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# The Ah Receptor in Stem Cell Cycling, Regulation and Quiescence

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## Abstract

Processes that regulate quiescence, self-renewal, and senescence of hematopoietic stem cells (HSCs) are not well understood. Due in part to the ability of xenobiotic ligands to have persistent effects on the immune system in experimental animals, there has been much work to define a physiological role of the aryl hydrocarbon receptor (AhR) and relationships to human disease. Persistent AhR activation by dioxin, a potent agonist, results in altered numbers and function of HSCs in mice. HSCs from AhR null-allele (KO) mice are hyperproliferative and have altered cell cycle. Aging KO mice show characteristics consistent with premature bone marrow exhaustion. We propose that the increased proliferation of HSCs lacking AhR expression or activity is a result of loss of quiescence, and as such, AhR normally acts as a negative regulator to curb excessive or unnecessary proliferation. Similarly, prolonged and/or inappropriate stimulation of AhR activity may compromise the ability of HSCs to sense environmental signals that allow these cells to balance quiescence, proliferation, migration, and differentiation. These data, and others, support a hypothesis that deregulation of AhR function has an important role in HSC regulation and in the etiology and/or progression of certain hematopoietic diseases, many of which are associated with aging.

### Keywords

Ah receptor; hematopoietic stem cells; quiescence

## Introduction: Ah Receptor (AhR) Biology and Immunity

The AhR is a basic helix-loop-helix transcription factor originally identified as mediating the toxicity of and adaptive response to a large group of xenobiotics including the dioxins and planar polychlorinated biphenyls (PCBs). Since that time, a large and diverse number of AhR ligands have been identified, some of which are naturally occurring as well as therapeutic compounds. Due in part to the ability of many of these ligands, especially the xenobiotics, to have potent and persistent effects on the immune system in experimental animals models, there has been much work to define a normal function of this protein and

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possible relationships to human disease. Indeed, many investigations over the past 10 years have implicated a role of the AhR at several levels in the immune response.[-] Furthermore, alteration of AhR activity has been associated with immune-related diseases as well as potential therapeutic activity.[-] Nevertheless, the cellular and molecular mechanisms that are responsible for these diverse responses are not well understood. Despite, and perhaps because of, the multidimensional immunomodulation activity of the AhR, further understanding of the AhR-regulated signaling pathways that may control immunity has great potential to provide us with new opportunities for diagnosis, prevention, and therapeutic targets.

## Hematopoietic Stem Cells and AhR in Hematopoietic Disease and Dysfunction

Hematopoietic stem cells (HSCs) are the source for the continuous replacement of 'worn out' differentiated cells in the immune system. Under normal homeostatic conditions, HSCs are mostly in a quiescent state so as to prevent premature exhaustion and their susceptibility to gene damage. Under conditions of stress, disease, tissue damage or pathogen exposure, HSCs can undergo extensive self-renewal, expansion and differentiation to mature lineage cells that fight infection and promote tissue repair. The appropriate balance between quiescence and proliferation of HSCs is an important aspect of their biology that allows for the preservation of the capability for long-term multi-lineage generation over the lifetime of an organism. Alterations in this balance may result in premature HSC exhaustion and subsequent bone marrow failure, or lead to senescence or malignant transformation (Figure 1).[-] It is known that age-related changes in this balance take place, and, as such, it is not unexpected that immune-related disease processes occur at a greater frequency with age. Loss of immune function and increased incidence of certain leukemias and other myelodysplastic syndromes are, in fact, some of the most clinically significant consequences of aging.[,] Age-related altered immune function also limits the success of therapies used to treat cancer and other disorders, and this further limits the life expectancy as well as quality of life in our aging population. As such, defining the regulatory networks and mechanisms that balance quiescence, self-renewal, differentiation and exhaustion and/or senescence of HSCs are crucially important in identifying biomarkers for early diagnosis as well as therapeutic targets to control and treat these diseases.

