. Although this may be due partly to effects on thymocyte proliferation (Laiosa et al., 2003), other data suggest that other upstream cellular targets exist that may contribute to this effect. In particular, the sensitivity of Lin^- $Sca\text{-}1^+$ $cKit^+$ (LSK) bone marrow cells, a population highly enriched for multipotent hematopoietic stem cells (HSC), to TCDD was

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[1] Current affiliation: Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania.

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ABBREVIATIONS: AhR, aryl hydrocarbon receptor; AhRR, aryl hydrocarbon receptor repressor; Arnt, aryl hydrocarbon receptor nuclear translocator; PAS, PER/ARNT/SIM (periodicity/Arnt/simple-minded); TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; LSK, Lin^- $Sca\text{-}1^+$ $cKit^+$; HSC, hematopoietic stem cells; HPC, hematopoietic progenitor cells; CMP, common myeloid progenitor; GMP, granulocyte macrophage progenitors; MEP, megakaryocyte erythrocyte progenitors; ZT, Zeitgeber time; HPP-CFC, high proliferative potential colony-forming cells; IL, interleukin; CSF, colony-stimulating factor; SCF, stem cell factor; ANOVA, analysis of variance; CFU-GEMM, colony-forming unit–granulocyte erythrocyte macrophage megakaryocyte; CFU-GM, colony-forming unit–granulocyte macrophage.

demonstrated (Staples et al., 1998; Murante and Gasiewicz, 2000; Sakai et al., 2003). This is the most immature population of hematopoietic cells observed to be altered by TCDD treatment. Although predominantly quiescent, HSC are characterized by a robust proliferative capacity and the ability for self-renewal. In addition, these cells are defined by the ability to differentiate into all of the blood lineages by first committing to hematopoietic progenitor cells (HPC) that are themselves committed to either the lymphoid or myeloid lineages, common lymphoid progenitors or common myeloid progenitors (CMP), respectively. Whereas common lymphoid progenitors further differentiate into B and T cells or natural killer cells (Kondo et al., 1997), CMPs differentiate to form granulocyte macrophage progenitors (GMP) and megakaryocyte erythrocyte progenitors (MEP) (Akashi et al., 2000).

A single dose of TCDD has been shown to increase the number of marrow LSK cells, although their ability to reconstitute an irradiated mouse is impaired (Murante and Gasiewicz, 2000; Sakai et al., 2003). Furthermore, the use of irradiation chimeras demonstrated that the presence of the AhR in the hematopoietic cells, and not supportive stroma, was essential for both the TCDD-elicited increase in LSK cells and their functional impairment (Staples et al., 1998; Sakai et al., 2003). This is particularly important because of the tightly regulated and critical interactions between hematopoietic precursors and the nonhematopoietic stroma. These findings, along with measurable levels of AhR mRNA and protein in these cells (van Grevenynghe et al., 2005), indicate that the AhR may have some normal function in HSC. In addition, human HSCs are AhR-dependent targets for carcinogenic polycyclic aromatic hydrocarbons (van Grevenynghe et al., 2005).

Many members of the PAS family, including the two murine Period homologues mPer1 and mPer2, are involved in controlling circadian rhythms (Zheng et al., 2001). These rhythms occur with an approximate 24-h period and are a temporal compartmentalization that governs much of vertebrate physiology. All aspects of hematopoiesis, including proliferation and differentiation, are controlled by circadian rhythms. For example, the number of human $CD34^+$ bone marrow cells, as well as those in S-phase, has been shown to be rhythmic (Abrahamsen et al., 1998). Rhythmicity in numbers of erythroid progenitors, burst-forming units (BFU-E) and colony-forming units (CFU-E), and myeloid progenitors, colony-forming unit granulocyte erythrocyte macrophage megakaryocyte (CFU-GEMM) and CFU-granulocyte macrophage (CFU-GM), have been identified (Aardal and Laerum, 1983; Stevold et al., 1988; Smaaland et al., 1992; Wood et al., 1998). Although it is likely that at least part of their rhythmicity is regulated by circadian-controlled circulating factors, these cells express all of the machinery required for an intrinsic circadian clock and maintain rhythmicity in vitro (Bourin et al., 2002; Tsinkalovsky et al., 2005).

The AhR itself has been linked to circadian physiology. Both mRNA and protein levels of AhR in rat liver oscillates in a circadian manner (Richardson et al., 1998; Huang et al., 2002). It is not surprising that the level of CYP1A1 mRNA, one of the AhR-regulated genes, has a rhythm that is antiphase to the AhR. A single exposure to TCDD also disrupted circadian-controlled rest/activity rhythms in mice, as well as the rhythms associated with the expression of circadian genes *Per1* and *BMAL1* in the hypothalamus (Miller et al., 1999). The purpose of these experiments was to test the hypothesis that dysregulation of the AhR signaling pathway resulting from exposure to a persistent AhR agonist TCDD could disrupt the circadian rhythms of antigenically and functionally defined hematopoietic precursors.

Materials and Methods

Animals. Five-week old female C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and allowed to acclimate for a period of 1 week. All animals were cared for under a protocol approved by the University Committee on Animal Resources. They were provided food and water ad libitum. Lights were maintained on a 12-h schedule, turning on and off at 6:00 AM and 6:00 PM, respectively. These times correspond with Zeitgeber times (ZT) 0 and 12, respectively.

TCDD Exposure. Mice were gavaged with 10 μg/kg TCDD in 5 μl of olive oil per 1 g of mouse weight, or oil alone 5 days before the first time-point. In all studies, time points consisted of ZT 6, 10, 14, 18, 22, and 2. The 5-day time point was predetermined to be optimal in generating an effect on the percentage of quiescent Lin^- $Sca\text{-}1^+$ cells in the BM; this effect was maximal on days 3 through 7 after exposure (data not shown).

