

IMPACT OF GESTATIONAL EXPOSURE TO
2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN
ON T LYMPHOCYTE DEVELOPMENT

by

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ABSTRACT

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Under the Supervision of Professor Michael D. Laiosa

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a ubiquitous environmental contaminant and the best characterized agonist of the arylhydrocarbon receptor (AHR), a transcription factor crucial to the detoxification of numerous xenobiotics. Studies in animals show that TCDD is immunosuppressive in adult exposures, and epidemiological studies have found an association between TCDD exposure and hematologic cancers. Additionally, developmental exposure to TCDD has been shown to increase the likelihood of autoimmunity and to impair immune response to later-life infections. The cells of the immune system are all descended from multipotent hematopoietic stem cells (HSCs) that originate in the fetus. This multipotency, defined as the ability to develop into several different cell types, suggests that any impact on HSCs potentially affects all cells arising from the original HSCs throughout the life course. During particularly sensitive timepoints such as puberty and pregnancy, alterations initiated by early life exposures can be triggered to cause immune disorder by shifts in hormone

levels, stress, or other endogenous means. The effect that developmental exposures have on later life immune disease is not completely understood, but epidemiological statistics suggest that the rise in immunological disease is associated with a changing environment, whether due to chemical, microbial, or as-yet-unknown contributing factors. We investigated the effects of developmental AHR activation on later-life immune outcomes and used a mouse model to examine T lymphocyte development following gestational exposure to environmentally relevant levels of TCDD. We found that developmental exposure to TCDD significantly decreased the lymphocyte differentiation potential of HSCs, and that this effect occurs through the fetal AHR. We also found that AHR signaling is involved in crosstalk with the Notch1 gene—a gene which has been linked to over half of the cases of T cell acute lymphoblastic leukemia. These studies support the role of developmental exposure to environmental contaminants in the development of immune disease and may lead to multiple public health focused interventions such as development of treatments, implementation of preventative measures, and changes in environmental policy.

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Dedication

In memory of my father,

Richard W. Kangas

(1940-2003)

Seldom a day goes past when I don't think of something that I wish I could share with my dad about my life—and often my research. I know that my natural curiosity and attraction to Science came from all that he taught my brother and me.

I strongly believe that things happen for a reason and that patterns emerge when we least expect them. It's really no surprise, then, that I joined a brand new program without any firm direction or research focus, but I landed in a laboratory that studies dioxin and immune system effects.

All my research has had a very personal motivation, due to my father's death from bone marrow cancer—following exposures when, as a Captain in the US Air Force, he flew transport in Vietnam.

I only wish he could be here to hear about our discoveries.

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LIST OF ABBREVIATIONS

1. 2.4G2 Monoclonal antibody specific to the extracellular domains of Fc γ RII and Fc γ RIII
2. AHR Aryl hydrocarbon receptor
3. AHRE Aryl hydrocarbon response element
4. APC Allophycocyanin
5. ARNT AHR nuclear translocator
6. bHLH basic Helix Loop Helix
7. CD Cluster designation
8. c-Kit Stem cell growth factor receptor; CD117
9. CSL CBF1, Suppressor of Hairless, Lag-1
10. Cy Cyanine
11. DL1 Delta-like 1
12. DN Double negative thymocyte
13. DOHaD Developmental Origins of Health and Disease
14. DP Double positive thymocyte
15. ELDA Extreme limiting dilution analysis
16. FITC Fluorescein isothiocyanate
17. FTOC Fetal thymic organ culture
18. GD Gestational day
19. HES Hairy/Enhancer of Split
20. HSC Hematopoietic stem cell
21. HZ Heterozygous genotype (+/-)

22. IACUC	Institutional Animal Care and Use Committee
23. ICN	Intracellular domain of Notch
24. KO	Knockout or null genotype (-/-)
25. LSK	Lineage negative, Sca-1 positive, c-Kit positive cells
26. MEM	Minimum essential media
27. MHC	Major histocompatibility complex
28. PAH	Polycyclic aromatic hydrocarbon
29. PAS	Period, AHR and ARNT, Single minded homology domain
30. PBS	Phosphate buffered saline
31. PCB	Polychlorinated biphenyl
32. PCR	Polymerase chain reaction
33. PE	Phycoerythrin
34. PerCP	Peridinin chlorophyll protein
35. PPD	Post parturition day
36. qPCR	Quantitative polymerase chain reaction; real-time PCR
37. Sca-1	Stem cell antigen-1
38. SEM	Standard error of the mean
39. SP	Single positive T cell
40. TCDD	2,3,7,8-Tetrachlordibenzo- <i>p</i> -dioxin
41. TCR	T cell receptor
42. WT	Wild type genotype (+/+) (C57BL/6 mice)

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Though I know that my greatest accomplishment will always be the role I played in raising my three beautiful children, and that nothing will ever compare to the twelve fabulous years where my only job was to be their mother, about six years ago I decided that I wanted to do something else, too. I knew I wanted to go back to school, and coincidentally, Mira's start in full day school coincided with admission of the first students to what would become the Joseph J. Zilber School of Public Health. I am so appreciative of the original faculty and staff who took a chance and admitted a student 17 years out of undergrad and to everyone from Zilber since then who has made this wild ride successful for me.

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At the risk of being teased, I want to extend a HUGE “thank you” to my Facebook friends—too plentiful to name individually. Over the last 5 ½ years, you have stuck with me through the minutiae of my Grad School life. You’ve rejoiced at my successes, comforted me through my failures, helped me troubleshoot MS WORD and computer issues, and most of all provided a social life when I had no other.

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Finally, the most important acknowledgement is to my husband and best friend, Greg Ahrenhoerster. There is literally no way to put into words my thanks for all that you have done for me through this entire journey. From the decision to go back to school and prepping for the GREs, through exams and presentations, despite having to miss out on conference trips to warm, sunny places while you held down the home, and your proofreading of so many of my papers that you really *do* deserve Continuing Ed. credits in Developmental Immunotoxicology. Thank you for cooking, cleaning, comforting... You have always told me that I could do it and you have been there, never saying “I told you so,” but being proud when I succeeded. You are my inspiration, my motivation, my ultimate love. Thank you.

“Knowing how to think empowers you far beyond those
who know only what to think.”

— Neil deGrasse Tyson

CHAPTER 1

GENERAL INTRODUCTION

I. Introduction

Disease development is multi-faceted, but increasingly it has become apparent that early life exposures—including those while still *in utero*—can have profound effects on later life disease progression. This concept, the Developmental Origins of Health and Disease (DOHaD), provides a potential explanation for the recent increase in hematological based diseases such as cancer, immune suppression, allergy, and autoimmunity. Common intrauterine exposures are nutritional or hormonal, but numerous other exposures are also known to impact the fetus's development. For example, stress during pregnancy has not only been linked to poor birth outcomes, which can lead to a host of health issues, but it can also result in later-life disease in offspring. Additionally, health can be affected by other factors such as parity or gender ratio in multiple births. It is now known that chemical and microbial environmental exposures during pregnancy can impact birth outcomes and fetal health. Many environmental contaminants exert their toxicity in adults through the arylhydrocarbon receptor (AHR). Given the increase in incidence of hematological based diseases, we wanted to determine whether developmental exposure to AHR agonists such as 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD) affects hematopoietic stem cells, the process of hematopoiesis, and later life hematological processes.

This research focuses on developmental exposure to the ubiquitous environmental contaminant TCDD, but many additional intrauterine effects can also impact the development of disease. While the studies described involve

responses in the immune system following hematopoietic stem cell (HSC) exposures, stem cells of other lineages might be similarly altered and tissues formed from those stem cells might also contribute to disease later in life. This effect is particularly important when applied to vulnerable populations who may be starting life already at a disadvantage in terms of nutrition or genetics.

Understanding the contribution of developmental influences to later life health is a Public Health priority. Not only is there a need for disease cures, but also means of disease prevention. Furthermore, better understanding of the connection between contaminants and diseases could inform future environmental regulation policies.

II. Human Development and the Life Course

In 1991, a connection was made between insufficient gestational growth due to inadequate maternal nutrition and later-life development of coronary heart disease (Barker, 2007, 1991). Since that discovery over two decades ago by the physician David Barker, what is now known as the Barker hypothesis or DOHaD has been applied to additional health effects including type 2 diabetes and cardiovascular disease (Power et al., 2013), neurodevelopment (Monk et al., 2013), reproductive function (Dupont et al., 2012), and immunological disease (Dietert, 2011, 2009; Faulk and Dolinoy, 2011). While Dr. Barker originally made his connections based on nutrition during pregnancy, other environmental exposures, including medications and toxic chemicals, can alter physiology during gestational development, particularly at sensitive timepoints (Fig. 1),

resulting in later life disease (Barouki et al., 2007; Susiarjo et al., 2013). Many immune diseases do not manifest until adulthood, making connection to developmental exposures complicated. However, associations are being discovered, and a recent study has connected prenatal exposure to several persistent environmental contaminants with an increased chance of developing asthma twenty years later—the longest range study of its type done to date (S. Hansen et al., 2013).

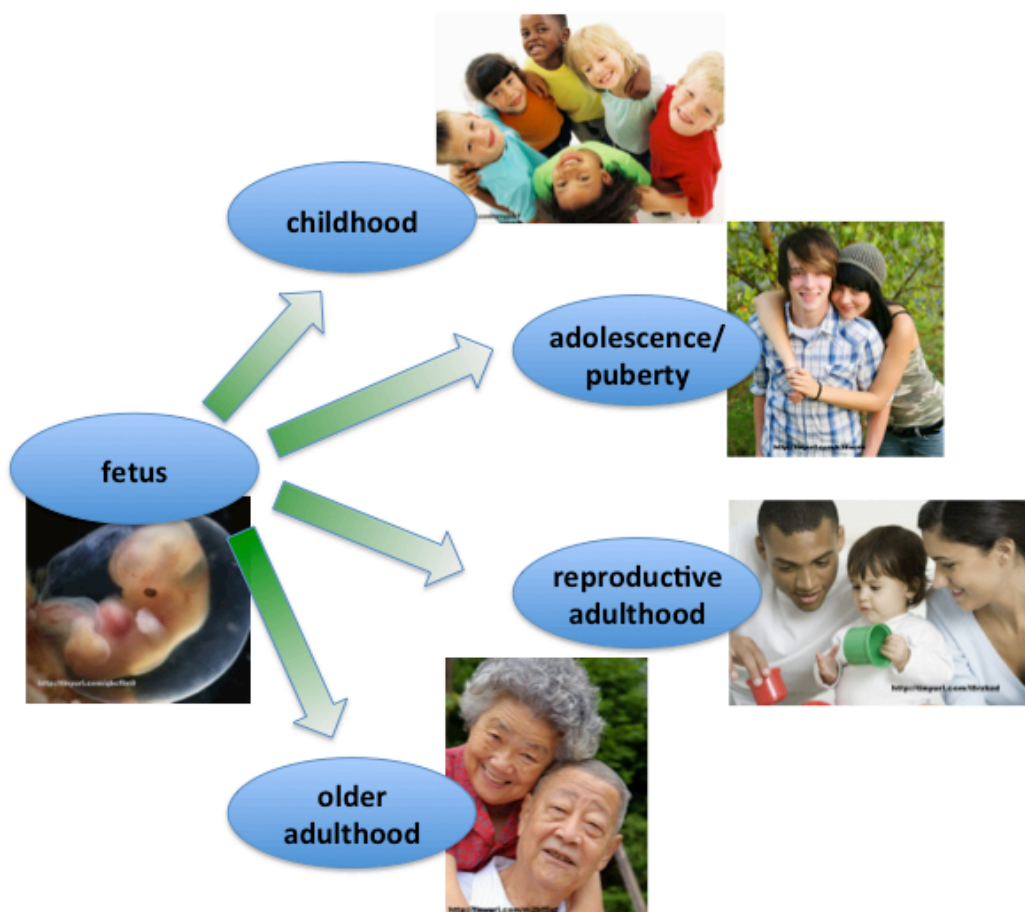


Figure 1. Sensitive timepoints exist in the life course. Experiences and exposures occurring during gestational development may impact health at later sensitive times of development, including childhood, puberty, during the childbearing years, or later.

A. Epidemiology of Immune Disease

The incidence of immune disorders such as asthma (Centers for Disease Control and Prevention, 2011), allergies (Jackson, KD et al., 2013), cancer, diabetes (Centers for Disease Control and Prevention, 2013), and autoimmune diseases is rising worldwide (Bach, 2002; Cooper et al., 2009), and while the impact on adult health is substantial, children are particularly susceptible. Leukemia, the most common childhood cancer, is responsible for 30% of childhood cancer (Belson et al., 2007; U.S. Cancer Statistics Working Group, 2013), and it is estimated that over 25% of children in developed countries have an immune disorder (Dietert and Zelikoff, 2010). While cure rates for childhood leukemia are on the rise (Belson et al., 2007) and many advances have been made in adult leukemia cure rates (Mathisen et al., 2013), most other immune disease treatments are minimally effective at best. Known inherited factors and single nucleotide polymorphisms have been linked to development of some immune diseases including autoimmunity (Perl, 2012) and cancer (Du et al., 2013; Han et al., 2013; Mosor et al., 2013; Perez-Andreu et al., 2013), and many immune disorders tend to run in families or are common in identical twins (Cooper et al., 2009; Rogler, 2011). However, the rise in rates is far too rapid to be attributable to genetic susceptibility alone (Barouki et al., 2012; Schmidt, 2011), which suggests an interaction between genes and the broader environment.

Environmental influence can refer to chemicals or infectious agents in the air, water, and other surroundings (Belson et al., 2007; Fazekas de St Groth, 2012; Selmi et al., 2012; Selmi, Carlo et al., 2011), or it can more specifically refer to exposures *in utero* and be compounded by maternal factors such as diet, tobacco use, or stress (Prescott and Noakes, 2007; Walker and Ho, 2012).

Given that the increase in immune disease rates correlates with the increase in industrialization of developing countries, a further examination of the gene-environment connection is warranted. Though the United States, through Public Health programs such as the Clean Air Act and Clean Water Act, has decreased emissions of environmental contaminants such as benzene, volatile organic compounds and particulates, many others persistent in the water and soil. Additionally, developing countries do not have as stringent environmental protections, and in addition to chemical contaminants, a new environmental risk has emerged. The United States and other developed countries send electronic waste, such as discarded computers, to developing nations in Asia and Africa where they are informally recycled by incineration, raising soil and air levels of dioxins, flame retardants and other toxic substances to dangerous levels (Tue et al., 2013).

A particular strength of our research model is that we use a relatively low dose of TCDD (3 μ g/kg) in our developmental exposures to mimic environmental levels. Many others who work with TCDD and immunity administer doses between 3 and 10 times higher (10-30 μ g/kg) and work with adult animals (Benson and Shepherd, 2011; Casado et al., 2011; Dere et al., 2011; Funatake

et al., 2008; Gasiewicz et al., 1983; Laiosa et al., 2003). We administer developmental doses through the mother, and given that less than 0.5% of TCDD crosses the mouse placenta (Van den Berg et al., 1994; Weber and Birnbaum, 1985), the fetuses receive even lower amounts than the dam. The maximum total TCDD exposure is approximately 30 ng/kg ($3\mu\text{g}/\text{kg} \times 2 \text{ doses} \times 0.5\% = 0.03\mu\text{g}/\text{kg}$ or 30 ng/kg). As a point of human comparison, total infant exposure to TCDD is estimated to be approximately 5.5 ng/kg in the infant's first six months (Gogal and Holladay, 2008). However, in more polluted areas, such as rapidly-developing areas in Asia, the infant would have an even higher exposure level.

B. Developmental Biology and Stem Cells

Developmental exposures cause heightened concern about environmental contaminants because an organism's time *in utero* is particularly sensitive to environmental insult due to the development of stem cells. Stem cells are undifferentiated cells responsible for replenishing themselves, and differentiating along explicit cell lineages. Because all tissues in the body are descendants of early stem cells, any effect on early stem cells is carried with the organism throughout its lifetime. Each organ system is formed during a particular timeframe (Fig. 2), during which that system is most vulnerable to outside influences. As an example, gestational week eight is when the immune system begins to develop, though initiated from stem cells formed even earlier in gestation.