In terms of hematopoietic disease, human exposure to the potent xenobiotic AhR agonists 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; dioxin) or the planar PCBs has been reported to be associated with increased incidence of several hematopoietic disorders including lymphoma and leukemia.[,,] AhR-mediated pathways regulating miRNAs have been suggested to be involved in acute myeloid leukemia, and the *Ahr* gene promoter was found to be hypermethylated in human acute lymphoblastic leukemia (ALL) cells. The exposure of mice to TCDD and prolonged activation of the AhR results in several changes in HSCs including altered circadian rhythms and expression of clock genes, as well as altered numbers and phenotypic characteristics of HSC and progenitor populations,[-] Although many studies have shown that altered phenotypic characteristics of HSCs do not always reflect altered function, TCDD exposure was also shown to inhibit the functional characteristics of HSCs as assessed

by their ability to engraft into irradiated mice.[9] Furthermore, studies using chimeric animals indicated that the alterations in both phenotypic and function were dependent on the presence of the AhR in hematopoietic cells but not in the stromal niche.[9] Although providing evidence that is indirect, together these data are consistent with the hypothesis that the AhR has a role in the regulation of HSCs and that the alteration of this function may be either lead to and/or be associated with hematopoietic disease and dysfunction.

## Cellular and Molecular Actions of TCDD in HSCs

Given the data suggesting the importance of the AhR in immunoregulation, and especially initial studies with TCDD indicated above, we engaged in studies to more critically define the mechanisms by which TCDD and AhR activation alter HSC activity. Further analysis of phenotypically-defined stem cell populations in bone marrow, showed that TCDD treatment to mice increased the relative percentages of cell subpopulations $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$(LSK)$CD48^{-}CD150^{-}$ and LSK$CD48^{-}CD150^{+}$ that have been correlated with short-term HSCs and multipotent progenitors and long-term HSCs, respectively. To address the apparent paradox that increased percentages of phenotypically-defined HSCs occurred with an apparent decreased functional ability of these cells to generate progeny,[9] we used a limiting dilution analysis to determine if TCDD affected the numbers of functional HSCs. Using this approach, it was determined that TCDD did not significantly affect the absolute proportions of functional long-term and short-term HSCs. Furthermore, the ability of LSK cells from TCDD-treated mice to undergo expansion and differentiation under *ex vivo* conditions was not different from cells taken from vehicle-treated animals. These data suggested that TCDD was affecting different steps in the engraftment process, e.g. migration to bone marrow, retention of these HSCs in vascular niches, and/or lineage differentiation. In order to address possible effects of TCDD on trafficking behavior of transplanted cells, we assessed the *in vivo* accumulation of cells in marrow after 24 h of transplantation, and, as a complementary study, measured the directional migration of TCDD-treated LSK cells through a semipermeable membrane to a chamber containing the chemokine CXCL12. Both studies demonstrated that TCDD-treatment decreases the migration and trafficking of phenotypically-defined HSCs. Thus, the observed decreased engraftment of cells from TCDD-treated mice is likely due to decreased migration rather than to decreased numbers of functional HSCs. A modified ability of HSCs and progenitors to move to and/or within the marrow niche may also contribute to the observed effects of TCDD on decreased thymic seeding and altered B cell numbers.[9]

Since the AhR is a transcription factor, we hypothesized that the phenotypic and cellular changes observed following TCDD exposure were consequences of AhR-mediated changes in gene expression. Microarray analyses of LSK cells from mice treated with TCDD were consistent with the phenotypic and functional changes observed. Significant gene changes occurred in pathways involved in antigen presentation, cell-to-cell signaling and interaction, cellular movement, hematological system development and function, and, in particular, immune cell trafficking. Among these, the most significant alterations occurred in genes expressing scinderin and metalloproteinase 8. Scinderin is a key regulator of chemotactic responses to CXCL12, and metalloproteinases are involved in the stability and function of cell surface proteins directing cell migration. Some of the other transcripts changed in the

data sets (e.g. *Fos*, *JunB*, *Ptgs2*, and *Egr1*) encode transcriptional regulators, and it seems likely that some of the functional changes are secondary to initial AhR signaling. Thus, although these gene changes are consistent with the functional changes observed, they do suggest a complex crosstalk of the AhR with several pathways associated with the ability of HSCs to sense their microenvironment and control their trafficking behavior. Notably, an Ingenuity Pathway Analysis of these data sets also indicated a significant association with 'cancer' in the hematopoietic system, suggesting that disruptions in these pathways may promote hematological disease. This also implies a plausible (and testable) molecular basis for functional changes in HSCs that could lead to the observed increased incidence of lymphoma and leukemias in several populations exposed to xenobiotic AhR ligands.