Bone Marrow Cell Isolation. Bone marrow cells were flushed from both femurs and tibiae with PBS + 2% FBS + 1% penicillin/streptomycin as described previously (Wyman et al., 2002; Laiosa et al., 2003). The bone marrow cells were placed in to a single-cell suspension before passing through an 80-μm nylon mesh (TETKO Inc., Briarcliff Manor, NY). Cell pellets were treated with erythrocyte lysis buffer (0.17 M NH_4Cl, 10 mM $KHCO_3$, and 1 mM EDTA) and the remaining cells were washed and counted.

Immunophenotyping. BM cells (10^6) from vehicle- and TCDD-treated mice were blocked with anti-FcR antibody in stain buffer (PBS + 1% FBS + 0.1% NaN_3) for 20 min on ice and then incubated for 30 min with predetermined saturating concentrations of the following fluorescently conjugated antibodies: fluorescein isothiocyanate-conjugated anti-CD34, Thy-1, Sca-1, Gr-1, and cKit; phycoerythrin-conjugated anti-Sca-1 and FcR; allophycocyanin-conjugated anti-c-Kit; Biotin-conjugated anti-Mac-1, Gr-1, CD8a, CD3ε, Ter119, B220, Sca-1, and IL-7R (BD Biosciences, San Jose, CA). Specific cell populations were detected using the following surface phenotypes as previously published by others (Kondo et al., 1997; Akashi et al., 2000): HSC (Lin^- $Thy\text{-}1^+$ $Sca\text{-}1^{hi}$ $c\text{-}Kit^+$), CMP (Lin^- $CD34^+$ FcR^{lo} $IL7R^-$ $Sca\text{-}1^-$ $c\text{-}Kit^+$), GMP (Lin^- $CD34^+$ FcR^{hi} $IL7R^-$ $Sca\text{-}1^-$ $c\text{-}Kit^+$), and MEP (Lin^- $CD34^-$ FcR^{lo} $IL7R^-$ $Sca\text{-}1^-$ $c\text{-}Kit^+$). In all cases, the lineage cocktail included antibodies to Mac-1, Gr-1, CD8a, CD3ε, Ter119, and B220. Streptavidin-PerCP was used as a secondary fluorescent agent. Cells were rinsed with staining buffer and fixed with 0.1% paraformaldehyde before running on the flow cytometer. Compared with our previous studies (Staples et al., 1998; Murante and Gasiewicz, 2000), the use of the Thy-1 marker allows further definition of the LSK population to include those cells that are phenotypically and functionally defined as long-term and short-term HSC. Cells were run (at least 50,000 events per sample) on a FACSCalibur flow cytometer (BD Biosciences), and data were analyzed with CellQuest software (BD Biosciences). Gates were set similarly as described previously (Kondo et al., 1997; Staples et al., 1998; Akashi et al., 2000; Murante and Gasiewicz, 2000), and data were taken as percentages of cells in the viable gates.

Cell Isolation for RNA Analysis. Total BM was harvested from both legs (femur + tibia) and blocked with anti-FcR in stain buffer. The cells were then stained with biotinylated anti-lineage (Mac-1, Gr-1, B220, CD8α, CD3ε, Ter119) followed by streptavidin-microbeads (Miltenyi Biotec, Auburn, CA). Lineage depletion was performed using an AutoMACS magnetic cell sorter (Miltenyi Biotec). For certain experiments, $Lin^-Sca\text{-}1^+$ and $Lin^-Sca\text{-}1^-$ cells were collected by staining the Lin^- fraction with anti-Sca-1 microbeads

(Miltenyi Biotec). Collection of Sca-1$^+$ and Sca-1$^-$ cells were performed using the sensitive positive sort function of the AutoMACS. Purity of fractions was assessed using flow cytometric analysis and determined to be >95%.

Colony-Forming Semisolid Assays. To measure high proliferative potential colony-forming cells (HPP-CFC), 2×10^4 BM cells were suspended in 1 ml of IMDM supplemented with 1% methylcellulose, 25% fetal bovine serum, 1% bovine serum albumin, 100 μM 2-mercaptoethanol, and 2 mM L-glutamine (Stem Cell Technologies, Vancouver, BC, Canada). To the media was added 250 ng/ml CSF-1, 50 ng/ml IL-3, 50 ng/ml IL-1α, and 50 ng/ml SCF (R&D System, Minneapolis, MN). CFU-GEMM and CFU-GM were measured using methylcellulose based media containing SCF, IL-3, IL-6, and erythropoietin or SCF, IL-3, and IL-6, respectively (Stem Cell Technologies). All plates were incubated at 37°C in 5% CO_2 and scored 14 days later. HPP-CFC were scored by the presence of macroscopic colonies (>0.5 mm in diameter). CFU-GEMM and CFU-GM were scored as colonies composed of granulocytes, erythrocytes, and monocytes or granulocytes and monocytes, respectively.

Cell Cycle Analysis. One million Lin$^-$ BM cells were stained with fluorescein isothiocyanate-conjugated anti-cKit and AF647-conjugated anti-Sca-1. Afterward, cells were stained with 7-aminoactinomycin D (Invitrogen, Carlsbad, CA) at room temperature for 20 min followed by Pyronin Y (PY) on ice in nucleic acid staining solution (0.1 M phosphate-citrate buffer + 0.15 M NaCl + 5 mM sodium EDTA + 0.5% bovine serum albumin + 0.02% saponin) for 10 min. Cells were analyzed using a FACSCalibur flow cytometer and data analyses were performed using CellQuest software.

Quantitative RT-PCR. Total RNA was isolated from Lin$^-$ Sca-1$^+$ and Lin$^-$ Sca-1$^-$ BM cells at the indicated time points 5 days after in vivo exposure to TCDD using the RNEasy Mini kit (QIAGEN, Valencia, CA) as instructed. RNA amplifications were performed using WTA (NuGEN Technologies, San Carlos, CA). cDNA was created using the Superscript First Strand Synthesis System (Invitrogen) as instructed and PCR was performed using a 7900 HT (Applied Biosystems, Foster City, CA). TaqMan gene expression assays (Applied Biosystems) were used to measure the following targets: AhR, Arnt, AhRR, mPer1, mPer2, p21, p27, and GAPDH. Data analyses were performed using the comparative Ct method.