Figure 2. Each organ system has specific sensitive developmental windows. The first 1000 days of life post-conception are particularly vulnerable timepoints in development, as the foundation of all organ systems is being formed. The various systems are illustrated above, along with the most sensitive times in their development (Altshuler, Berg et al. 2003).

C. Immune System Development

Hematopoiesis, the process by which immature precursor cells or HSCs develop into mature blood and immune cells, is initiated in the yolk sac in both mice and in humans (Fig. 3) (Orkin and Zon, 2008; Samokhvalov et al., 2007). In mice, the anatomical location and timing of hematopoiesis proceeds in such a way that the HSCs migrate, and the site of hematopoiesis moves to the aorta-

gonads-mesonephros (gestational day (GD) 8-9), then the fetal liver and spleen (GD11), and finally to the bone marrow, which remains the site of hematopoiesis throughout adulthood (Dzierzak and Medvinsky, 1995; Orkin and Zon, 2008; Robin, C et al., 2003). Though lengths of gestation differ, human hematopoietic development proceeds in the same sequence as in mice. All HSCs, regardless of location, are capable of self-renewal and differentiation in both the fetus and adult organisms (Orkin and Zon, 2008).

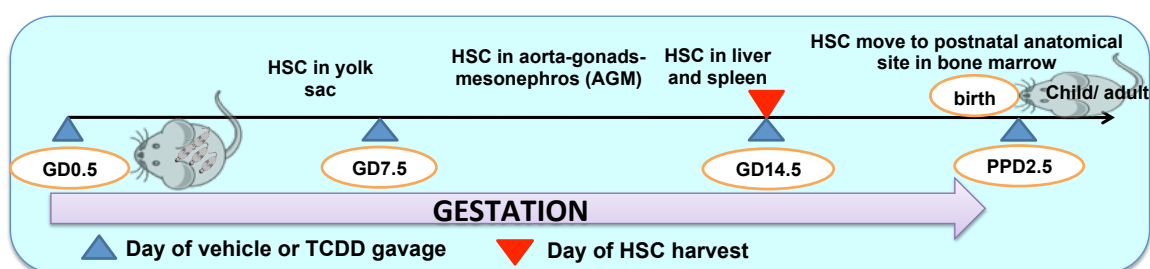


Figure 3. HSC location migrates throughout gestation. Timeline showing location of hematopoiesis, including dosing regimen, for mice used in this research. For HSC harvest, mice were administered vehicle or 3 $\mu\text{g}/\text{kg}$ TCDD on GD 0.5 and 7.5. For adult experiments, treatments continued for GD14.5 and PPD2.5.

All mammalian blood and immune cells develop from hematopoietic stem cells and originate in the fetal liver or the bone marrow of an adult. At the top of the hierarchy (Fig. 4) are a very rare population of multipotent long-term (LT) HSCs, which are self-renewing and capable of differentiation into any cells of the blood and immune system. Identifiable by the presence of several surface markers, including CD150, along with the absence of CD48 and CD244 in the mouse, LT HSCs comprise approximately 0.01% of whole bone marrow (Oguro et al., 2013) and 0.10% of the fetal liver (Kim et al., 2006). Multipotent HSCs must maintain a balance between differentiation into the many mature blood and

immune cells, and quiescence (resting in the G₀ cell cycle) as self-renewing, undifferentiated HSCs.

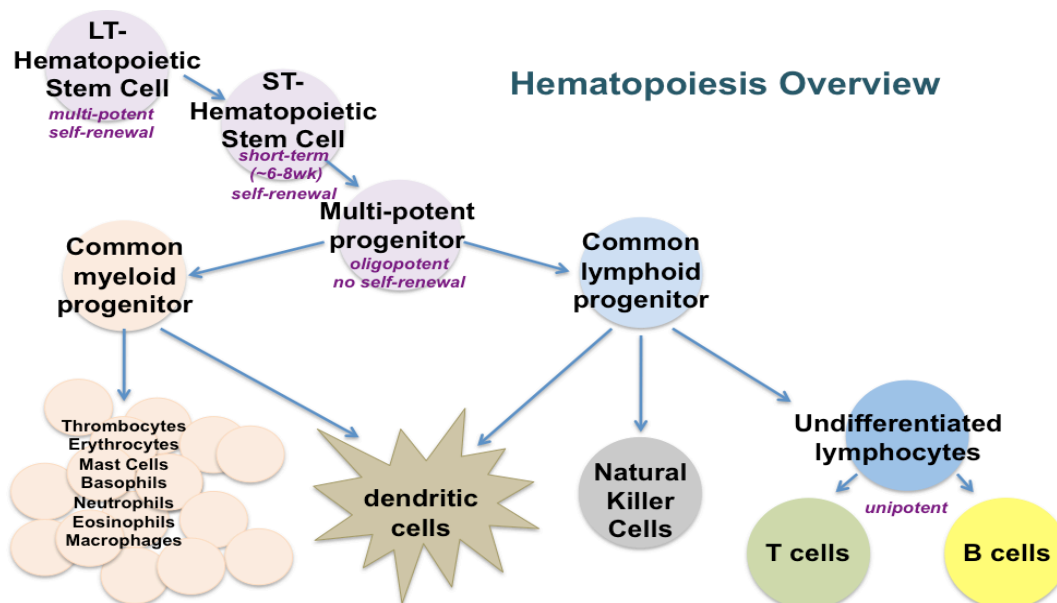


Figure 4. All blood cells descend from hematopoietic stem cells (HSCs). HSCs gradually lose potency (differentiation potential) and renewal potential, becoming more specialized. The first split is between myeloid or lymphoid lineages.

A larger but less specific subset of HSCs are identifiable by the absence of cell surface markers for lineage-committed cells (Table 1) along with the presence of surface markers Sca-1 and cKit (Li et al., 1995; Oguro et al., 2013; Spangrude, G J et al., 1988). This heterogeneous population of short-term (ST) HSCs, known as LSKs (Lin-Sca1+cKit⁺), are easily identifiable and commonly employed in HSC research. LSKs are the HSC population isolated and used in the experiments described here.

In a healthy system, each HSC will form two daughter cells—for replacement and differentiation. In response to micro-environmental signals in the stem cell niche, a proportion of HSCs begin differentiation along myeloid or

lymphoid pathways (Fig. 4), and become committed to progressively more specific lineage fate options (Luc et al., 2008). Myeloid committed precursors can become thrombocytes (platelet-forming cells), erythrocytes (red blood cell forming), mast cells, basophils, neutrophils, eosinophils, macrophages, or dendritic cells. The lymphoid committed precursors can become dendritic cells or can take one of two lymphocytic paths. Some of the undifferentiated lymphocyte progenitors travel to the thymus, where they receive signals from a transmembrane protein known as Notch1, resulting in maturation to T lymphocytes (T cells). The other progenitors remain in the bone marrow, do not receive Notch1 signals, and mature into B lymphocytes (B cells) (Deftos et al., 2000; Zúñiga-Pflücker, 2004).

Table 1. Cell surface markers included in LSK panel

Abbreviation	Function	Cell type it identifies
CD3 ²	T cell co-receptor	T lymphocytes
LY-76/Ter-119 ²	Lymphocyte antigen 76	Erythroid cells
CD45R/B220 ²	Protein tyrosine phosphatase, receptor type, C	B lymphocytes
CD11b ²	Integrin α M chain	Macrophages
LY6G/LY6C/GR-1 ²	Lymphocyte antigen 6 complex, locus G	Granulocytes
Sca1 ³	Stem cell antigen-1	Hematopoietic stem & progenitor cells
cKit ³	Stem cell growth factor receptor	Hematopoietic stem & progenitor cells

¹LSK refers to lineage negative, Sca1 positive, cKit positive cells

²Cell types excluded in LSK

³Cell types included in LSK

Both B and T cells show specificity in responding to foreign antigen but differ in how they recognize it. Whereas B cells recognize soluble antigen, T cells

can only recognize antigen presented by other cells—bound by the cell surface molecule major histocompatibility complex (MHC) which forms a complex with the T cell receptor (TCR) on the T cell. While in the thymus, pre-T cells undergo selection to ensure that their TCRs can recognize MHC presented by self, without reacting too strongly to self (self-tolerance). If the affinity between pre-T cell and MHC presentation is not strong enough, the cell is destined to exit the thymus as a naïve T cell, since these recognize only foreign proteins. If the affinity is too strong, the pre-T cell would be self-reactive, so it is selected against and undergoes apoptosis. But if the level of affinity is “just right,” that cell becomes a regulatory T cell (T_{reg} cell; Sakaguchi et al., 2008). After selection, T cells leave the thymus competent and are able to recognize and react with foreign antigen. When in the periphery, the TCR identifies a specific antigen, triggering a cascade of events causing an immune response, and at this point the T cell is activated.

Lymphocytes, developing from HSCs, can potentially be affected by intrauterine effects resulting in later-life disease. While traditional toxicology looks at adult exposures to potentially harmful substances, our research examines how developmental exposures to immunotoxicants differ from adult exposures. Because hematopoiesis maintains the functional cells of the immune system throughout the life course (Seita and Weissman, 2010; Yamada et al., 2013), there is a growing recognition that factors that disrupt the balance of HSC self-renewal and lineage differentiation into immune effector cells can result in

immune system diseases (as reviewed in Seita and Weissman, 2010) including myeloproliferative disorders and leukemia (K. P. Singh et al., 2013).

III. Epigenetics

One of the mechanisms proposed to explain DOHaD is epigenetic modification (Barouki et al., 2007; North and Ellis, 2011; Susiarjo et al., 2013). An organism's cells each possess a copy of the same DNA, which is the blueprint for the organism, written in genes made up of base pairs. At different developmental timepoints and in different tissues, genes are turned "on" or "off." But there is evidence that dietary, chemical, or other environmental exposures can impact the timing of the expression of genes. Epigenetics provides an explanation of tissue-specific gene regulation—especially throughout development. Common epigenetic alterations include DNA methylation and histone modifications. DNA methylation occurs in cytosine rich areas (CpG islands) on regions of DNA, and results in inactivation of genes due to an inability of transcription factors to bind (Aluru et al., 2011; Mulero-Navarro et al., 2006). Histone modifications include acetylation, methylation, phosphorylation, ubiquitination and sumoylation of amino acids located on the histones. They, too, result in a change in expression of associated genes without an alteration of the DNA sequence. Increasingly, epigenetics has come to be accepted as a means by which adult stem cells respond to the balance of quiescence and differentiation in mature tissues (Rinaldi and Benitah, 2014). However, environmental exposures can alter the

epigenetic mechanisms in terms of timing or location of gene expression, and improper methylation or histone modification in stem cells can be particularly detrimental. Epigenetic alterations in stem cells have been linked to cancer, and cancer stem cells have now been identified due in part to their altered ability to self-renew and proliferate (Easwaran et al., 2014; Jordan et al., 2006). Additional epigenetic alterations, such as methylation changes, have been shown to impact both self-renewal and differentiation of HSCs, and alterations have been observed in leukemic cell lines (B. Liu et al., 2013; Prasad et al., 2014).

Developmental epigenetic modifications, such as those induced by environmental exposures, affect not only the adult, but offspring and even grandchildren of the organism initially exposed (Anway et al., 2005; Burton and Metcalfe, 2014). Fortunately, some of these environmentally-influenced epigenetic modifications can be altered with diet (Carlin et al., 2013), thus illustrating why this research is so important to public health. Dietary interventions could be easily incorporated into maternal standards of care, thus modulating the environmental damage.

IV. 2,3,7,8 tetrachlorodibenzo-*p*-dioxin

TCDD, commonly known as dioxin, has been linked to numerous adverse human health effects, including endometriosis (Bellelis et al., 2011), neurological and vascular issues (Pelclova et al., 2009), decreased fertility (Eskenazi et al., 2010), endocrine disruption (Yoon et al., 2014), diabetes (Michalek and Pavuk, 2008) and cancer (Ansbaugh et al., 2013; Michalek and Pavuk, 2008; Warner et

al., 2011). Many of these diseases are immunological in origin, multi-generational, and potentially result from developmental exposures during gestation or the perinatal period (Dietert, 2009; Mustafa et al., 2009, 2008).

A. Structure and Mechanism of TCDD Toxicity

TCDD exerts its toxicity through a transcription factor known as the arylhydrocarbon receptor (AHR). Due to its high binding affinity to AHR ($K_d=10^{-10} - 10^{-9}M$) (Funatake et al., 2005) and because of steric hindrance (Fig. 5), TCDD is poorly metabolized (Stevens et al., 2009), and all toxicity is mediated through the AHR (Van den Berg et al., 1994) without confounding due to local metabolic effects such as inflammation. Though numerous other environmental toxicants also activate the AHR, in the laboratory setting TCDD is the most commonly used AHR agonist.

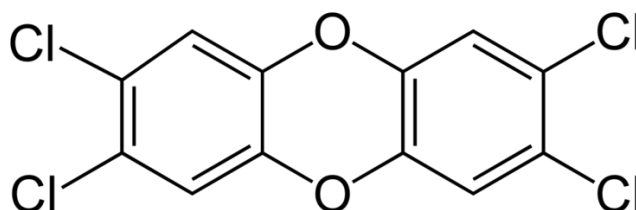


Figure 5. The chemical structure of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin illustrates its resistance to metabolism. (image public domain)

B. Human TCDD Exposures

TCDD has long been known to be toxic, and most research regarding exposures has used animal models. However, a few cases of acute or chronic human exposure to TCDD have occurred and these populations have been very well studied.

Perhaps the best-known case of human TCDD exposure was in Vietnam War military veterans who flew aircraft from 1962-71 transporting and spraying the herbicide Agent Orange or who worked on the ground where it was used. Agent Orange, so named for the orange-colored barrels it was contained in, was contaminated with TCDD. A longitudinal health study known as The Air Force Health Study has followed veterans of this unit, Operation Ranch Hand, since 1982 (Pavuk et al., 2005). Among the health outcomes potentially linked to Agent Orange exposure are prostate cancer, melanoma, leukemia, malignancy, neurological disorders, and immune suppression (Institute of Medicine, 2011; Michalek and Pavuk, 2008; Pavuk et al., 2005).

In 1976, a chemical manufacturing plant outside of Seveso, Italy experienced a factory accident which released high levels of TCDD in the neighboring communities (Bertazzi et al., 1998). Some of the health outcomes potentially found following the accident include chloracne, neurologic and reproductive effects, and changes in liver and immune function (Bertazzi et al., 1998). Limited information exists on developmental exposures, but Seveso cohorts showed connections between TCDD exposure and metabolic syndrome in girls exposed pre-pubertally (Warner et al., 2013), and neonatal thyroid function impairment 25 years after maternal exposure (Baccarelli et al., 2008).

There is disagreement on the strength of the epidemiological data surrounding human TCDD exposures, due to great variation in the population exposed and the concentration and length of time of exposure. Furthermore, unlike with animal studies, human epidemiological studies are frequently

underpowered. Finally, given the latency period associated with non-fatal exposures, many environmental exposures are likely never associated with health outcomes.

V. The Arylhydrocarbon Receptor

AHR, a member of the Per-Arnt-Sim (PAS) superfamily of proteins, is an evolutionarily-conserved, basic helix-loop-helix, ligand-activated transcription factor responsible for xenobiotic metabolism as well as normal development and cell cycle regulation. It promiscuously binds to multiple endogenous and exogenous ligands. AHR can be activated by naturally occurring ligands such as tryptophan derivatives, bilirubin, prostaglandin G (Stevens et al., 2009) and 6-shogaol, an extract of ginger (Yoshida et al., 2014). AHR is activated by numerous synthetic ligands, such as aromatic hydrocarbons, aromatic amines, and indolecarbozoles, which are anthropogenic environmental toxicants (Aluru et al., 2011; Beedanagari et al., 2010a, 2010b). It has been hypothesized that the ability to bind ligands—especially those created by industry—is a secondary, acquired function of the AHR and that its original, evolutionarily conserved purpose has yet to be fully elucidated.