## Loss of AhR Results in HSCs with Abnormal Characteristics and Functions

To better understand the physiological role of the AhR, *Ahr*-null allele (AhR-KO) mice have been produced by several investigators.[9] These animals have abnormal vascular development and develop other lesions in several tissues, some of which only become manifest with aging. The immune system also develops abnormally, with decreased mass and cellularity of liver, spleen and lymph nodes at one to two weeks of age.[9–11] Although some of these alterations resolve by three weeks, these mice exhibit and earlier onset of spontaneous neoplasms including lymphomas. Nevertheless, up until just recently there has been only a limited characterization of the immune system, especially of HSCs, in these animals.

Further examination of the hematopoietic system in AhR-KO mice revealed several changes consistent with significant alterations in the HSC/progenitor cell populations. Young adult animals had enlarged spleens with increased B220+ and Mac-1+ populations. There was also a near doubling in the number of white cells in peripheral blood that was mainly due to increased numbers of lymphocytes, but a relative decrease in red blood cells. The total number of bone marrow cells, lineage cells, and numbers of phenotypically-defined HSCs/progenitor populations as well as functional HSCs, defined by competitive repopulation into irradiated mice, were also increased in KO mice. The most striking finding was that HSCs from young adult KO mice have inherently high rates of cell division as determined by BrdU incorporation, cell cycle analysis, and expansion under *ex vivo* conditions. That the splenomegaly in KO mice is transplantable by KO HSC/progenitor cells into wild-type mice, also indicates the dysfunctionality of these cells, and further suggests that the hematopoietic phenotype in these animals is a function of the increased cycling of HSCs with an increased throughput of lineage cells.

Together, these data support a contention that the AhR functions directly in HSCs to regulate their balance between quiescence and proliferation. In bone marrow, this could occur through a loss of a proliferation block and/or increased sensitivity to proliferative signals. The hypothesis is also consistent with a previous postulate, based on the finding that the *Ahr* promoter is silenced by hypermethylation in human acute lymphoblastic leukemia, that the AhR is a cell-specific negative regulator of proliferation. This role of the receptor is also supported by the finding that AhR antagonists promote the expansion of human HSCs, and

that the *Ahr* gene is expressed during periods of quiescence but turned off during the proliferation and self-renewal of these cells.

## Loss of AhR Results in Premature Hematopoietic Stem Cell Exhaustion and Development of a Myeloproliferative Disorder in Aging Mice

Based on the above findings, we further hypothesized that that prolonged loss of AhR and a lifetime of excessive stem cell cycling (i.e. loss in quiescence), would result in sensitivity to stress conditions, premature exhaustion of HSCs, and hematopoietic disease. Indeed, the analyses of aging AhR-KO mice up to two years of age supported this hypothesis. Aging AhR-KO mice showed decreased survival, splenomegaly, increased circulating white cells, and hematopoietic cell accumulation in tissues. Although, like younger KO mice, aging mice showed an increased number of phenotypically-defined stem/progenitor cells in bone marrow, there was a significant decrease in self-renewal capacity as determined by competitive repopulation and serial transplantation. HSCs also showed increased levels of reactive oxygen species (ROS), increased staining for γ-H2A.X (an indicator of DNA damage), but decreased $p16^{INK4a}$. The latter observation was of particular interest given that the expression of $p16^{INK4a}$ is increased in most cells with aging, and is believed to confer protection against mutations by inducing senescence. However, a recent report also indicated a decreased expression of p16 with age in acute myeloid leukemic cells, suggesting that this loss of protection may facilitate oncogenesis.

These data in aging mice are further consistent with the hypothesis that AhR has some role in regulating the balance between quiescence and proliferation. A prolonged lack of AhR expression and/or activity allows HSCs/progenitors to escape quiescence, and a lifetime of cycling ultimately results in premature stem cell aging and exhaustion. All of these events increase the risk of DNA damage from ROS and other stressors that may also result in hematopoietic disease. Other studies have also shown that loss of quiescence can lead to HSC engraftment defects, HSC exhaustion, and eventually to myeloproliferative disease and leukemia. A previous report indicated an earlier onset of neoplasms, including lymphomas, in AhR-KO mice.