Statistical Analysis. A two-way ANOVA (Excel) was performed on each set of 24 h data to determine treatment-dependent differences. When the ANOVA indicated differences between treatment groups, the Student's t test was used to determine differences between control and experimental groups at designated time points. Values of $p \leq 0.05$ were considered statistically significant.

Results

TCDD Disrupts the Circadian Rhythm of Hematopoietic Precursor Numbers. The numbers of HSC and HPC have been established to fluctuate with a circadian period (Abrahamsen et al., 1998). With this in mind and because prolonged activation of the AhR by TCDD increases the number of murine LSK BM cells (Murante and Gasiewicz, 2000; Sakai et al., 2003), we measured the effect of AhR activation on the circadian rhythms associated with these cells. Five-week old female C57BL/6 mice received by gavage a single 10 μg/kg dose of TCDD. Five days later, bone marrow was collected every 4 h for 24 h and stained with fluorescently labeled antibodies. As indicated in Fig. 1, Thy-1^{lo} LSK cells, a population that contains all of the murine HSC, are characterized by a near 3-fold difference between the minimum value and the maximum value in vehicle-treated mice. After treatment with TCDD, there is an overall increase in the number of Thy-1^{lo} LSK cells (Fig. 1) throughout the 24-h period (ANOVA $p < 0.001$). There were no statistical differences observed in total bone marrow cellularity at any time point (data not shown). A comparable TCDD-induced increase in this population of progenitor cells was observed in similar experiments carried out over the course of approximately 1 year. These studies were carried out because of known seasonal variations in these diurnal patterns. Occasionally, these experiments conducted at different seasons also suggested abnormal period and/or phase shifts after exposure to TCDD (data not shown).

Because there is a considerable body of literature demonstrating circadian rhythmicity in myeloid and erythroid precursors (Aardal and Laerum, 1983; Stevold et al., 1988; Smaaland et al., 1992; Wood et al., 1998), these studies were extended to examine effects on these populations. CMP, GMP, and MEP were all characterized by apparent rhythms in control animals (Fig. 2). Five days after TCDD exposure, the rhythms of all three populations were altered (ANOVA $p < 0.001$), demonstrating changes in phase and magnitude that are specific to both time of day and cell type (Fig. 2). In addition to measuring antigenically defined hematopoietic precursors, we performed methylcellulose-based ex vivo assays to determine effects on functional capacity by measuring HPP-CFC, CFU-GEMM, and CFU-GM. As seen in those antigenically defined cells, these functionally defined populations were all characterized by diurnal rhythms (Fig. 3). Although time-of-day-specific changes may have occurred after TCDD treatment, there were no overall significant treatment-dependent differences when the 24-h data sets for these colony assays were compared. Together, these data demonstrate that a single oral dose of TCDD is sufficient to cause a modification in the circadian rhythms of murine hematopoietic precursors, as well as overall changes in their numbers. These changes can be both positive and negative, with time points during a daily cycle that are absent of differences between treatment groups.

TCDD Disrupts the Circadian Rhythm of LSK Quiescence. Simultaneous staining for RNA and DNA allows

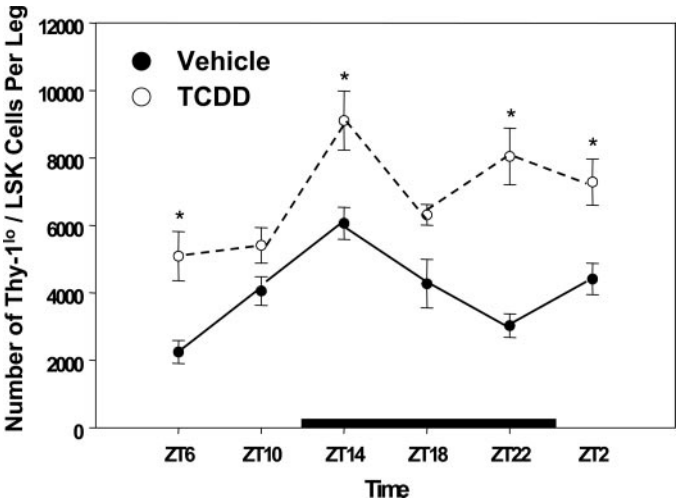

Fig. 1. TCDD disrupts the circadian rhythms of the numbers of antigenically defined (LSK) hematopoietic stem cells. Bone marrow cells isolated from control or TCDD-treated animals ($n = 6$ per group per time point) at ZT 6, 10, 14, 18, 22, and 2 on day 5 after treatment. Flow cytometry was performed as described under *Materials and Methods*. The data shown are representative of three separate experiments. Data are presented as mean values ± S.E. There was a treatment-dependent difference for this pattern (ANOVA $p < 0.001$), and * indicates statistically different ($p < 0.05$) from the vehicle-treated value at the same time point.

discrimination between G_0- and G_1-phase cells. This is an important distinction to make when investigating HSC because of their relatively high representation in quiescence (G_0) under normal homeostatic conditions. In addition, G_0 human $CD34^+$ cells, a population enriched for human HSC, were found to be more highly enriched for long-term culture-initiating capacity, indicating functional differences between cells differing in their cell cycle status (Gothot et al., 1997). As indicated in Fig. 4, the number of G_0 LSK cells in control mice exhibits a pattern suggesting a biphasic effect, with two periods of diminished quiescence occurring at the beginning of both perceived day and perceived night. After TCDD treatment, the daily cycle of quiescent cells was disrupted (ANOVA $p < 0.001$). The most obvious difference is at the period of late perceived night when the greatest percentage of the control LSK cells is quiescent. Here, more LSK cells in