A. Activation by Ligands

TCDD is the best-characterized ligand of the AHR, and is used in Figure 6 to illustrate the mechanism by which gene transcription follows AHR activation. In the absence of a ligand, AHR binds to the AHR complex (which includes the

chaperones heat shock protein 90 (HSP90), protein 23, and hepatitis B virus X-associated protein) in the cytosol. When bound to ligand such as TCDD, AHR dissociates from HSP90, which reveals the AHR's nuclear localization signal, causing a conformational change and allowing entry into the nucleus. In the nucleus, AHR heterodimerizes with aryl hydrocarbon receptor nuclear translocator (ARNT) via mediation by the PAS domain, and other proteins of the complex are removed. The AHR/ARNT heterodimer recognizes DNA binding motifs known as arylhydrocarbon response elements (AHREs—also known as xenobiotic or dioxin response elements). AHREs are located in the promoter region of AHR-responsive genes and binding by the AHR/ARNT heterodimer results in differential changes in gene expression, which yield phenotypic variation. Some of the pathways triggered by AHR activation include phase I and II metabolism, inflammation, and cell cycling (Aluru et al., 2011; Beedanagari et al., 2010a, 2010b; Casado et al., 2010; Cui et al., 2009; Stevens et al., 2009).

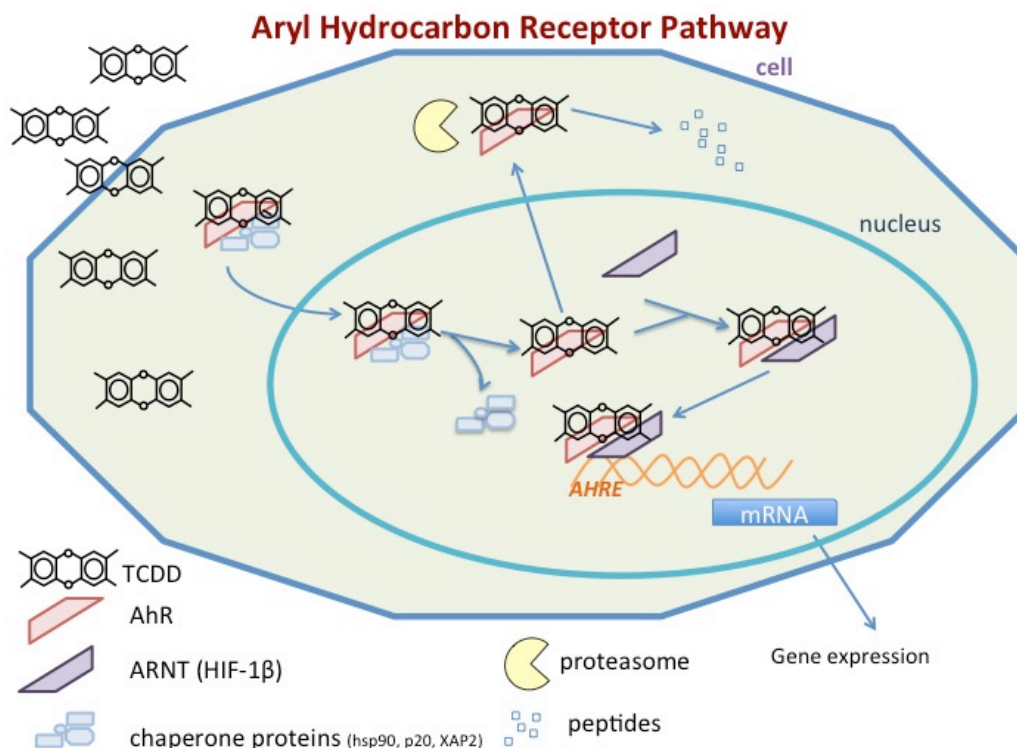


Figure 6. TCDD induces differential gene expression through the AHR pathway.

The AHR is a bHLH, ligand-activated transcription factor primarily responsible for xenobiotic metabolism. Agonists such as TCDD pass through the cell membrane and bind to the AHR complex in the cytosol. Binding of the ligand reveals the AHR's nuclear localization signal, causing a conformational change and allowing entry into the nucleus. In the nucleus, AHR heterodimerizes with ARNT and other proteins of the complex are removed. The AHR/ARNT heterodimer is recognized by DNA binding motifs known as AHREs.

One of the best-studied and most important multi-gene families of enzymes that are located downstream of AHR is the cytochrome P450 (CYP) family (Beedanagari et al., 2010a, 2010b; Stevens et al., 2009). The CYP family is instrumental in metabolizing xenobiotic compounds into intermediary phase I compounds, which are then further broken down by members of the glutathione-S transferase family (GST) and excreted (Fig. 7) (Suter et al., 2010).

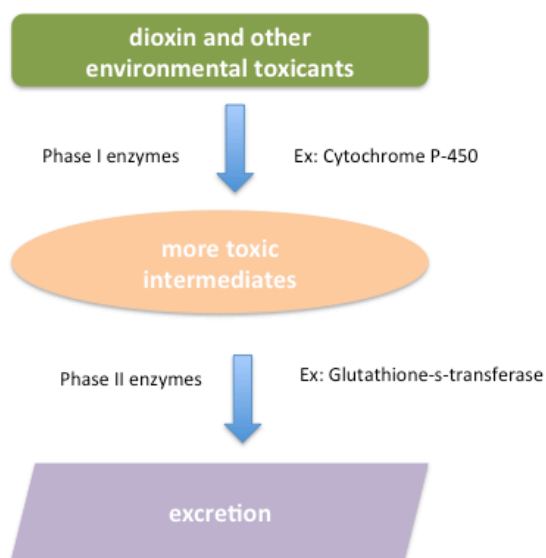


Figure 7. Phase I and Phase II enzymes are involved in detoxification. The cytochrome P450 (CYP) enzymes often produce a more toxic intermediate which is not readily excretable. Glutathione-S-transferases (GST) will metabolize the toxic intermediate into a water-soluble compound which can then be excreted.

The AHR is a principle player in the detoxification pathway of many environmental contaminants—specifically the halogenated aromatic hydrocarbons, such as polychlorinated biphenyls (PCBs), aromatic amines, polycyclic aromatic hydrocarbons (PAHs), and other dioxins. Though our research focuses on TCDD as the prototypical AHR agonist, the likelihood exists that many other environmental contaminants can cause similar responses in the AHR and downstream gene cascades. Furthermore, combinations of AHR agonists and/or other exposures can act in an additive manner to increase likelihood of disease. For example, mice exposed *in utero* to TCDD and also fed a high fat diet after birth were twice as likely to develop mammary tumors than those exposed to TCDD *in utero* but fed a regular diet (La Merrill et al., 2010).

AHR signaling leads to three different biological pathways—adaptive, toxic or developmental (Stevens et al., 2009). In addition to the more familiar xenobiotic-induced detoxification, AHR is also associated with maturation, cell proliferation, cell cycle control and homeostasis of multiple organ systems, including the liver, vascular system, lungs, gastrointestinal and immune system (Cui et al., 2009; Mulero-Navarro et al., 2006; Stevens et al., 2009).

Down-regulation of AHR associated genes can occur by degradation of AHR by the ubiquitin/proteasome pathway or via negative feedback by another PAS protein known as AHR repressor (AHRR) (Oshima et al., 2009). These evolutionarily distinct methods prevent over-activation of AHR associated genes (Stevens et al., 2009). However, genes can also be regulated by epigenetic mechanisms such as hypermethylation of the promoter region, which has been found in both mouse and human cancer cell lines and in samples from human acute lymphoblastic leukemia (ALL) tumors (Beedanagari et al., 2010a; Mulero-Navarro et al., 2006).

Prenatal exposure to TCDD even prior to implantation has an effect on the methylation of some imprinted fetal genes, thus impacting their expression (Wu et al., 2004). However, this may be dose-dependent and only observable at comparatively high doses (ie: 10 ng/kg/day = 0.01 μ g/kg/day but not 2ng/kg/day) (Somm et al., 2013). Although not directly affecting HSCs, AHR activation does epigenetically modify immune cells that are derived from HSCs. Specifically, it has been suggested that epigenetic regulation is responsible for a decrease in the number of CD4⁺ T lymphocytes and an increase in the number of T_{reg} cells in

adult animals following developmental AHR activation and challenge with influenza virus (Boule et al., 2014).

B. AHR and the Immune System

AHR is essential for immune development, including regulation of hematopoiesis (Casado et al., 2010), induction of auto-immune fighting T_{reg} cells (K. P. Singh et al., 2011; Wu et al., 2011), and maintenance of HSCs in the quiescent or resting stage (Oguro et al., 2013). In AHR knock-out mice (AHR^{-/-}), multiple immunological defects are found, including abnormal HSCs with decreased self-renewal potential (K. P. Singh et al., 2011), altered spleen and thymus lymphocyte counts (Casado et al., 2010), enlarged spleen in females (Esser, 2009), and perinatal extramedullary hematopoiesis in the liver (Harrill et al., 2013). Additionally, myeloproliferative hematopoietic diseases are more common in mice lacking AHR (K. P. Singh et al., 2013).

Though AHR is necessary for regular development, over-activation is as detrimental as lack of AHR. Many environmental contaminants that are byproducts of combustion exert their toxicity through the AHR. The potent AHR agonist TCDD has been linked to numerous poor health outcomes in adult animals, including gastrointestinal hemorrhage, liver toxicity, thymic atrophy, and death (Sorg, 2013). AHR activation also results in direct immunosuppression (Esser, 2009; Hao and Whitelaw, 2013), reduces B cell maturation, and increases the severity of some autoimmune diseases (Hao and Whitelaw, 2013). Laboratory research has shown suppression of humoral and cellular immune

response, increased susceptibility to infection, and thymic involution all following TCDD exposure (Stevens et al., 2009). Since the thymus is the site of T cell development, the direct effects of thymic involution are thymocyte loss and premature thymic emigration of T cells. Therefore alterations in the AHR and downstream metabolic target genes of AHR have been implicated as susceptibility factors for numerous cancers (Mulero-Navarro et al., 2006) and autoimmunity (Stevens et al., 2009).

Despite the negative health effects connected to adult AHR activation, one interesting inconsistency exists. Adult humans and animal models exposed to AHR agonists have shown improvement in numerous autoimmune diseases, including psoriasis (Di Meglio et al., 2014), Crohn's disease (Benson and Shepherd, 2011), colitis (Furumatsu et al., 2011), Type 1 diabetes (Kerkvliet et al., 2009), uveoretinitis (Zhang et al., 2010), and a murine model of multiple sclerosis (Hamza and Abdullah, 2013). The mechanism by which AHR activation in adult murine models improves autoimmune disease symptoms involves T_{reg} cells (Benson and Shepherd, 2011; Funatake et al., 2008; Gandhi et al., 2010; Marshall and Kerkvliet, 2010; Mezrich et al., 2010; Zhang et al., 2010). Conversely, exposure to AHR agonists during fetal development has shown that prenatal AHR activation by TCDD increases immune disorder (Gogal and Holladay, 2008; Holladay et al., 2011; Mustafa et al., 2009), specifically leading to a loss of tolerance in later life.

Given the paradox in disease outcomes related to the AHR and the potential for benefits following AHR activation, a recent focus of research

involves non-toxic dietary AHR agonists such as those derived from *Lactobacillus bulgaricus* (Takamura et al., 2011), resveratrol (Rimmelé et al., 2014) and multiple tryptophan derivatives (Bock, 2013). These endogenous ligands differ most notably from TCDD in their transient activation of the AHR. These and other non-toxic AHR agonists have shown promise in combatting both autoimmune and inflammatory diseases (Busbee et al., 2013).

In addition to an increase in autoimmunity, developmental AHR activation has been linked to impairment of multiple cell-mediated immune functions (Holladay and Smialowicz, 2000) including response to influenza infection (Boule et al., 2014; B A Vorderstrasse et al., 2004). In mice, while exposure to TCDD throughout gestation and lactation does not initially affect T cell ratios, when the same mice were infected with influenza, the frequency of CD4+ T_{reg} cells increased and activated CD4+ T cells decreased (Boule et al., 2014). Similarly, in humans, maternal AHR activation has been linked to immunosuppressive response to measles vaccine in offspring three years later (Hochstenbach et al., 2012). Given the drop in vaccination rates in developed countries for non-medical reasons, adding a similar drop in vaccine effectiveness due to environmental exposures could allow a return of many nearly eradicated diseases.

Furthermore, while adult AHR activation results in transient immune effects, developmental activation has been linked to more persistent functional effects (Hogaboam et al., 2007). This emphasizes the importance of timing of AHR activation—the specifics of which have yet to be fully elucidated.

AHR is just one of the signaling pathways involved in immune system development and maturation. Pathway crosstalk is common, and the AHR is known to crosstalk with pathways including protein kinases (Esser et al., 2009), which are important for immune system regulation and function. Furthermore, dysregulation in protein kinases has been linked to disease development, including leukemia and myeloproliferative neoplasms (Gery and Koeffler, 2013). Discovery of additional pathway crosstalk with AHR could help explain the recent increase in immune disorders.

VI. Notch

Notch is necessary for direct cell-to-cell contact involved in regulation of cell development in the embryo and during adulthood. Notch proteins are highly conserved receptors found on the cell surface (Fig. 8) (Alam et al., 2010; Borggreffe and Oswald, 2009; Osborne and Minter, 2007; Schmitt and Zúñiga-Pflücker, 2002). The four Notch proteins (Notch 1, 2, 3, & 4) have been found in all animal species to date (Lai, 2004). The Notch ligands in vertebrates are made up of members of the Jagged (Jagged 1 & 2) and Delta-like (DL1, DL3, DL4) families. Both the receptors and the ligands are single-pass transmembrane proteins and each possess an extracellular epidermal growth factor (EGF) repeating motif necessary for direct contact mediation between the two (Ferrando, 2009; Lai, 2004).

A. The Notch Signaling Pathway

It has been suggested that AHR-mediated environmental signals can contribute to over-stimulation of Notch, resulting in tumors, T cell acute lymphoblastic leukemia lymphoma (T-ALL), and other cancers (Alam et al., 2010).

The thymus is rich in Delta-like Notch ligands (Zúñiga-Pflücker, 2004) which play a role in hematopoietic cell fate decisions. Ligands such as DL1 interact with the extracellular portion of Notch. In addition to the extracellular, EGF-rich portion, Notch also has an intracellular portion (ICN). The intercellular domain is responsible for binding to transcriptional repressor CBF1-suppressor of hairless-Lag1 (or CSL). Even before encountering ligand, Notch receptors undergo activation in preparation for signaling. Notch receptors are housed in the endoplasmic reticulum in an inactive form. In the Golgi complex, the receptor is constitutively cleaved by a furin-like protease, forming a non-covalently bound heterodimer composed of the extracellular and intercellular portions, and reassembled into a cell surface receptor (Lai, 2004). The extracellular portion is then glycosylated (Osborne and Minter, 2007).

When a Notch receptor on a lymphocyte progenitor physically interacts with a Notch ligand such as a Delta-type ligand present on a stromal cell, EGF repeats on the ligand interact with the Notch receptor and trigger the second cleavage—which results in release of the extracellular portion of Notch. The third step involves cleaving of ICN by gamma secretase (Schmitt and Zúñiga-Pflücker, 2002), which allows the ICN to travel to the nucleus, where it associates with a

constitutive CSL transcription factor (CBF1-Su(H)-LAG-1 in humans; RBP-J in the mouse), as a co-activator which drives T cell commitment (Borggrefe and Oswald, 2009; Lai, 2004; Osborne and Minter, 2007; Pajeroski et al., 2009). CSL is associated with co-repressors when Notch is not present, which prevent Notch target gene transcription (Lai, 2004) and drive B cell commitment (Osborne and Minter, 2007; Zúñiga-Pflücker, 2004). ICN, upon association with the co-activators, recruits transcriptional co-activators from the mastermind-like (MAML) family, which allow the expression of Notch target genes (Pajeroski et al., 2009). MAML1 may also serve to mediate early T cell development, as it has been shown in vitro to increase phosphorylation and subsequent Notch receptor degradation when combined with CSL (Osborne and Minter, 2007).

Several Notch target genes have been identified to date. These include the highly evolutionarily conserved hairy/enhancer of split (HES) family—basic helix-loop-helix (bHLH) proteins, which are essential for T cell development and signaling, and a related family—HES with YRPW motif (HEY—also known as HERP). Both HES and HEY members can be activated by constitutive Notch1 and act as transcriptional repressors. HES1 and HES5 over-expression also inhibits B cell development. In addition to T cell development and signaling, Notch target genes have been associated with T cell activation, proliferation, cytokine production and protection from apoptosis (Osborne and Minter, 2007).