## Gene Changes in AhR-KO HSCs are Consistent with the Phenotype

To determine gene changes in AhR-KO HSCs that may lead to excessive cycling and premature exhaustion, we assessed the global gene expression profile in HSCs ($LSKCD34^{-}CD48^{-}CD150^{+}$) cells from young adult KO and wild-type mice. The most significant gene changes (increased expression of *Srpk2*, *Mir170*, *Creb1*, *Hes1*, *mTOR*, *Rad50*, *Pdp1*, but decreased expression of *Stra13*) observed in KO mice have also been associated with HSC hyperproliferation, leukemia, and accelerated aging, and are consistent with the phenotype observed in these animals. Pathway analyses also indicated genes changes enriched for signaling pathways associated with oxidative stress, acute myelogenous leukemia, and aging. Notably, there was also an enrichment for genes possessing putative AhR response elements.

## Summary: A Role of the AhR in HSCs

Together, these studies and others support a critical role of the AhR in signaling pathways controlling the balance between HSC quiescence and proliferation. It seems likely that this occurs through both a direct regulation of gene expression and a complex cross talk of the AhR with other factors. Notably, a very recent study identified the AhR as one of several transcription factors, in addition to Egr1, Sox4, and Stat1, playing critical roles in HSC regulation and function. It is tempting to speculate further that the dysregulation of AhR function, and thus disruption of the quiescence-proliferation balance, has an important role in the etiology and progression of hematopoietic disease. While this has been suggested for humans exposed to particular xenobiotic AhR ligands,[9] the potential that genetic and/or epigenetic changes could lead to this dysregulation has not been previously recognized.

Clearly more work is needed to define these relationships in terms of the signaling pathways directly regulated by the AhR that define HSC function or phenotype, the endogenous ligands which regulate AhR activity within the marrow compartment, and how expression of the AhR is itself regulated. It is of interest that kynurenine, which has immune regulatory activity, has been found to be a potent AhR ligand.[9] It is also noteworthy, that expression of the AhR appears to be differentially expressed in quiescent versus proliferating HSCs, again suggesting a direct involvement of this transcription factor in regulating this balance. Finally, it is essential to consider and dissect the possible, indeed likely, role of stromal elements and the marrow niche in the regulation of the AhR either through the synthesis of endogenous AhR ligands and/or other factors that regulate AhR expression or its activity. The definition of the latter may be challenging especially if one of the roles of the AhR is to regulate how HSCs ‘sense’ the marrow microenvironment.

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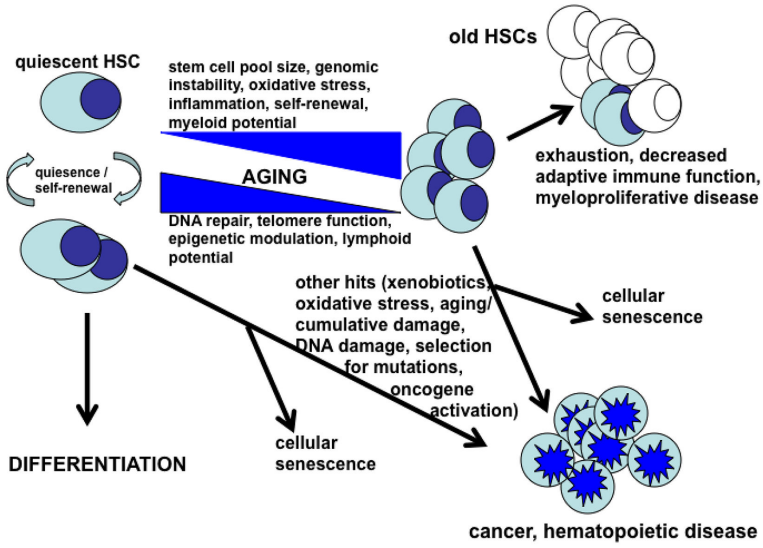


**Figure 1.**

A simple proposed model of relationships among stem cell quiescence, self-renewal and differentiation, exhaustion, senescence and development of disease. Modified from Jacob and Osato, and van Zant and Liang.