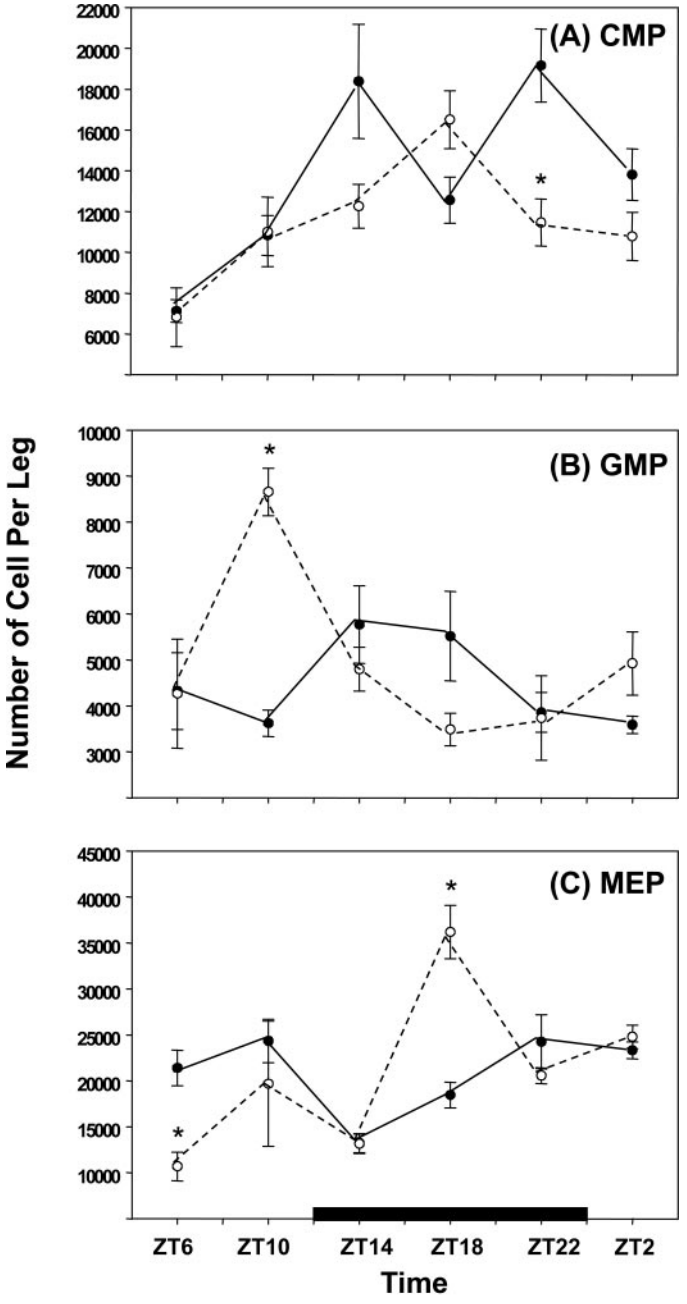

Fig. 2. TCDD disrupts the circadian rhythms of the numbers of antigenically defined myeloid progenitors. Bone marrow cell were isolated from control (●) and TCDD-treated (○) animals (n = 6 per group per time point) at ZT 6, 10, 14, 18, 22, and 2 on day 5 after treatment. Flow cytometry was performed as described under *Materials and Methods*. A, CMP; B, GMP; C, MEP. The data shown are representative of three separate experiments. Data are presented as mean values ± S.E. There was a treatment-dependent effect for CMP, GMP, and MEP (all ANOVA $p < 0.001$), and ∗ indicates statistically different ($p < 0.05$) from the vehicle control value at the same time point.