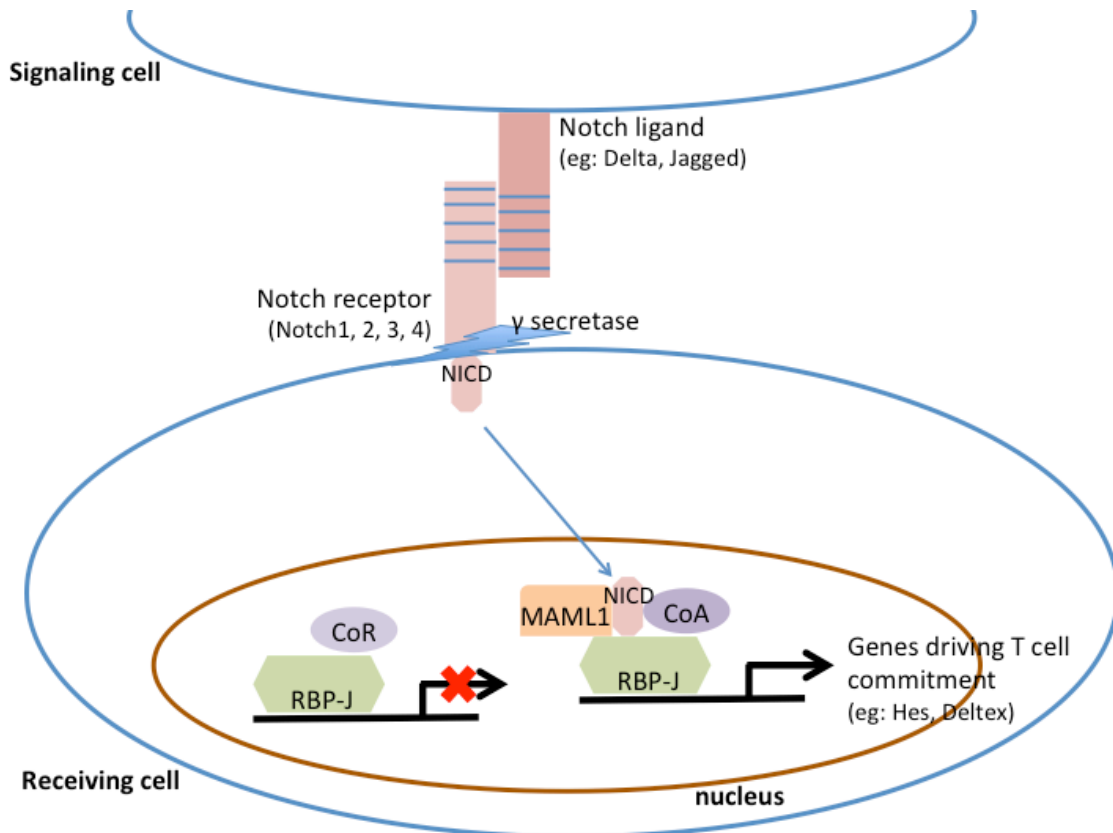


Figure 8. Notch activation by ligands induces differential gene expression driving T cell commitment. The Notch receptors are composed of an extracellular portion with EGF repeats, and an intercellular portion. When the EGF repeats on a Notch ligand (Delta or Jagged families) contact the extracellular portion of a Notch receptor, gamma secretase is responsible for lysis of the intercellular (NICD) portion of Notch. NICD then moves into the nucleus of the cell where it interacts with a CSL (RBP-J in the mouse) co-activator to drive expression of genes, including those involved in T cell commitment.

While Notch is also involved in development of CD4⁺ and CD8⁺ single positive (SP) T cells from CD4⁺CD8⁺ double positive (DP) precursor thymocytes, the definitive link between Notch signaling and T cell development concerns the T cell vs. B cell fate decision at the progenitor step of development.

B. *In vitro* Culture of T cells, Using Notch

Early lymphocyte differentiation research most often focused on B cells because they were relatively easy to grow in the lab from HSCs in a co-culture system with murine bone marrow stromal cells. Since B cells differentiate in the bone marrow, these stromal cells provided all the signaling needed for maturation from HSC to effector B cells. However, T cells differentiate in the thymus, and until 2002, the only way to model HSC differentiation into T cells was by using the much more involved fetal thymic organ culture (FTOC). While it was known that Notch played a necessary role in T cell commitment, the component of the Notch pathway that could be isolated from the thymus and used to help grow T cells *in vitro* was still unknown.

Schmitt and Zúñiga-Pflücker were the first to successfully engineer a cell line that could support the commitment, development, and growth of T cells in the lab (Schmitt and Zúñiga-Pflücker, 2002; Zúñiga-Pflücker, 2004). They began with the bone marrow stromal cell line derived from OP/OP mice (and therefore named OP9) which had previously been used to support the growth of B cells and NK cells from both bone-marrow derived HSCs and also from embryonic stem cells (ESCs). Both OP9 cells and thymic stromal cells were found to transcribe Jagged-1 and Jagged-2, but only the thymus transcribed Delta-like-1 and Delta-like-4.

Schmitt and Zúñiga-Pflücker developed a line of OP9 stromal cells which were retrovirally transduced to express the Notch ligand Delta-like-1. The resulting cell line was named OP9-DL1. The OP9-DL1 stromal cell line and an

OP9-GFP non-Delta-like-1 expressing control stromal line were used to test the growth of hematopoietic progenitor cells (HPCs). LSKs harvested from fetal livers were co-cultured on both OP9 cell lines in the presence of interleukin-7 (IL-7) and Fms-related tyrosine kinase 3 (FLT3) ligands (Schmitt and Zúñiga-Pflücker, 2002; Zúñiga-Pflücker, 2004). Development was followed via flow cytometry analysis for up to 17 days (Fig. 9).

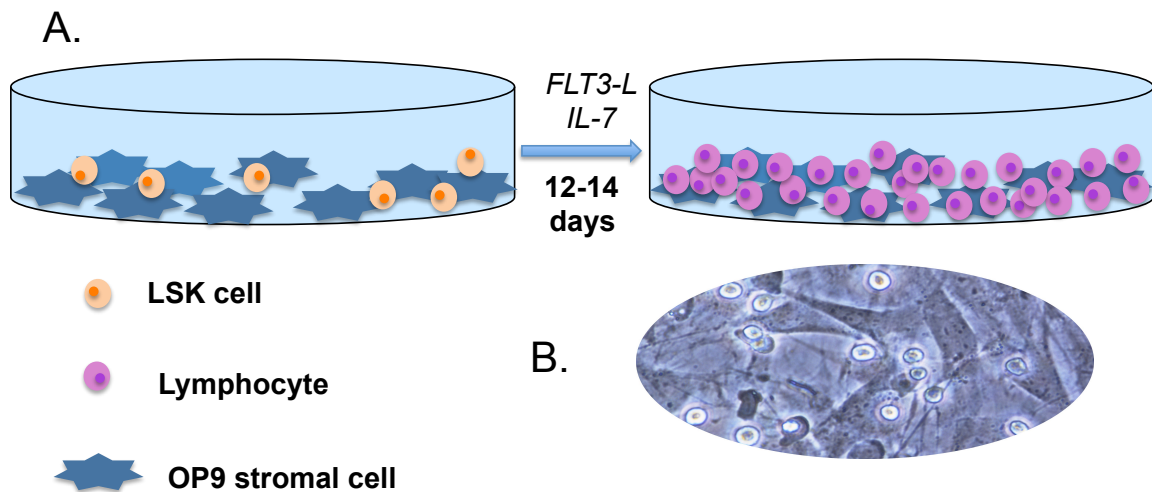


Figure 9. The OP9-LSK co-culture system includes bone marrow stromal cells and HSCs. A) LSK cells isolated from GD14.5 fetal livers are grown in co-culture with OP9 bone marrow stromal cells in the presence of FLT3-L and IL-7 for 12 (T cell) or 14 (B cell) days. B) Mature lymphocytes grown in co-culture with OP9 bone marrow stromal cells. Cells were harvested from co-culture and analyzed by flow cytometry for B or T lineage development.

The control OP9-GFP cells resulted in mature B cells and natural killer (NK) cells starting on day 4 and continuing through day 12, and did not produce any T cells. However, the LSKs cultured on OP9-DL1 cells gave rise to DP immature T cells starting on day 7 and developing through day 12 (Schmitt and Zúñiga-Pflücker, 2002). The pattern of development follows that of HSCs in FTOC, including maturation from double negative (DN; $CD4^{-}CD8^{-}$) to double

positive (DP; CD4+CD8+) and single positive (SP; CD4+ or CD8+) T cells, as well as generation of both $\gamma\delta$ -TCR and $\alpha\beta$ -TCR T cells. In addition, the T cells grown were found to be functionally mature, as they produced IFN γ when stimulated by CD3 and TCR (Schmitt and Zúñiga-Pflücker, 2002). Finally, the HSCs were obtained from mouse fetal livers, which are considerably larger and easier to obtain than the fetal thymus. Chapters three and four of this dissertation rely on the OP9 co-culture system to measure HSC differentiation potential.

C. Notch in T-ALL

Notch mutations have been correlated with the occurrence of T cell acute lymphoblastic leukemia (T-ALL) (Ferrando, 2009; Gordon et al., 2009; Kumar et al., 2014; N. Liu et al., 2013; Paryan et al., 2013; Tosello and Ferrando, 2013; Weng et al., 2004). Over half of the diagnosed cases of T-ALL have activating Notch1 mutations. Overexpression of the intracellular portion of Notch1 (ICN) drives CD8+ commitment at the expense of CD4+ commitment and transgenic mice that overexpress ICN are prone to T cell thymic tumors (Fowlkes and Robey, 2002). We use thymocyte specific *lck*-proximal promoter over-expressing ICN transgenic mice in the experiments described in chapter five.

VII. Study Rationale

Given that the AHR has been linked to immunological disease in adult models and it is recognized that developmental exposures are frequently more detrimental due to impact on stem cells, we hypothesized that developmental

exposure to AHR agonists such as TCDD alter HSCs in a manner that negatively impacts later-life immunity.

An organism's time in utero is developmentally unique, as it transforms from a fertilized egg into a multi-cellular organism. The initial multi-potent stem cells, able to differentiate into all types of cells, gradually become committed to particular lineages, such as the hematopoietic lineage. Because the directions for development of the mature immune system is directed by DNA in the HSCs, any resulting impact on HSCs during gestation—such as the failure to differentiate correctly—can reprogram the genetic code, leading to immunodeficiencies later in life. The immune system formed from imperfect HSCs may exhibit immunosuppression (an insufficient ability to fight infection), may not correctly develop self-tolerance (resulting in autoimmunity), or may not balance replenishment and differentiation correctly (resulting in cancer). The overall goal of this research was to determine what role developmental exposure to AHR agonists such as TCDD has on later-life immunity.

The effect that developmental exposure to TCDD has on the ability of HSCs to differentiate into lymphocytes was determined using a novel limiting dilution analysis assay. HSCs were isolated on GD14.5 from fetal livers of C57BL/6 mice exposed to either vehicle control or 3µg/kg TCDD throughout gestation. HSCs were co-cultured with OP9 bone marrow stromal cells and the ability to differentiate into B or T lymphocytes was measured using Extreme Limiting Dilution Analysis (ELDA) for stem cell research (Hu and Smyth, 2009).

Developmental TCDD exposure was found to decrease by two and a half fold the ability of HSCs to become mature B cells or thymocytes.

Given that TCDD is the most potent AHR agonist known, we hypothesized that developmental TCDD exposure induces differentiation effects by fetal AHR activation. The role that fetal AHR plays in HSC development after gestational exposure to TCDD was determined by repeating the limiting dilution assay using HSCs from transgenic mice lacking an AHR. We found that AHR in the fetus is responsible for the effects caused by developmental TCDD exposure, which results in a decrease in the potential of HSCs to differentiate into mature lymphocytes. Mice lacking an AHR do not experience the TCDD-induced decreased thymocyte differentiation effect.

The role that developmental exposure to TCDD plays in T cell development in adults was determined using transgenic mice that overexpress Notch1, which is implicated in over half the cases of T-ALL. Notch has been associated with the maintenance of undifferentiated HSCs (Zhou et al., 2010) and is downregulated as HSCs differentiate (Duncan et al., 2005). We exposed Notch transgenic mice to TCDD throughout gestation and lactation, then followed the mice through adulthood, tracking blood cell phenotype weekly. We found that the crosstalk between AHR, as developmentally activated by TCDD, and later Notch1 signaling does not act in an additive manner, but rather in what appears to be a competitive manner, resulting in more typical T cell ratios and better health outcomes in the transgenic mice developmentally exposed to TCDD.

These studies contribute significantly to our knowledge of the AHR during development, the effect of environmental insult on genetically-susceptible populations, DOHaD specific to the immune system, and the mechanism by which these effects occur. This research provides an important contribution to public health because of the need to elucidate reasons for increasing disease rates in order to drive policy and to develop preventative health strategies (Barouki et al., 2012). Prevention plays a critical role because unlike polymorphisms such as BRCA1, epigenetic alterations are reversible with dietary interventions (Carlin et al., 2013).

While research findings support the impact of dioxin-like chemicals as factors increasing immune disorders, and efforts are being made to decrease environmental contaminants, the genetic component is equally important to complete understanding of this health crisis. Given that the increase in immune disorders is likely the result of a combination of genetic and environmental causes (Faulk and Dolinoy, 2011), determining at which stage(s) developmental AHR activation causes immune dysfunction would allow clinical intervention at an early time point. The ability to test stem cells for genetic or epigenetic alterations could identify susceptible populations and focus potential preventative efforts at an early stage in particularly vulnerable people and to counsel patients at risk on avoiding additional risk factors. Furthermore, elucidating the genetic aspect of susceptibility provides a therapeutic focus (Quintana, 2012).

Finally, an underdeveloped immune system can not only increase development of immune-related diseases, but also result in an immune system

less likely to respond favorably to immunization (Hogaboam, Moore et al. 2008). Though public health practice aims for 100% immunization, in order for effective population immunity, vaccination rates need only meet the herd immunity threshold, which varies depending on the vaccine and population (Fine et al., 2011). However, even if the threshold is reached, if not all vaccinated are achieving full immunity from vaccines due to deficient immune systems, a public health crisis could emerge.

CHAPTER 2

MATERIALS AND METHODS

Solutions and Buffers

MEM: Minimum Essential Medium alpha (α -MEM; Life Technologies, Carlsbad, CA); 1 packet, combined with 2.2 g NaHCO_3 , topped off with 1 L ddH_2O , stirred for 10 minutes and filter sterilized using a $0.2\mu\text{m}$ filter (aPES membrane), then stored at 4°C in the dark.

OP9 media: MEM supplemented with 20% FBS (lot tested for supporting OP9 co-cultures; Life Technologies) and 0.05mg/mL gentamycin (Life Technologies), filter sterilized and stored at 4°C in the dark.

DMEM complete: Dulbecco's Modified Eagle Medium (Mediatech, Manassas, VA); supplemented with 10% fetal bovine serum (FBS; Invitrogen, Grand Island, NY), 1% L-glutamine (Invitrogen), 1% 1 M HEPES (Invitrogen), 0.01% 0.5 M 2-Mercaptoethanol (EMD, Gibbstown, NJ), and 0.001 mg/mL gentamycin (Life Technologies), filter sterilized and stored at 4°C in the dark.

Serum-free DMEM: Dulbecco's Modified Eagle Medium (Mediatech); supplemented with 1% L-glutamine (Invitrogen), 1% 1 M HEPES (Invitrogen), 0.01% 0.5 M 2-Mercaptoethanol (EMD), and 0.001 mg/mL gentamycin (Life Technologies), filter sterilized and stored at 4°C in the dark.

FACs buffer: Fluorescence activated cell sorting buffer; consisting of Hank's buffered saline solution (HBSS; Corning CellGro, Herndon, VA), supplemented with 0.5% FBS (Invitrogen) and 0.1% sodium azide (J.T. Baker/Avantor, Center Valley, PA), then stored at 4°C in the dark.

RBC lysis buffer concentrate: Red blood cell lysis buffer, consisting of 8.02 g (155 mM) NH_4Cl (ammonium chloride) + 0.84 g (12 mM) NaHCO_3 (sodium

bicarbonate) + 0.37 g (0.1 mM) EDTA (10X concentration), then filled to 100ml with Millipore water. Diluted 1:10 before use.

Experimental Animals

All animal procedures were conducted according to NIH's *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011) and with the approval of the Institutional Animal Care and Use Committee (IACUC) at the University of Wisconsin-Milwaukee. C57BL/6J (B6) and B6.129-*Ahr*^{tm1Bra}/J (AHR) mice used were obtained directly from the Jackson Laboratory (Bar Harbor, ME), or were offspring from original Jackson mice. Due to reproductive deficiency of AHR^{-/-} females, this strain was maintained by either HZ x HZ (AHR^{+/-} x AHR^{+/-}) or KO (AHR^{-/-}) male x WT (C57BL/6J; AHR^{+/+}) female pairings. C57BL/6-Tg(LckNotch1)9E mice, hereafter referred to as Notch1^{ICN-TG} mice, were offspring of original pups that were a generous gift from B.J. Fowlkes, PhD, at the National Institute of Allergy and Infectious Disease (NIAID), Bethesda, MD. These mice were maintained as homozygous stock prior to breeding to C57BL/6 to generate heterozygous Notch1^{ICN-TG} used for all experimental procedures.

After overnight pairings, the presence of a vaginal mucus plug was used as evidence of mating and designated "gestational day (GD) 0.5." For breeding resulting in live birth offspring, mice were placed together for 4-7 days and checked for presence of vaginal plug each morning. However, for time-sensitive breeding, animals were placed together between 3:00 pm and 5:00 pm and presence of vaginal plug was determined between 7:00 am and 9:00 am the next

morning to ensure that fetuses were as close to similar developmental age as possible. This was a change in protocol from week-long breeding periods as it was determined that fetuses from the same day vaginal plug observations still appeared varied in size and developmental stage.

For breeding resulting in live birth offspring after gestational exposure to TCDD, all multiparous mothers were used. This change in protocol came about due to a high percentage of neonatal deaths, possibly due to either a lack of mothering experience or a failure of mammary gland differentiation after exposure to TCDD (Lew et al., 2009; Beth A Vorderstrasse et al., 2004).

All mice were housed in micro-isolator cages in a specified pathogen-free facility at the University of Wisconsin-Milwaukee, provided food and water ad libitum, and maintained on a 12:12-h light cycle.

Genotyping of Transgenic Mouse Strains

Tissue samples were collected as ear punches from adult mice and tail samples from fetal mice. Ear tissues were digested with rotation at 55°C overnight in 190µl DirectPCR Lysis Reagent (ViaGen Biotech, Los Angeles, CA) plus 10 µl Proteinase K (ViaGen Biotech). Fetal tail tissues were digested in 160µl Direct PCR lysis buffer plus 40 µl Proteinase K for 20 minutes with intermittent vortexing in a locking-cap 1500µl microcentrifuge tube (Eppendorf, USA). Proteinase K in samples tubes was then inactivated for one hour at 85°C (ear) or a 1:5 diluted sample for 20 minutes at 95°C (fetal tail). Primers (Table 2) were thawed over ice. Reaction samples were mixed, each containing 12.5µl

MyTaq, 0.5µl each primer, 9.5µl DNase free water, and 2 µl of genomic DNA.

Samples were run in thermocycler according to specifications listed in Table 3.

Table 2. qPCR SybrGreen primers used

Gene name	Direction	Sequence
AHR	forward	CAGTGGGAATAAGGCAAGAGTGA
	reverse	AGGGAGATGAAGTATGTGTATGTA
HgH	forward	CAACAGGGAGGAAACACAACAG
	reverse	GTTTGGATGCCTTCCTCTAGGT
SrY	forward	TCATGAGACTGCCAACCACAG
	reverse	CATGACCACCACCACCACCAA
Myog	forward	TTACGTCCATCGTGGACAGC
	reverse	TGGGCTGGGTGTTAGTCTTA

Table 3. Thermocycler specifications

Gene name	Stage	Temp	Time	Note
AHR	1	95°C	3 min.	
	2	95°C	5 sec.	Repeat stage 2 for 30 cycles
		58°C	5 sec.	
		72°C	10 sec.	
	3	72°C	5 min.	Hold
		4°C	-	

HgH	1	94°C	5 min.	
	2	4°C	30 sec.	Repeat stage 2 for 35 cycles
		63°C	1 min.	
		72°C	1 min.	
	3	72°C	7 min.	Hold
		4°C	-	
Sry & Myog	1	94°C	3 min.	
	2	94°C	5 sec.	Repeat stage 2 for 30 cycles
		57°C	5 sec.	
		72°C	15 sec.	
	3	72°C	1 min.	Hold
		4°C	-	

PCR samples were run on a 2.2% Lonza FlashGel single tier DNA cassette (Lonza, Allendale, NJ) at 225 V for 15-20 minutes, while MovieMaker (Microsoft, Redmond, WA) ran continuously, recording band migration.

TCDD Preparation and Treatment Protocol

TCDD (Cambridge Isotopes, Andover, MA) was diluted in 1,4-dioxane (Sigma-Aldrich, St. Louis, MO) to a working stock concentration of 0.2 mg/ml. Appropriate volume of TCDD in 1,4-dioxane was subsequently transferred to a sterile 15 ml conical tube and the liquid was evaporated in a chemical fume hood. The remaining TCDD residue was then suspended in olive oil (Filippo Berio,

Hackensack, NJ) to a concentration of 0.3µg/ml, 1.0µg/ml or 3.0µg/ml and was mixed by rotation overnight at room temperature. For dosing of vehicle control mice, olive oil was added to a tube from which an equal volume of 1,4-dioxane had been evaporated.

For experiments where LSKs were isolated from GD 14.5 fetal livers, pregnant mice in the treatment group were exposed to either 3µg TCDD/kg body weight by oral gavage on gestational days 0.5 and 7.5, or olive oil vehicle control (0.1ml per 10g) on the same days. Doses were given 7 days apart to insure a relatively constant level of TCDD throughout because the half-life of TCDD in a C57BL/6 mouse is approximately one week (Gasiewicz et al., 1983; Hogaboam et al., 2007; Miniero et al., 2001; Weber and Birnbaum, 1985).

For experiments where developmentally-exposed mice were allowed to grow to adulthood, pregnant mice in the treatment group were given 3µg TCDD/kg body weight by oral gavage on GDs 0.5, 7.5, 14.5, and post-partum day (PPD) 2.5, while the control mice received an equivalent volume of olive oil (0.1ml per 10g) on the same days. This dosing regimen included not only *in utero* exposure, but also lactational exposure, mimicking the pattern of exposure in the environment. Such methodology was chosen to accurately represent environmental exposure as TCDD has been found to be more detrimental when maintained throughout development (Hogaboam et al., 2007).

For some experiments rather than a developmental exposure, four week old naïve mice were exposed to a single dose of 10µg or 30µg TCDD/kg body

weight or an equal volume of vehicle control (0.1 ml per 10g) by oral gavage and tissues were analyzed 10 days after exposure.

Antibodies Used for HSC/HPC and Lymphocyte Staining

Primary fluorochrome-conjugated monoclonal antibodies were used in flow cytometry analysis and cell sorting (Table 4) on a BD FACSAria III, DIVA version 6.1.3.

Table 4. Monoclonal antibodies, clones and fluorochromes used in fluorescence activated cell sorting and flow cytometry analysis

Antibody	Clone	Fluorochrome*
B220	RA3-6B2	PE
CD3	145-2C11	biotin
CD3	145-2C11	PE-CF594
CD4	RM4-5	APC-H7
CD4	RM4-5	PECy5
CD4	RM4-5	PE
CD5	53-7.3	biotin
CD11b	M1/70	biotin
CD11b	M1/70	Alexa488
CD19	1D3	FITC
CD25	7D4	biotin
CD44	IM7	PECy5
CD45	30-F11	PE
CD45R/B220	RA3-6B2	biotin
CD8 α	53-6.7	PE-Cy7
CD8 α	5H10	Alexa647
cKit ¹	2B8	Alexa647
GR-1	RB6-8C5	APC-Cy7
LY-76	TER119	biotin
LY6G/LY6C/GR-a	RB6-8C5	biotin
Sca-1	E13-161.7	PE
Streptavidin	-	FITC
Streptavidin	-	PE-TR

*FITC=fluorescein isothiocyanate; PE=phycoerythrin; Cy=cyanine; APC=allophycocyanin; TR=Texas Red

¹Purchased from Life Technologies

HSC/HPC populations were identified by the absence of lineage markers (CD3, LY-76, CD45R/B220, CD11b and LY6G/LY6C/GR-a1) and presence of two stem cell markers (Sca-1 and cKit).

Immature thymocytes and B cells resulting from OP9 co-culturing were identified based on expression of CD8 α , CD4, CD25, CD44, B220 and CD19.

Weekly blood and tissue cells were identified based on expression of CD19, CD11b, CD45, CD3, CD4, CD8 and GR-1. Tumor cells were identified by the presence of CD5 after gating on CD3⁺ populations and within CD4⁺ or CD8⁺ subsets.

For thymocyte and splenocyte cellular analysis, living cells were first identified by staining with 10 μ l of the vital dye Sytox Blue (Life Technologies), 1mM, diluted 1:80, to label nuclei from dead or dying cells with permeable membranes. Sytox Blue negative cells were then identified by the presence of CD4 and/or CD8.

For adult high-dose exposures, total cellularity was determined by dilution in viable cell determining trypan blue and manual counting using a hemocytometer (Fisher Scientific, Pittsburg, PA). Cells were then identified by the presence of CD4, CD8, CD44, CD3 and CD25.

For cell-cycle experiments, co-cultures were incubated in the presence of 10 mM 5-bromo-2'-deoxyuridine (BrdU) for one hour prior to harvest. Cells were then stained with appropriate surface markers followed by fixation and permeabilization using the BD FITC BrdU flow cytometry kit according to the manufacturer's instructions (BD Biosciences). Thymocyte precursors were

identified by the presence of both CD4 and CD8 α surface markers. B cell precursors were identified by presence of both B220 and CD19 surface markers.

All antibodies were used at titrated concentrations and were purchased from BD Biosciences unless otherwise noted.

Fetal Liver HSC Isolation and Cell Sorting

Pregnant C57BL/6J or AHR+/- mice were euthanized by CO₂ asphyxiation according to the AVMA Guidelines on Euthanasia (AVMA, 2013) on GD 14.5. Dissections and tissue harvest were carried out within a 2 hour window (7:00 am-9:00am CST) each day to minimize time-of-day fluctuations in HSC numbers (K. P. Singh et al., 2011). Fetuses were removed to culture dishes containing cold DMEM complete media.

For all HSC experiments, fetal livers were removed from individual fetuses, mechanically dispersed in DMEM complete, and passed through sterile Nitex nylon mesh (80 μ m; Wildco, Yulee, FL) to form single-cell suspensions. Mature fetal liver cells were eliminated by CD24-mediated (clone J11d.2) complement-dependent lysis with low tox Rabbit Complement (Cedarlane, Burlington, NC) at 37 $^{\circ}$ C for 30 minutes with gentle agitation. After centrifugation at 300 g for 5 minutes, pellet was then re-suspended in serum-free DMEM and depleted of dead cells by centrifugation over Lympholyte Mammal (Cedarlane) at 800g for 20 minutes (Carlyle and Zuniga-Pflucker, 1998). Remaining cells were harvested from the buffy layer, washed three times in DMEM complete, and sorted.

Sorting of hematopoietic stem cells was first based on the absence of mature lineage markers in a cocktail of antibodies including CD3, LY-76, B220, CD11b and GR-1, as described above. Lineage negative (Lin-) cells were further identified by the presence of Sca-1 and cKit, also described above.

Cells were sorted on a BD FACSAria III, DIVA version 6.1.3, equipped with four laser (violet 407 nM, blue 488 nM, yellow/green 561 nM and red 633 nM), four way sorting capacity and an automated cell deposition unit capable of sorting a single cell into an individual well on a 96 well tissue culture plate. Just prior to cell sorting, 10 μ l of the vital dye Sytox Blue (Life Technologies), 1mM, diluted 1:80, was added to label nuclei from dead or dying cells with permeable membranes. Sytox Blue negative cells were electronically gated on the 407 violet B detector. Subsequently, doublets were excluded using FSC Height X Width and SSC Height x Width discrimination. Lin- cells were detected on the Blue B detector with a 502LP mirror and 530/30 filter. Sca-1+ cells were detected on the yellow-green D detector with a 582/15 filter set. c-Kit+ cells were detected on the Red B detector with a 660/20 filter set. Lineage-Sca-1+cKit+ (LSK) cells were directly sorted into individual wells on a Costar tissue culture treated 96 well plate (Corning, Corning, NY) at concentrations ranging from 1-30 cells per well, or sorted into a single tube and then divided into aliquots of 4000 cells per well in a 24 well plate (Corning). Both 96- and 24-well plates were pre-seeded with mitomycin C treated OP9-GFP or OP9-DL1 adherent bone marrow stromal cells at 75-85% confluency (described below).

Blood and Tissue Harvest and Analysis

For analysis of immune cell ratios, mice were weighed and blood was harvested weekly, beginning at 5 weeks of age and ending at 12 weeks of age. For maxillary blood collection, mice were restrained by hand, then the right cheek was nicked with a 5 mm Goldenrod animal lancet (Medipoint, Inc., Mineola, NY). Approximately 100 μ L of blood was collected from the maxillary vein and deposited into a 2000 μ L microcentrifuge tube (Eppendorf) containing 50 μ L of heparin (Sagent Pharmaceuticals, Schaumburg, IL).

100 μ L of blood/heparin were washed in 1 ml Hank's buffered saline solution (HBSS; Corning CellGro), supplemented with 0.5% FBS (Invitrogen) and 0.1% sodium azide (J.T. Baker/Avantor). Prior to analysis, F_c receptors were blocked with 2.4G2, red blood cells were lysed with BD Pharm Lyse (BD Biosciences) and then cells were stained and analyzed for surface expression of CD4, CD8, CD19, CD11b, CD-3, Gr-1 and CD45 (see antibody section for details). Because CD4+CD8+ cells are normally found only in the thymus, any blood samples with populations of CD4+CD8+ were eliminated from our analyses (Fig. 10).

Throughout the experiment, tissue samples were harvested from mice at varying times. Mice were observed daily, and if a mouse appeared visibly ill (slow-moving, apparent tumor growth), it was euthanized by CO₂ inhalation followed by cervical dislocation according to the AMVA guidelines (AVMA, 2013). For some experiments, half of the mice were euthanized at week 8, and all remaining mice were euthanized at completion of the experiment on week 12.

After euthanasia, spleen and thymus were removed from individual mice, mechanically dispersed in HBSS (Corning CellGro), supplemented with 0.5% fetal bovine serum (Invitrogen) and 0.1% sodium azide (J.T. Baker/Avantor), and passed through sterile Nitex nylon mesh (80 μ m; Wildco, Yulee, FL) to form single cell suspensions. The cells were then depleted of red blood cells with lysis buffer, F_c blocked with 2.4G2, then stained and analyzed for surface expression of CD3, CD4, CD8, CD11b, and CD5.

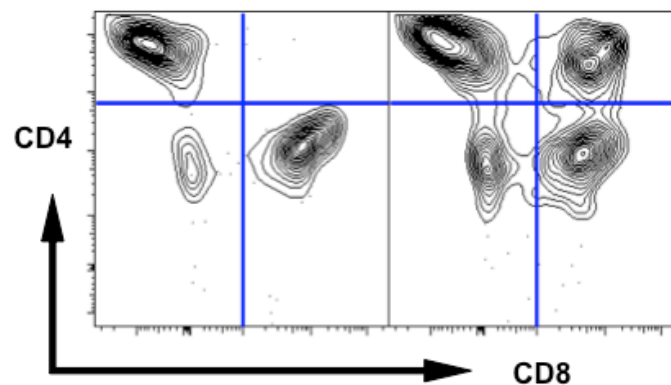


Figure 10. One measure of sickness in mice is presence of DP (CD4+CD8+) cells in peripheral blood. A) Flow cytometry plot of blood illustrating normal DP population; B) Flow cytometry plot of blood illustrating DP population in excess of 2.5%; Mice resembling B were eliminated from our analysis of bloods based on sickness, with DP cells indicating presence of a metastatic thymoma. *Figure originally included as supplementary for Chapter 5 manuscript.*

Placenta Harvest and Histology

Placentas were placed individually into processing/embedding cassettes (VWR, West Chester, PA) and preserved in 10% Neutral Buffered Formalin (VWR) for 48-72 hours, then transferred to 70% EtOH (Pharmco-AAPER, Brookfield, CT). Sample mounting and hematoxylin and eosin (H&E) stain were

completed at the CRI Histology Core Facility at the Medical College of Wisconsin, Milwaukee, WI. Slides were examined by a minimum of two readers, and areas of labyrinth and total placenta were calculated using ImageJ software (Schneider et al., 2012), following published guidelines (Susiarjo et al., 2013).