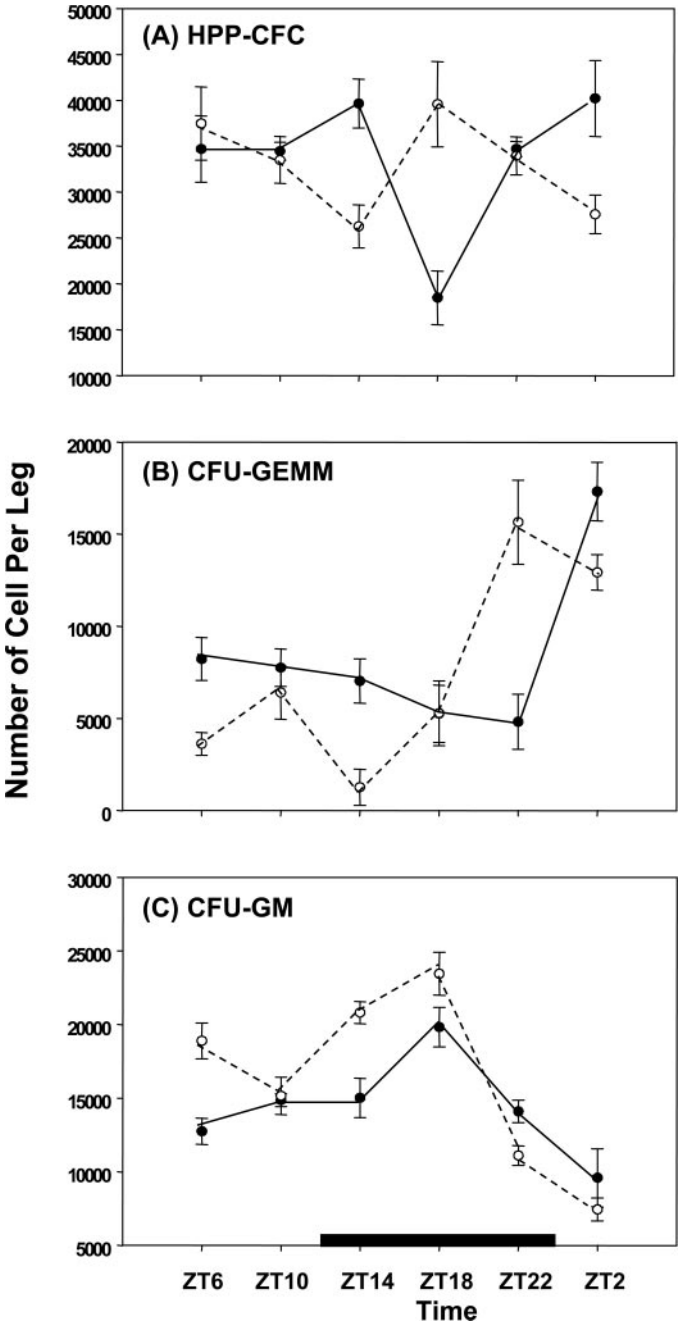

Fig. 3. TCDD disrupts the circadian rhythms of the numbers of functionally defined progenitors. Bone marrow cells were isolated from control (●) and TCDD-treated (○) mice (n = 6 per group per time point) at ZT 6, 10, 14, 18, 22, and 2 on day 5 after treatment. Colony-forming assays were performed as described under *Materials and Methods*. A, HPP-CFC; B, CFU-GEMM; C, CFU-GM. The data shown are representative of two separate experiments. Data are presented as mean values ± S.E. ANOVA p values were 0.14, 0.07, and 0.09 for HPP-CPC, CFU-GEMM, and CFU-GM, respectively.

TCDD-treated mice exit quiescence at ZT 22 such that the ratio of quiescent cells to actively cycling cells is at its lowest of anywhere on the two curves. Comparing the data specifically between ZT 18 and ZT 2 also suggests the possibility that the period of the rhythm has been compressed. Together, these data suggest that one possible mechanism by which TCDD-induced AhR activation alters the numbers and rhythms of primitive hematopoietic progenitors is the disruption of their movement in and out of quiescence. These data are also consistent with other studies from our lab showing increased BrdU incorporation into LSK marrow cells after TCDD treatment (A. Wyman, K. P. Singh, R. W. Garrett, and T. A. Gasiewicz, manuscript submitted).

TCDD Disrupts the Circadian Rhythm of Lin$^-$ Sca-1$^+$ Gene Expression. Circadian rhythms are controlled by tightly regulated molecular clocks, at the heart of which are several PAS transcription factors, including the murine Period homologues mPer1 and mPer2, that are structurally similar to the AhR and its dimerization partner Arnt (Zheng et al., 2001). These proteins act as important positive regulators of the circadian clock. A second putative AhR DNA response element has been identified upstream of several genes including mPer2 (Boutros et al., 2004). We therefore sought to determine whether exposure to TCDD could disrupt the expression of these genes in Lin$^-$ Sca-1$^+$ bone marrow cells. The data presented in Fig. 5 agree with those from previous studies demonstrating that mPer1 and mPer2 are expressed in murine marrow Lin$^-$ Sca-1$^+$ cells and that this expression is itself circadian in manner (Chen et al., 2000; Tsinkalovsky et al., 2005). Both mPer1 and mPer2 are characterized by rhythms that have maximal expression near ZT 14 and ZT 23, respectively. These patterns were disrupted by TCDD treatment (ANOVA $p < 0.05$) with peaks being truncated by nearly 50%. The cyclin-dependent kinase inhibitors p21 and p27 also have been demonstrated to be both TCDD-responsive and regulated with circadian rhythmicity (Enan et al., 1998; Bjarnason et al., 1999; Kolluri et al., 1999; Cheng et al., 2000). However, analysis of their mRNA expression over 24 h revealed only modest rhythmicity in Lin$^-$ Sca-1$^+$ cells and no changes with TCDD exposure (data not shown). Together, these data support previous findings that hematopoietic precursors express circadian-related genes. These data also indicate that this expression is susceptible to dysregulation by TCDD.

Finally, it has been demonstrated by others that both AhR mRNA and protein expression are circadian regulated in several other tissues including rat liver (Richardson et al., 1998; Huang et al., 2002). We sought to determine whether the AhR and Arnt are expressed with circadian rhythmicity in immature hematopoietic cells. Total RNA was isolated from Lin$^-$ Sca-1$^+$ cells collected over a 24-h period, and qRT-PCR was used to measure AhR and Arnt mRNA. Both have maximal expression occurring during subjective midday (near ZT 10) with minimal expression occurring during the hours surrounding the transition from dark to light (Fig. 6). These curves were not significantly modified after TCDD exposure (data not shown). The AhR repressor (AhRR) is a recently characterized TCDD-inducible member of the AhR

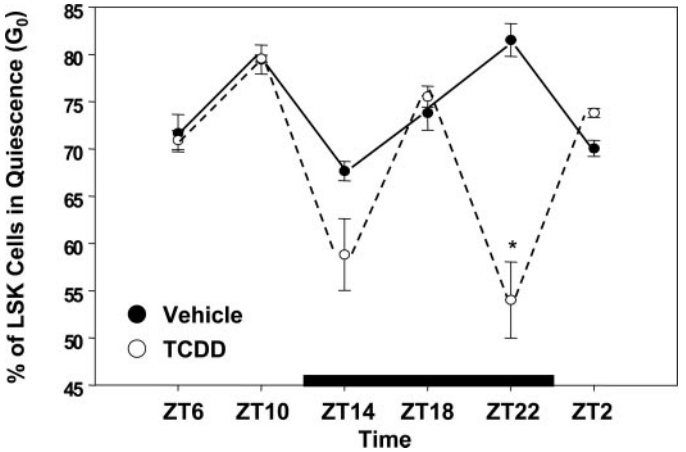

Fig. 4. TCDD disrupts the circadian rhythm of relative LSK bone marrow cell quiescence. Bone marrow cells were isolated from control and TCDD-treated mice ($n = 6$ per group per time point) at ZT 6, 10, 14, 18, 22, and 2 on day 5 after treatment. Cell cycle analysis was performed as described under *Materials and Methods*. The data presented are the percentage of total number of LSK cells in quiescence (G_0) ± S.E. and is representative of two separate experiments. There was a treatment-dependent difference for this pattern (ANOVA $p < 0.001$), and * indicates statistically different ($p < 0.05$) from the vehicle control value at the same time point.