***In vitro* LSK Short-Term Self-Renewal Assay**

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

OP9 Co-Culture System

OP9 bone marrow-derived stromal cells transduced with either GFP (OP9-GFP) or Notch ligand delta-like 1-GFP-IRES (OP9-DL1) were a generous gift from Dr. Juan Carlos Zúñiga-Pflücker (Department of Immunology, University of Toronto, Sunnybrook, Ontario, Canada). From original cell lines, cells were expanded and then frozen in 1.2 ml of sterile 90% FBS+10% DMSO. Approximately one week prior to experiment, OP9-DL1 or OP9-GFP cells were

thawed from liquid nitrogen and grown in OP9 complete media, expanding to additional culture flasks (Corning) as needed to maintain sufficient growth space and confluency. Three days prior to establishment of co-culture, OP9-DL1 or OP9-GFP cells were plated into either 24-well or 96-well tissue culture treated plates (Corning) and allowed to grow to approximately 80% confluency, in a humidified 37°C incubator maintained with 5% CO₂. Prior to introduction of LSK cells, OP9 medium was added to the wells, supplemented with 5ng/ml Flt3L (PeproTech, Rocky Hill, NJ) and 5ng/ml Interleukin-7 (PeproTech). Sorted LSK cells were maintained in co-culture system for up to 15 days with media replacement (complete OP9 medium, supplemented with 5ng/mL Flt3L and 5ng/mL Interleukin-7) occurring every three days of co-culture.

For initial measurement of lymphocyte differentiation, 4000 LSK cells were plated in each well of a Costar 24 well cell culture plate (Corning) on day 0 and were harvested from triplicate wells every other day starting on day 5 and ending on day 15, diluting and expanding into new 80% confluent OP9-adherent wells when counts exceeded 1×10^6 cells per well.

For limiting dilution analyses, OP9 stromal cells were treated with 5µg/mL Mitomycin C (Acros Organics, Thermo-Fisher Scientific, Waltham, MA) 4-24 hours prior to initiation of co-culture. For Mitomycin C treatment, growth media was removed from 80% confluent OP9-GFP or OP9-DL1 cells and replaced with 100 µL of complete OP9 medium with 0.1% Mitomycin C. After 30 minutes of incubation at 37°C in darkness, the Mitomycin C/OP9 media was removed and cells were washed five times with sterile Phosphate Buffered Solution (PBS)

containing 2.5% FBS. On day 0, either 1, 3, 6, 10 or 30 cells were sorted into individual wells of a Costar 96 well cell culture plate (Corning). Sorted LSK cells were maintained in co-culture system for up to 15 days with media replacement (complete OP9 medium, supplemented with 5ng/mL Flt3L and 5ng/ml Interleukin-7) occurring every three days of co-culture. All wells were harvested and analyzed for presence of cell surface markers on day 12 for OP9-DL1 co-cultures and day 14 for OP9-GFP co-cultures as described above.

RNA Preparation

Hematopoietic cells from vehicle or TCDD-treated fetuses were isolated and sorted in TRIzol (Life Technologies) from GD 7.5 yolk sac, GD 11.5 AGM, and GD 14.5 fetal liver. 1-bromo-3-chloropropane (Sigma-Aldrich) was used to separate RNA into the aqueous phase after centrifugation. Resulting pellet was re-suspended in molecular-biology grade water. Yield and purity were quantified spectrophotometrically on a Nanoquant™ plate using OD260/OD280 ratio (Tecan) and was either made into cDNA or stored at -80°C. RNA was subjected to reverse transcription using Tetro cDNA Synthesis kit (Bioline) using both anchored-oligo(dT) 18 priming and random hexamer priming options and was stored at -20°C until day of assay.

Real-Time Quantitative Reverse Transcriptase PCR

Primers were selected using the Universal ProbeLibrary version 2.5 for Mouse (Roche) and checked for specificity using Primer-Blast (NCBI). cDNA was

used as a template in 20 μ l reaction consisting of 10 μ M of forward and reverse primers and 10 μ l SensiFAST SYBR No-ROX (Bioline). Gene names, accession numbers, and sequences are provided in Table 5. Relative expression change was determined using $2^{\Delta\Delta Ct}$ with standardization to housekeeping genes.

Samples were run in triplicate for each result on a Roche LightCycler480 system (Roche Applied Science, Indianapolis, IN). Cycling conditions were 95°C for 2 min, followed by 45 cycles of 95°C for 5s, 60°C for 10s, and 72°C for 10s.

For reference genes B-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Universal ProbeLibrary Mouse Gene Assays (Roche Applied Science, Indianapolis, IN) were used according to the manufacturer's directions.

Table 5. Gene name, accession number and primer sequence used for qRT²PCR

Name	Accession	5'→3' Forward	5'→3' Reverse
Cyp1A1	NM_009992.4	ccaagaggaagggcatagg	gagcagctctgtggctgac
Cyp1B1	NM_009994.1	agccaggacaccctttcc	gcctgaacatccgggtatc
AHR	NM_013464.4	cgggtccctgctcagta	ccagaagtcaaagctccaaa
ARNT	NM_001037737.2	ccagatttcacgtcactcca	ttggaagaacgagctctgg
TBP ^{1,2}	NM_013684.3	ggcggttggctaggttt	gggtatcttcacacacatga
SDHA ^{1,3}	NM_023281.1	gggaatggttgaacacag	tgcgcacagcatcagatt
HPRT ^{1,4}	NM_013556.2	gtcaacgggggacataaaag	caacaatcaagacattcttcca

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

² TBP housekeeping gene used on GD 7.5

³ SDHA housekeeping gene used on GD 11.5 and 14.5

⁴ HPRT housekeeping gene used on GD 7.5 and maternal liver

Apoptosis of Thymocytes via APO-BRDU™ TUNEL Assay

Thymocytes were harvested from Notch1^{ICN-TG} mice developmentally exposed to vehicle control or 3µg/kg TCDD. One million thymocytes per well were incubated overnight in 24 well plates (Corning) at 37°C and 5% CO₂ in RPMI (Life Technologies) supplemented with 10 mg/mL gentamycin (Gibco, Grand Island, NY), 10% FBS (Gibco), and 0.5M 2-Mercaptoethanol (EMD). Thymocytes were then stained for surface markers CD4 and CD8 as described above. After a 15 minute fixation with 1% paraformaldehyde, thymocytes were resuspended in 70% EtOH and frozen at -20° C overnight. A Terminal deoxynucleotide transferase dUTP Nick End Labeling (TUNEL) assay was performed following manufacturer's protocol outlined in the Phoenix Flow Systems, Inc. APO-BRDU™ Kit (San Diego, CA), eliminating the addition of Propidium Iodide/RNase A Solution. Cells were analyzed on BD FACSAria III as described above, using FSC-Area by SSC-Area for doublet exclusion, followed by identification of thymocytes by presence of both CD4 and CD8. Anti-BrdU cells were identified within the thymocyte population by FITC fluorescence.

Statistical Analysis

Statistical significance was determined and is denoted on graphs and tables as a single asterisk for $p \leq 0.05$, two asterisks for $p \leq 0.01$ or three asterisks for $p \leq 0.001$.

For precursor frequency calculations, we first determined an acceptable threshold value for a positive response using wild type (C57BL/6) mice. The

percentage of CD4+CD8+ cells for each well (Fig. 11A) was used to discriminate responsive from non-responsive wells. Using the extreme limiting dilution analysis (ELDA) program (Hu and Smyth, 2009), T cell precursor frequencies were calculated for CD4+CD8+ percentages at every point from 0.1% to 5.0% of the total cell population. The ELDA program returns a Chi Square value for each vehicle/TCDD frequency comparison. Chi Square values were plotted at each percentage point against the matching cell population percentage (Fig. 11B). The peak of this graph, where the Chi Square value indicated the greatest difference between control and exposed wells, was determined to be a range including the three highest peaks (2.0-2.8). The highest peak value was chosen (2.8%) and used to determine progenitor frequency for both B and T lymphocyte precursors. Data for the limiting dilution was analyzed and graphed according to the “limdil” function contained in the “statmod” package in Rstudio (RStudio, 2012). Our calculations are based on a compilation of all data from three replications of the OP9-DL1 co-culture system and three of the OP9-GFP co-culture system.

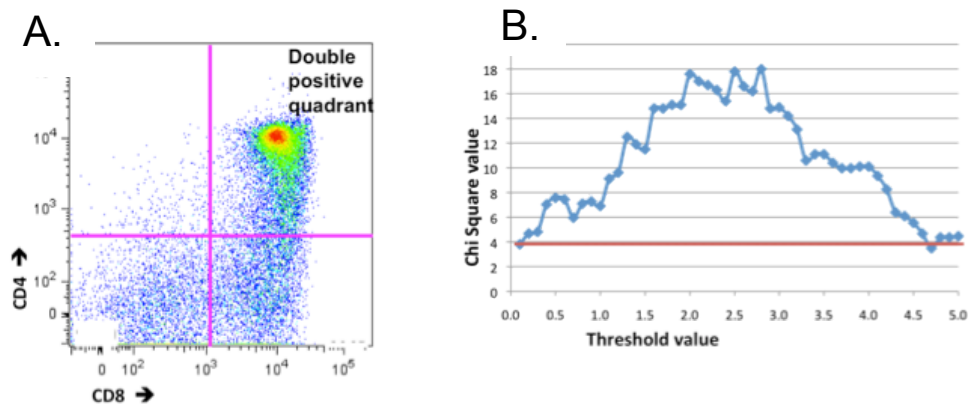


Figure 11. Positive growth score cut-off value was determined by graphing Chi Square values.

A. The percent of DP (CD8+CD4+) cells were graphed against B. Chi Square values. The highest peaks represent the appropriate range for labeling a positive result. Positive counts were made per well and associated Chi Square values calculated by Hu, et al's ELDA software. Images show CD8+CD4+ quadrant (T cells), but similar methods were employed for CD19⁺B220⁺ (B cells). *Figure originally included as supplementary for Chapter 3 publication.*

For limiting dilution analyses using AHR transgenic mice, data sets were cleaned prior to combination for final analysis. Because our yield of LSK cells from KO fetuses was occasionally very low, we eliminated repeats that had fewer than 20 wells of 30 cells per well. We then compared the remaining experiments to the 95% confidence interval previously determined for TCDD-exposed C57BL/6 mice (Ahrenhoerster et al., 2014). We eliminated any ELDA experiments for which progenitor frequency exceeded 1 in 49.9, using that number as indication that insufficient growth was achieved in the co-culture system for unclear reasons.

Graphpad Prism (Graphpad Software, LaJolla, CA) was utilized for analysis of variance with Dunnett's t post-hoc tests, Log-rank (Mantel-Cox) test of survival curve comparison, and graphical presentation of all other data.

CHAPTER 3

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

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ABSTRACT

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

inappropriate developmental AHR activation in HSC/HPCs adversely impacts lymphocyte differentiation and may have consequences for lymphocyte development in the bone marrow and thymus later in life.

INTRODUCTION

Hematological malignancies are the fifth most common cancer in the world and include lymphomas, acute lymphoblastic leukemias, and myeloproliferative neoplasms (Rodriguez-Abreu et al., 2007). These malignancies are the most common childhood cancer and the incidence rate for both children and adults has increased during the last 40 years (Howlader et al., 2013). One risk factor identified for leukemia comes from epidemiological analysis of Vietnam War military veterans exposed to the defoliant Agent Orange that was contaminated with 2,3,7,8 Tetrachlorodibenzo-p-dioxin (TCDD) (Institute of Medicine, 2011). In present day China, children living near electronic waste recycling facilities experience daily exposure to a spectrum of dioxin congeners (Han et al., 2011; Leung et al., 2011; Shen et al., 2010). Moreover, humans in industrialized countries can be exposed to dioxins through daily intake of meat, dairy and seafood (Kvalem et al., 2012; Liem et al., 2000). Thus, dioxin exposure continues to represent a significant global public health issue with potentially greater adverse effects for populations exposed during development because the effects can be longer lasting and may occur at lower developmental doses (Dietert et al., 2010; Hogaboam et al., 2007).

TCDD is the most potent agonist of the Aryl hydrocarbon receptor (AHR) and is known to impact a spectrum of adverse health conditions including malignancy, neurological disorders, and immune suppression (Institute of Medicine, 2011). We and others have shown that exposure to TCDD leads to thymic atrophy and impairs T lymphocyte development (Laiosa et al., 2003,

2002). Recently, the AHR has been implicated in mechanisms required for maintenance and differentiation of adult hematopoietic stem cells (Boitano et al., 2010; Casado et al., 2010; Gasiewicz et al., 2010; Lindsey and Papoutsakis, 2012; Smith et al., 2013). However, AHR-dependent effects on fetal hematopoiesis have received little recent attention despite the possibility that exposure to AHR agonists early in life could lead to long-term defects of hematopoietic stem cell self-renewal or differentiation.

Hematopoiesis is the process by which immature hematopoietic stem and progenitor cells (HSC/HPCs) develop into mature blood and immune cells, and during development this process is initiated in the yolk sac (Orkin and Zon, 2008; Samokhvalov et al., 2007). In fetal mice, the anatomical location and timing of hematopoiesis proceed such that the site of hematopoiesis migrates from the yolk sac to the aorta-gonads-mesonephros (gestational day (GD) 8-9), followed by the fetal liver and spleen (GD11-GD16). Ultimately HSC/HPCs localize to the bone marrow, which remains the site of hematopoiesis throughout adulthood (Orkin and Zon, 2008; Robin, C et al., 2003). In response to micro-environmental signals in the stem cell niche, a proportion of the long-term HSCs begin differentiation along multiple blood cell lineages, and become committed to more specific effector cell fates (Luc et al., 2008). For example, progenitors referred to as common lymphoid precursors that receive a signal through the trans-membrane protein Notch1, further differentiate into T lymphocytes whereas B lymphocytes are generated from common lymphoid precursors that fail to engage Notch1 (Deftos et al., 2000; Zúñiga-Pflücker and Schmitt, 2005).

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

While there is considerable evidence connecting adult TCDD exposure to immune suppression, the target hematopoietic cell population and the mechanism by which immune suppression after developmental exposure is occurring have not yet been fully elucidated. Therefore, we set out to test the hypothesis that developmental aryl hydrocarbon receptor activation by TCDD modulates the capacity of hematopoietic stem cells to undergo lymphocyte differentiation. In order to test this hypothesis, we co-cultured fetal liver HSC/HPCs in an *in vitro* co-culture system designed to drive differentiation along either the B or the T lymphocyte lineage. Our method of determining B or T lymphocyte differentiation capacity is particularly innovative because we have created an assay that is a hybrid of stem cell limiting dilution analysis and traditional toxicological assay. Importantly, we can accomplish this while approaching environmentally-relevant levels of TCDD exposure *in utero*. Using this quantitative *in vitro* co-culture system, we found a significant decrease in the

lymphocyte differentiation potential of HSC/HPCs developmentally exposed to TCDD. This decrease in the ability of fetal HSC/HPC to complete normal hematopoietic differentiation may increase risk for later-life hematological diseases such as malignancies or stem cell exhaustion (Lento et al., 2013; Stein and Baldwin, 2013).

RESULTS

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

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

exposed to TCDD (Fig. 12). Furthermore, the absolute number of LSK cells per fetus and the mean fluorescence intensity of Sca-1 containing cells is also significantly increased in fetal livers of mice exposed to TCDD transplacentally (Fig. 12C & 12D; $p \leq 0.01$).