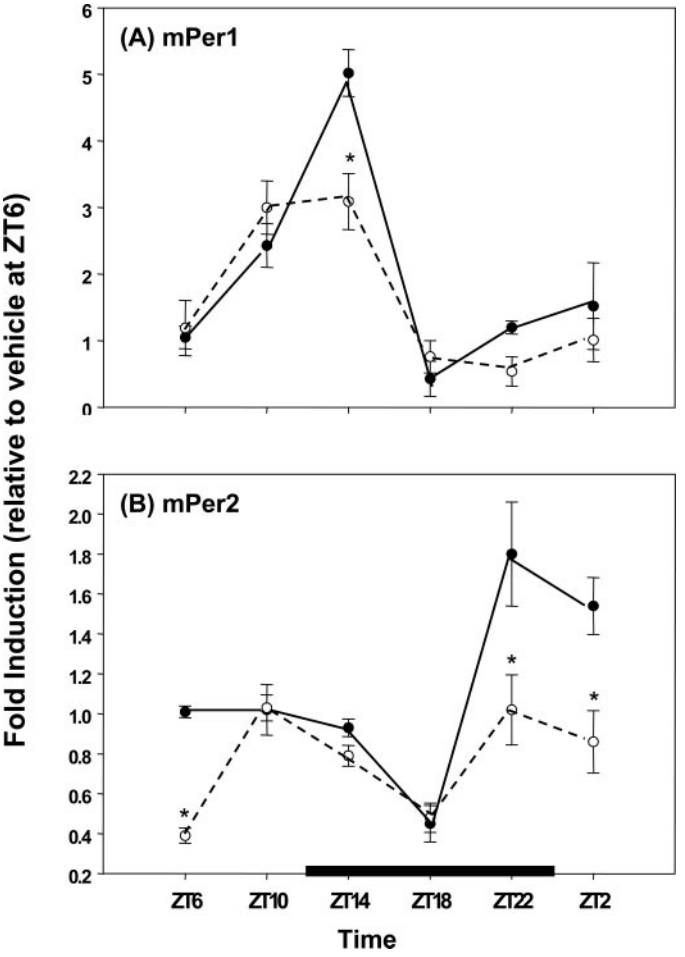

Fig. 5. TCDD disrupts the circadian rhythms of Per1/Per2 mRNA levels. Lin$^-$ Sca-1$^+$ bone marrow cells were isolated from control (●) and TCDD-treated (○) animals ($n = 6$ per group per time point) at ZT 6, 10, 14, 18, 22, and 2 at day 5 after treatment. Total RNA was isolated as described under *Materials and Methods*. Quantitative RT-PCR was performed using primer/probe sets specific to mPer1 (A) and mPer2 (B). Both mPer1 and mPer2 demonstrated treatment-dependent differences in the diurnal patterns (ANOVA p values were 0.049 and <0.001, respectively), and * indicates statistically different ($p < 0.05$) from the vehicle control value at the same time point.

signaling pathway that competes with the AhR for binding to both AhRE and Arnt and acts as a negative regulator of AhR-elicited transcriptional activity (Baba et al., 2001). TCDD exposure up-regulated AhRR in murine Lin$^-$ Sca-1$^+$ BM cells 20-fold in a time-of-day–dependent manner. In addition, although the pattern of AhRR mRNA expression is characterized by a single, but very modest, maximal time of expression near ZT 10 in control animals, TCDD-treated animals have an additional peak of high expression occurring approximately 12 h later (Fig. 7). Together, these data demonstrate that the members of the AhR signaling pathway are expressed in a circadian manner in murine hematopoietic precursors and that the expression of the AhRR is susceptible to TCDD-induced dysregulation.

Discussion

Processes regulating the numbers of hematopoietic stem and progenitor cells are under the control of circadian timing mechanisms (Aardal and Laerum, 1983; Stevold et al., 1988; Smaaland et al., 1992; Abrahamsen et al., 1998; Wood et al., 1998). As such, the number of murine hematopoietic progenitors can change as much as 2- to 3-fold during the course of a single day. Although these rhythms demonstrate a change in a specific antigenically or functionally defined cell type, it is important to understand that this probably does not represent changes in the number of actual cells. Instead, these daily cycles are likely to represent changes in the expression of HSC- and HPC-specific proteins, as well as the functional potential of those cells, two events that are not mutually exclusive. For example, rhythmic expression of cKit, the receptor for SCF, would be observed as oscillating numbers of Lin$^-$ Sca-1$^+$ cKit$^+$ cells. This daily plasticity for the complement of proteins expressed on the surface of HSC and HPC would in turn create a time-dependent in vivo functional potential. This would also affect their ability to respond to in vitro assay conditions that include SCF, creating measurable rhythms in the numbers of SCF-dependent colony-forming cells. Diurnal rhythms have been demonstrated for other growth factor receptors, such as the surface expression of interferon-α/β receptor on hepatocytes, lymphocytes, and implanted melanoma cells (Takane et al., 2001, 2002). The maximal expression of interferon-α/β receptor on tumor cells corresponded with an increase in the percentage of those cells in S-phase, indicating a relationship between cell cycle transit and specific growth factor signaling (Takane et al., 2001). The circulating levels of soluble receptors for tumor necrosis factor, IL-6, and IL-2 are all rhythmic (Haack et al., 2004). The responsiveness of murine myeloid progenitors to IL-3, GM-CSF, and G-CSF was also found to be circadian-dependent (Perpoint et al., 1995).