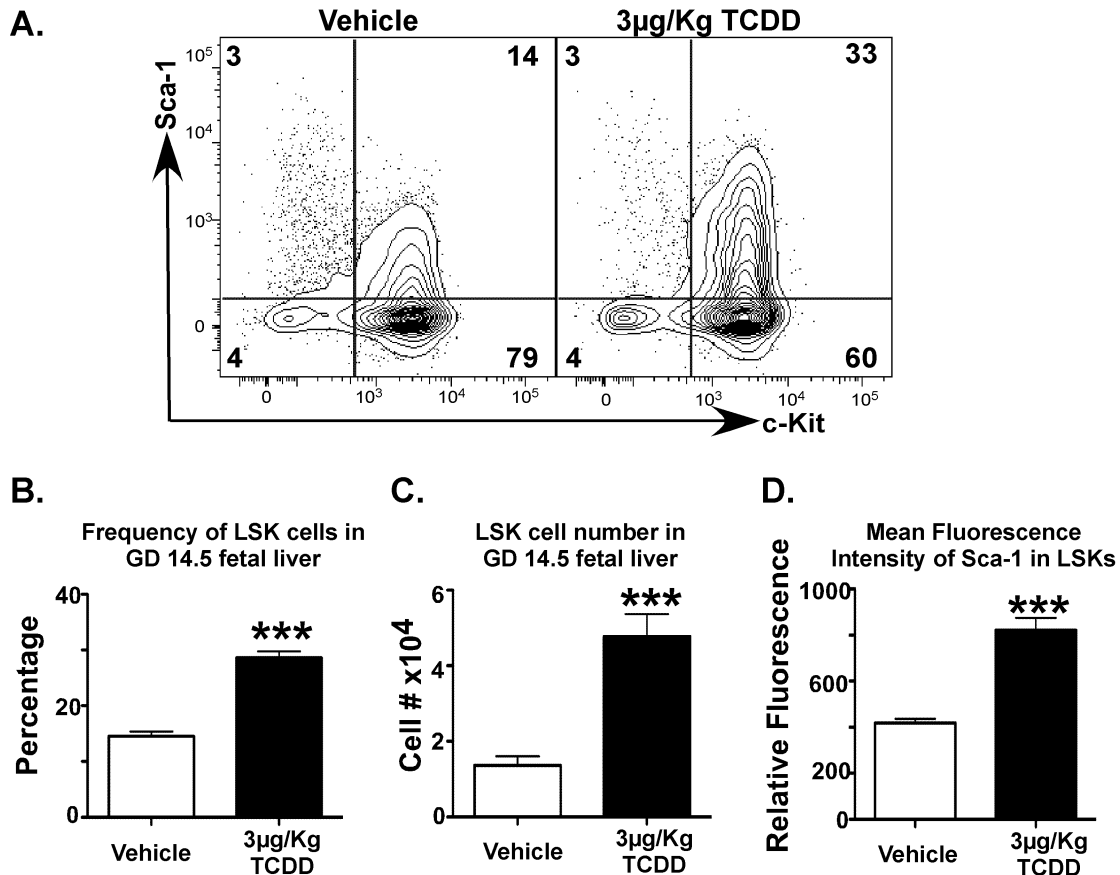


Figure 12. Hematopoietic progenitor cell frequency and number increase following developmental TCDD exposure *in vivo*. Pregnant C57BL/6 dams were exposed to 3µg/Kg TCDD or vehicle control throughout gestation and fetal liver cells were isolated on GD 14.5. A) representative fetal liver c-Kit versus Sca-1 flow cytometry plots are shown from vehicle- and TCDD-exposed dams. The numbers in each quadrant represent the percentage of Lin⁻ cells expressing cKit, Sca-1 or both. B) The percentage of fetal liver LSK cells from vehicle- and TCDD-exposed dams are shown. C) The percentage of LSK cells was multiplied by total fetal liver cell number to determine absolute number of LSK cells. D) The mean fluorescence intensity of Sca-1⁺ cells. Open histograms represent vehicle and shaded histograms represent TCDD. Data are presented as the mean \pm SEM (vehicle n=9; TCDD n=10). Statistical differences were determined by ANOVA and Dunnet's t post-hoc test. An *** is used to denote statistical significance with $p \leq 0.001$.

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

Alterations in the proportion of fetal HSC/HPC cells following TCDD exposure is predicted to be due to AHR expression and transcriptional activation in hematopoietic cells. Thus, gene expression of the AHR and its heterodimerization partner ARNT were analyzed in hematopoietic cells obtained from yolk sac on GD 7.5, the aorta-gonads-mesonephros (AGM) on GD 11.5 or GD 14.5 fetal liver during early fetal development. The AHR was expressed at all three time points and its gene expression was significantly increased in TCDD-exposed fetuses relative to control fetuses on GD 11.5 (Fig. 13A; $p < 0.01$). In comparison, ARNT was relatively unchanged except in the maternal liver where its expression was down modulated following 14.5 days of exposure (Fig. 13B). Maternal liver was used as a control to demonstrate the dose and method of TCDD-exposure (gavage) was sufficient to activate the adult AHR throughout the 14.5 day exposure window. Furthermore, induction of Cyp1A1 by TCDD exposure in GD 11.5 AGM HSC/HPC cells suggests the AHR-ARNT heterodimer is transcriptionally active in the fetus (Fig. 13C). Taken together, these data demonstrate the potential of the ligand-activated AHR to impact hematopoiesis in the fetus through transcriptional changes in gene expression.

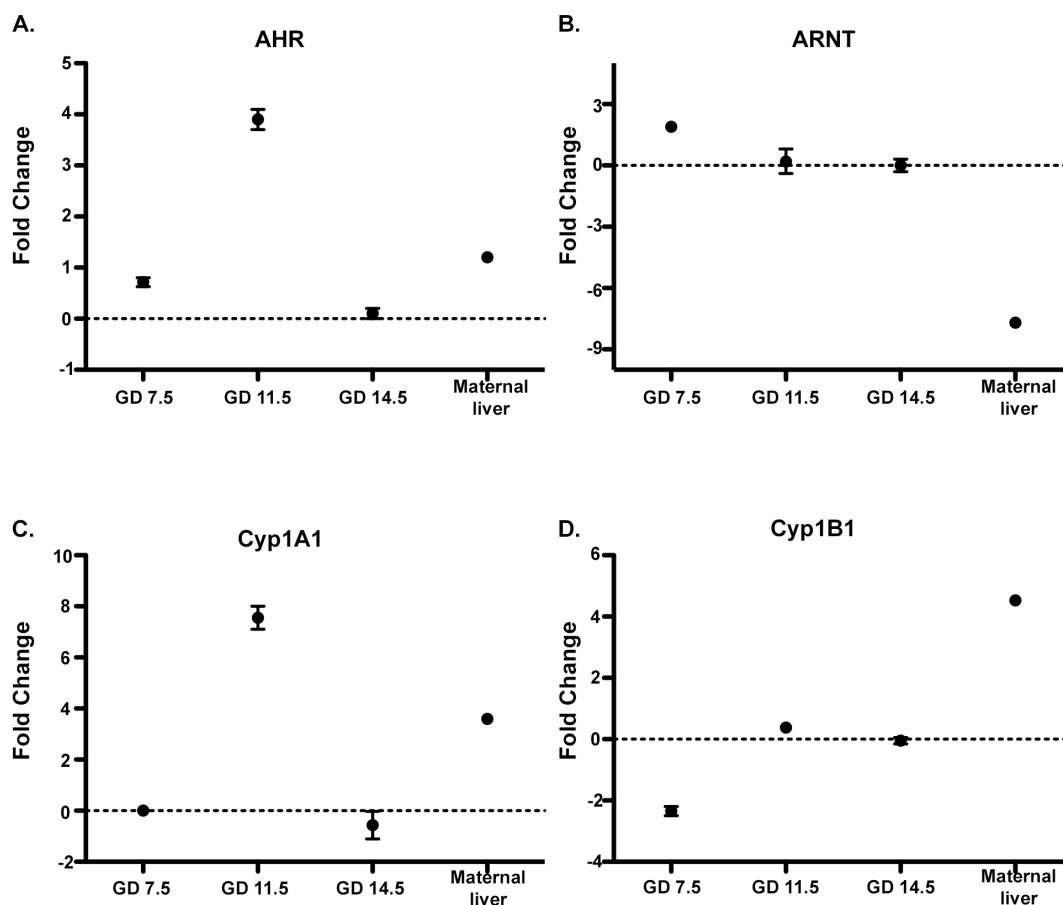


Figure 13. AHR and ARNT are expressed in fetal HSC/HPC cells and are transcriptionally active. Pregnant C57BL/6 dams were exposed to 3 μ g/Kg TCDD or vehicle control throughout gestation and HSC/HPC cells were sorted and RNA obtained from the maternal liver or fetal yolk sac on GD 7.5, aorta-gonadal-mesonephros on GD 11.5 and fetal liver on GD 14.5. RNA was reverse-transcribed and cDNA PCR amplified by quantitative real-time PCR. A) The TCDD-induced fold change of AHR gene expression is shown for the three gestational time points and in maternal liver. The housekeeping genes used for each developmental time point are indicated in Table 1. B) TCDD-induced fold change for ARNT. C and D) The TCDD-induced fold change for AHR-ARNT target genes Cyp1A1 and Cyp1B1. The black dot represents the average fold change from two independent experiments with the error bars indicating the fold change from each individual experiment. RNA was pooled from HSC/HPCs sorted from an entire litter.

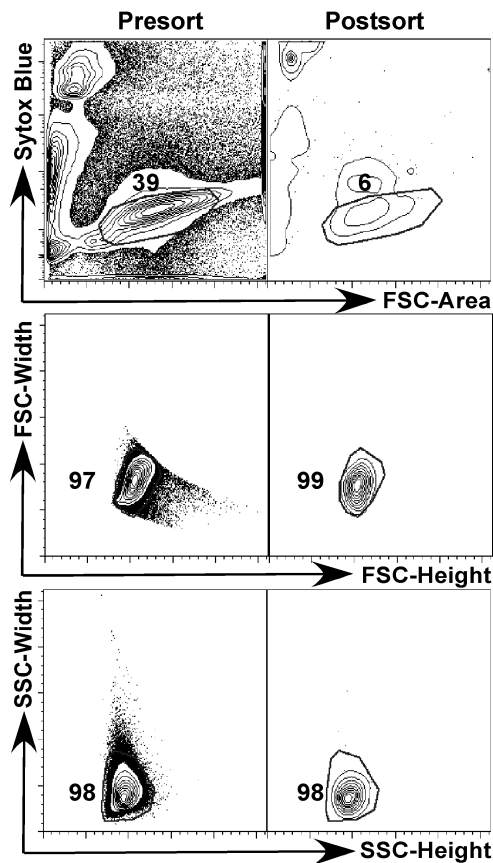
LSK cells obtained from GD 14.5 fetal livers exposed to TCDD have normal growth potential in vitro

In order to determine if the increase in the LSK cell population in TCDD-exposed mice was attributed to differences in their proliferative potential, LSK

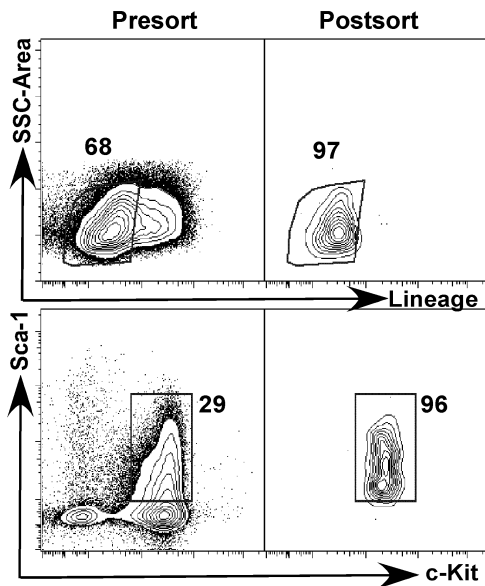
cells were sorted and cultured *in vitro* using serum free media optimized for expansion of short-term self-renewing HSCs and HPCs. To obtain a pure population of LSK cells, fetal livers from GD 14.5 mice were dissected and mature CD24⁺ cells were depleted by J11d mediated complement dependent lysis as previously described (Schmitt and Zúñiga-Pflücker, 2002). Subsequently, cells were prepared for fluorescence activated cell sorting and isolated with greater than 95% purity (Fig. 14A & 14B).

Once placed in culture, LSK cells were supplemented with the stem cell growth factor (SCF), where there is an approximately 1000 fold expansion of LSK cells over the course of a two-week culture period (data not shown). During the initial phase of the culture period, there appeared to be a slight increase in the proportion of LSK cells in the culture but this was not statistically significant (Fig. 14C-14D). Moreover, by the end of the culture, there were no differences between the frequency and number of LSK cells obtained from vehicle or TCDD exposed fetuses (Fig. 14C-14D). These data indicate that the process of short-term self-renewal that is facilitated by this *in vitro* culture system is not a primary target of *in utero* TCDD exposure.

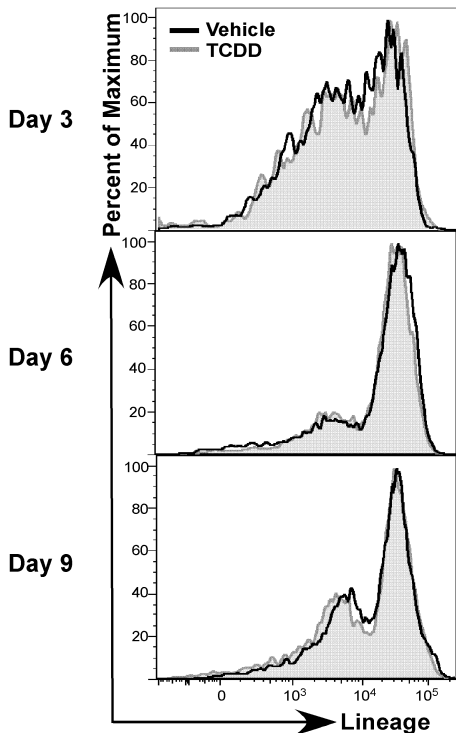
A.



B.



C.



D.

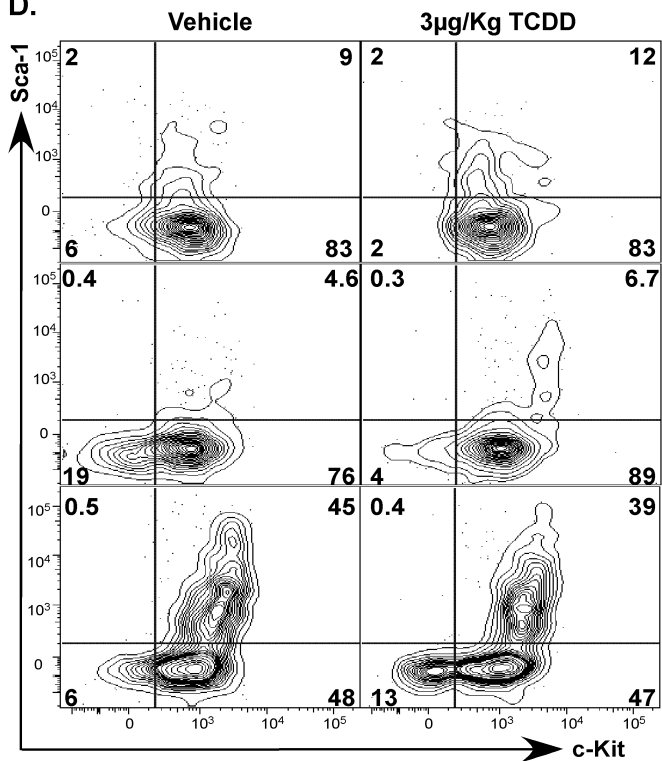


Figure 14. LSK cells obtained from GD 14.5 fetal livers exposed to TCDD have normal growth potential *in vitro*. LSK cells were sorted on the FACS Aria III following J11d mediated complement-dependent lysis. A) The gating strategy for sorting is shown and results from a pre- and post-sort representative sample are displayed. Briefly, viable cells based on Sytox blue dead cell exclusion were first gated, followed by doublet discrimination using Forward and Side scatter (FSC, SSC) height by width gating. Fluorescence gating strategy is shown with top panels showing lineage staining versus SSC-area. B) c-Kit+Sca-1+ events were sorted using the FACS Diva version 6.0 software purity mask. This sorting strategy was employed and purity was determined (>95%; data not shown) for all subsequent experiments. After cell sorting, five hundred LSK cells were grown in StemSpan SFEM media + 100 ng/ml SCF and cultured for 15 days. C) Representative histograms of lineage stained cells (CD11b, Ter-119, Gr-1, B220, CD3) are shown for days 3, 6 and 9. LSK cells from Vehicle-exposed dams are shown with a black line, and LSK cells from TCDD-exposed dams are illustrated in gray. D) cKit versus Sca-1 expression in LSK growth cultures is shown for Vehicle (left hand contour plots) or TCDD (right hand contour plots). The numbers in each quadrant represent the percentage of viable Lin- cells. Experiment was repeated three times.