We have presented data that for the first time indicate circadian rhythms in the numbers of specific antigenically defined populations of murine hematopoietic progenitor cells. Thy-1^{lo} LSK cells, a population of cells enriched for murine HSC, as well as three populations of Lin$^-$ cKit$^+$ myeloid precursors, CMP, GMP, and MEP, all seemed to have diurnal rhythms in vehicle-treated C57BL/6 mice (Figs. 1 and 2). Data for MEP and GMP suggest rhythms that were antiphase to each other, supporting data that differentiation into the different lineages is separated in time (Wood et al., 1998). In theory, a measurable plasticity may exist between erythroid and myeloid progenitors, with a single cell changing surface levels of proteins such as FcR and CD34 so that it meets the criteria for both GMP and MEP at different times of the day. The antiphase nature of the curves for those two populations supports this notion (Fig. 2). This kind of temporal compartmentalization provides for unique periods of specific timing and duration when cellular and extracellular factors required for a specific differentiation program are likely to be up-regulated. Properly timed circadian rhythms are hypothesized to be energetically advantageous to the organism, allowing production of signaling molecules and receptors only when they are required. Five days after TCDD

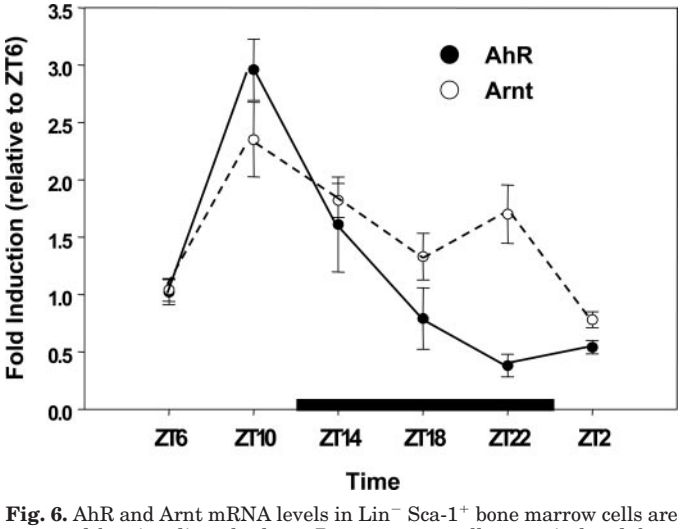

Fig. 6. AhR and Arnt mRNA levels in Lin$^-$ Sca-1$^+$ bone marrow cells are governed by circadian rhythms. Bone marrow cells were isolated from control mice (n = 6 per time point) at ZT 6, 10, 14, 18, 22, and 2 on day 5 after treatment of animals with vehicle (olive oil). Total RNA was isolated as described under *Materials and Methods*. Quantitative RT-PCR was performed using primer/probe sets specific to AhR (●) and Arnt (○).

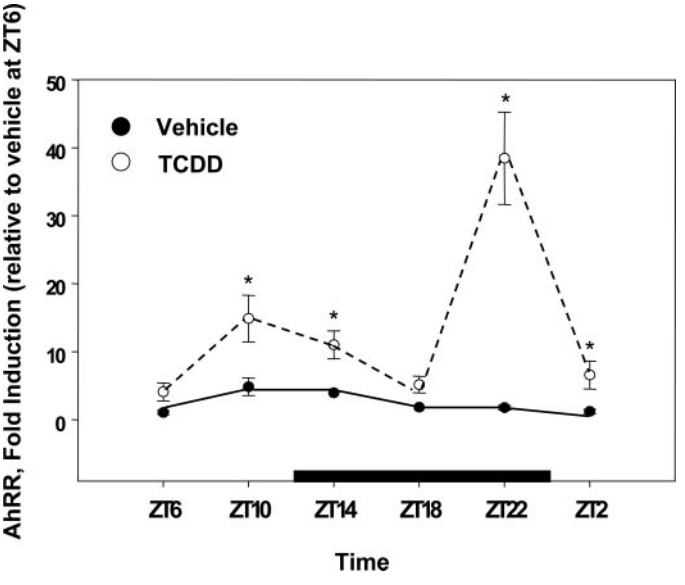

Fig. 7. TCDD up-regulates AhRR mRNA in Lin$^-$ Sca-1$^+$ bone marrow cells. Bone marrow cells were isolated from control and TCDD-treated mice (n = 6 per group per time point) at ZT 6, 10, 14, 18, 22, and 2 on day 5 after treatment. Total RNA was isolated as described under *Materials and Methods*. Quantitative RT-PCR was performed using primer/probe sets specific to AhRR. There was a treatment-dependent difference in this pattern (ANOVA $p < 0.001$), and $*$ indicates statistically different ($p < 0.05$) from the vehicle control value at the same time point.

exposure there seemed to be a shift in the timing of the GMP and MEP rhythms; although those two specific curves remained antiphase to one another, adding support to our hypothesis that these are indeed the same cells. Although the absolute minimum numbers of these two populations do not change after TCDD treatment, the maximal values increase roughly 2-fold (Fig. 2). This increase in the number of myeloid-erythroid progenitors does not necessarily precede increased hematopoietic potential. It is likely that in control bone marrow, the expression of progenitor-specific markers is timed to correspond with extracellular signals required for further differentiation. TCDD may decouple these two sets of events, and depending on whether those signals are positive or negative regulators, the increased numbers of GMP and MEP can be characterized by more, less, or unchanged function. It is therefore critical that future experiments investigate the direct consequences of shifted circadian rhythms to the outcome of hematopoietic progenitors (i.e., their ability to differentiate into more committed lineage cells). In this regard, it is noteworthy that other data suggest a TCDD-elicited shift toward the granulocyte/macrophage lineage at the expense of lymphoid and erythroid lineages in murine bone marrow (A. Wyman, K. P. Singh, R. W. Garrett, and T. A. Gasiewicz, manuscript submitted). This is consistent with published data showing a decrease in the production of immature B and T cells in marrow after TCDD exposure (Fine et al., 1990; Thurmond and Gasiewicz, 2000), but increased numbers of $Lin^-/Sca\text{-}1^+/cKit^+$ stem/progenitor cells as indicated in Fig. 1. At present, whether or how modulated hematopoietic rhythms may be related to these outcomes remains unknown.

In addition to numbers of specific cells, the apparent function and proliferation of HSC and HPC is also temporally dependent. The cell cycle of both primitive hematopoietic progenitors and other cell types progresses with a 24-h period and is linked to the coexpression of circadian-associated genes. It is noteworthy that the ability of transplanted murine bone marrow to engraft is influenced by circadian time (D'Hondt et al., 2004). Thus, cell cycling and functional potential are probably linked. Consistent with this, investigators have shown that HSC isolated during the active phases of the cell cycle are less able to home to bone marrow and spleen (Jetmore et al., 2002). It is therefore not surprising that the rhythm of S-phase HSC and HSC engraftment are antiphase to each other (D'Hondt et al., 2004) and that the number of CFU-spleen are measured with a circadian period (Stevold and Laerum, 1988). We have shown a TCDD-elicited disruption in the quiescence of LSK cells (Fig. 4). These data are important for several reasons. First, the regulation of HSC division is tightly regulated, with the majority (70–85%) of cells quiescent under normal 'resting' conditions; a phenomenon that is critical for the maintenance of hematopoietic potential throughout the lifespan of the organism. Second, this alteration in the functional composition of the bone marrow may explain reported data indicating that TCDD, although increasing their number, diminished the ability of murine $LSK/CD34^-$ cells (functionally defined as long-term reconstituting HSCs) to reconstitute an irradiated recipient mouse (Sakai et al., 2003). Again, this result may not be surprising if TCDD treatment increases the cycling of HSCs and actively cycling cells are less able to home to bone marrow (Jetmore et al., 2002).

The proteins mPer1 and mPer2 are important positive arms of the circadian clock. Targeted mutation of their genes results in abnormal circadian rhythms (Zheng et al., 2001). However, because the expression of *mPer2* normally increases and decreases daily, measuring the effect of TCDD at just one time point can lead to a misinterpretation of the data. When mPer1 and BMAL1 mRNA expression were monitored in the hypothalamus from TCDD-treated mice at multiple time points over 24 h, it was found that the overall rhythm was phase-shifted (Miller et al., 1999). Rather than a change in the phase of their rhythms, we observed changes in the magnitude of both mPer1 and mPer2 mRNA in Lin^- $Sca\text{-}1^+$ BM cells exposed in vivo to TCDD (Fig. 5). In particular, their respective maximal expression were reduced by roughly 50%. Although it is unclear what effect this reduction would have on these cells, it is plausible that this transient modulation in mPer mRNA is significant enough to disrupt normal cellular function. In addition, although TCDD has been identified as a tumor promoter, no current data provide a convincing mechanism by which this might happen. It is noteworthy that mPer mutant mice are more susceptible to γ-irradiation induced tumorigenesis (Fu et al., 2002). The incidence of lymphoma and tumors in several tissues was nearly 15-fold higher in *mPer2* mutant animals, leading to early mortality. In addition, a chromosomal translocation fusing ETV6 with the *hPer1* gene is associated with acute myeloid leukemia (Penas et al., 2003). It is noteworthy that TCDD exposure in humans has been associated with increased incidence of lymphoma and leukemia (IARC, 1997). It would be important to investigate further the specific effects of AhR-mediated dysregulation of *mPer* on murine hematopoietic progenitors.

Although we and others have referred to the action of TCDD on the AhR as activation, evidence suggests this may not always be functionally the case. When many types of cells are exposed to TCDD, both in vitro and in vivo, there is a rapid and sustained proteosome-mediated degradation of AhR protein (reviewed in Pollenz, 2002). In many cases, a single dose of TCDD is sufficient to reduce AhR protein in vivo to 10 to 30% of control animals in multiple tissues for up to 14 days (Pollenz et al., 1998). However, despite this decrease in the level of AhR, CYP1A1 and/or CYP1B1 increase with TCDD exposure, indicating some continued AhR function or the presence of 'spare' AhR protein. Thus, TCDD may cause a rapid and sustained reduction in AhR protein while directing the remaining active AhR to particular TCDD-inducible genes, including the AhRR (Baba et al., 2001), adding an additional level of negative regulation. The possible nullification of endogenous AhR activity in HSC/HPC by TCDD is important when considering the following points. First, a putative second AhRE has been identified upstream of the *mPer2* gene (Boutros et al., 2004). Second, we observed a time-of-day–dependent elevated number of LSK cells in AhR-null allele mice (R. W. Garrett and T. A. Gasiewicz, unpublished observations) of a magnitude similar to the increase after TCDD exposure. Third, we observed that TCDD treatment substantially induces AhRR in Lin^- $Sca\text{-}1^+$ marrow cells (Fig. 7). Thus, a plausible and testable explanation for some of the present data are that TCDD is inducing, either through the ability to elicit down-regulation of AhR protein and/or increased AhRR expression, an AhR-null–like state for signaling pathways regulating HSC/HPC function.

Future experiments will investigate the mechanisms by which TCDD might affect distinct AhR functions in murine LSK cells.

In summary, this article describes a novel effect of AhR activation on immature murine hematopoietic progenitors. Significant changes in the circadian rhythms associated with both the number of antigenically and functionally defined cell populations, as well as their movement into and out of quiescence, were detected. These disruptions include changes in phase, period, and magnitude and may be related in part to altered expression of mPer1 and mPer2. Experiments are planned to delineate those mechanisms responsible for these changes, including establishing the relationship between mPer expression and signaling through the AhR. In addition, experiments will be designed to gain an understanding of the consequences of disrupted circadian rhythms on both normal and leukemic hematopoiesis. Finally, these data emphasize the need to evaluate daily cycles when determining effects of AhR agonists, therapeutic agents or other chemicals, on bone marrow functions.

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Address correspondence to: Dr. Thomas A. Gasiewicz, Department of Environmental Medicine, University of Rochester Medical Center, 601 Elmwood Ave., Box EHSC, Rochester, NY 14642. E-mail: tom_gasiewicz@urmc.rochester.edu