TCDD induces cell cycle arrest in in vitro generated thymocytes

We have previously demonstrated that thymocytes obtained from adult mice exposed to TCDD undergo cell cycle arrest within hours of exposure (Laiosa et al., 2003), and that this effect is due to activation of the AHR in the hematopoietic cell population directly (Staples et al., 1998). Given the importance of AHR signaling in HSCs (Boitano et al., 2010; Gasiewicz et al., 2010), and thymic atrophy, a critical question that remains is what effect TCDD exposure has on HSC differentiation into cells of the lymphoid lineage. Up until now, answering this question has proven difficult because of the different anatomical locations where hematopoiesis and thymopoiesis occur. However, by using the OP9-DL1 *in vitro* system of thymocyte differentiation (Schmitt and Zúñiga-Pflücker, 2002), we can track the transition of hematopoietic stem and progenitor cells into thymocytes and determine the effect of AHR activation on this process. The first step in this process was to determine if OP9-DL1 *in vitro* generated

thymocytes have TCDD sensitivity similar to that of thymocytes obtained *in vivo*. This was accomplished by sorting unexposed LSK cells harvested from murine fetal livers into OP9-DL1 co-cultures and maintaining cells for 12 days. On day 11, co-cultures were exposed to various concentrations of TCDD or DMSO vehicle control. Twelve hours after exposure, BrdU incorporation within each stage of thymocyte development was analyzed. We observed an equivalent percentage and number of cells expressing CD44 and CD25 twelve hours after *in vitro* TCDD exposure indicating the distribution of early thymocyte progenitor populations within the co-cultures are unaffected by this short exposure (Fig. 15A). However, BrdU incorporation is significantly attenuated in the CD44-CD25+ (DN3) population of thymocyte progenitors (Fig. 15B; $p \leq 0.05$). Moreover, significant reduction in BrdU incorporation can be detected in cultures exposed to as low as 0.001nM TCDD (Fig. 15C; $p \leq 0.05$). Importantly, the nearly 50% reduction in BrdU within the CD44-CD25+ and CD44-CD25- populations is highly correlated with the TCDD-induced cell cycle inhibition observed in these specific thymocyte progenitor populations *in vivo* (Laiosa et al., 2003).

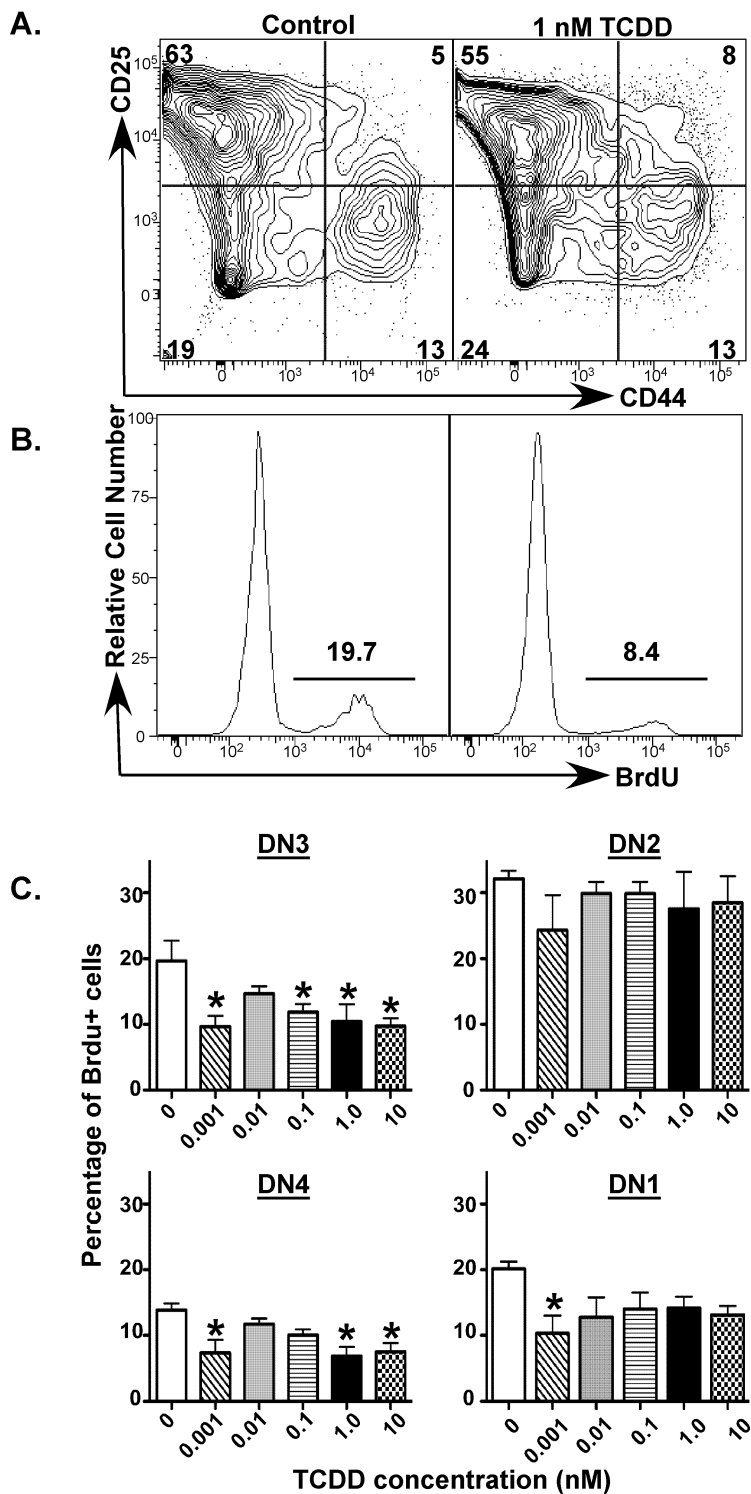


Figure 15. BrdU incorporation is decreased in TCDD-exposed thymocyte progenitors derived from LSK cells grown in OP9-DL1 co-cultures. A) Representative CD25 versus CD44 flow cytometry plots for day 12 OP9-DL1 co-cultures exposed *in vitro* to vehicle or 1nM TCDD for 24 h. Numbers inside each quadrant

represent the percentage of CD4-CD8- (DN) cells. B) Intracellular BrdU incorporation was measured in each thymocyte progenitor population and representative BrdU histograms are shown for the CD44-CD25+ population from one vehicle and one 1nM TCDD exposed culture. C) The percent of BrdU+ cells for each population of thymocyte progenitors 12 h after *in vitro* exposure to TCDD or vehicle. Cultures were dosed on day 11, and surface staining and intracellular BrdU staining was conducted on day 12. Experiment was repeated two times. Data are presented as the mean \pm SEM with 6 wells analyzed per group. Statistical differences are determined by ANOVA and Dunnet's t post-hoc test. An * is used to denote statistical significance with $p \leq 0.05$.

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

The demonstration that *in vitro* TCDD exposure in the OP9-DL1 – LSK co-culture system models the *in vivo* response to TCDD suggested that this system could be employed to follow the lymphocyte differentiation potential of LSK cells exposed to TCDD *in utero*. Thus, pregnant dams were exposed to vehicle or 3 μ g/Kg TCDD on GD 0.5 and 7.5 followed by cell sorting of LSK hematopoietic stem and progenitor cells on GD 14.5. LSKs were then placed into OP9-DL1 or OP9-GFP control co-cultures and thymocyte or B lymphocyte differentiation was tracked every two days in cultures seeded with either vehicle or TCDD-exposed LSK cells. Following developmental exposure to TCDD, no statistically significant difference was found in the rate of LSK maturation into thymocytes in OP9-DL1 co-culture when 4000 cells were plated per well (Fig. 16). Similar results were found with B cell progenitors grown in co-culture with OP9-GFP cells (Fig. 17). Notably, a slight but not statistically significant difference in the frequency of CD4+CD8+ thymocytes produced by OP9-DL1 co-cultures was detectable between the vehicle- and TCDD-exposed wells at the end of the culture period.

This slight difference led us to conclude that any subtle variation may have been obscured by the high number (4000) of starting LSK cells per well.

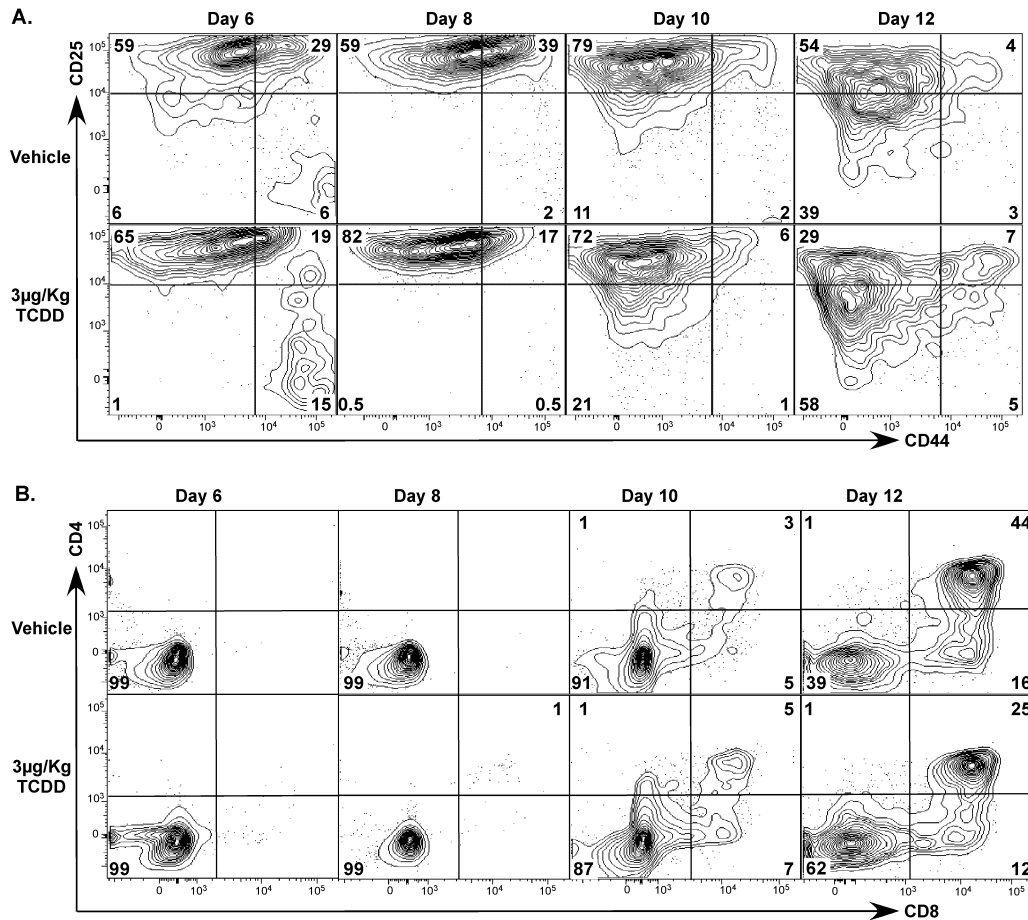


Figure 16: Fetal liver LSK cells co-cultured with OP9-DL1 bone marrow stromal cells undergo thymocyte differentiation with similar kinetics following developmental vehicle or TCDD exposure. Fetal liver LSK cells were isolated on GD14.5 as described and placed into OP9-DL1 co-cultures. Beginning on day 6 of co-culture, and continuing every other day, cells were harvested, counted, and stained with fluorochrome-labeled antibodies directed against CD4, CD8, CD44, and CD25. A) Representative CD4-CD8- gated cells were analyzed for the percentage of CD44 versus CD25 from vehicle- and TCDD-exposed dams. The numbers in each quadrant represent the percentage of viable cells within the CD4-CD8- gate. B) CD8 versus CD4 expression on each day of the differentiation assay is shown. The numbers in each quadrant represent the percentage of viable cells. Experiment was repeated five times.

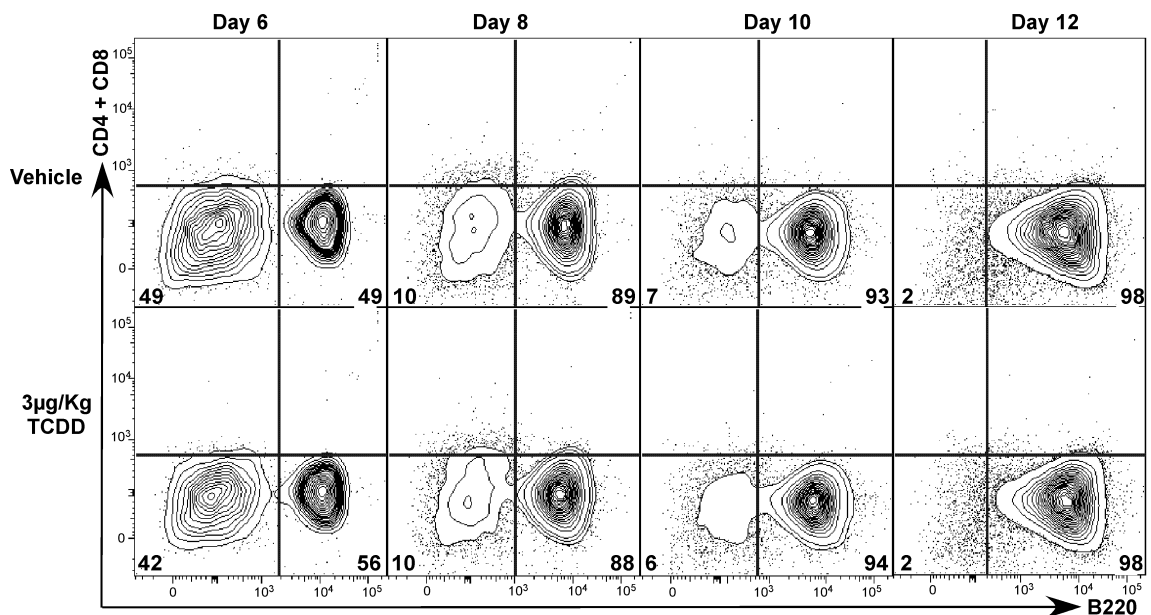


Figure 17: Fetal liver LSK cells co-cultured with OP9-GFP bone marrow stromal cells undergo B lymphocyte differentiation with similar kinetics following developmental vehicle or TCDD exposure. Fetal liver LSK cells were isolated on GD14.5 as described and placed into OP9-GFP co-cultures. Beginning on day 6 of co-culture, and continuing every other day, cells were harvested, counted, and stained with fluorochrome-labeled antibodies directed against CD4 and CD8, and B220. Representative flow cytometry plots were analyzed for the percentage of B220 versus CD4 and CD8 from vehicle- and TCDD-exposed dams. The numbers in the bottom right quadrants represent the percentage of viable B lymphocytes. Experiment was repeated five times. *Figure originally included as supplementary.*

Developmental TCDD exposure decreases lymphocyte differentiation potential of LSK cells

In order to precisely quantify the lymphocyte differentiation potential on a per cell basis following developmental vehicle- or TCDD-exposure, a limiting dilution approach was employed to the OP9 – LSK co-culture system. Following prenatal TCDD exposure, exactly 1, 3, 6, 10, or 30 LSKs from GD 14.5 fetuses were deposited by FACS into individual wells in 96 well plates containing OP9-DL1 or OP9-GFP stromal cells. On day 12 for the OP9-DL1 co-cultures, cells from individual wells were harvested and stained with the thymocyte surface

markers CD4 and CD8 and the percentage of wells containing CD4+CD8+ cells was recorded. We found 1 in 16.9 (95% CI: 14.2-20.2) LSK cells have T cell potential compared with nearly 1 in 39.6 LSK (95% CI: 31.5-49.9) cells possessing T cell potential from the developmentally TCDD-exposed fetuses (Fig. 18A; Table 6; $p \leq 0.01$). Developmental TCDD exposure was observed to also reduce by nearly two-fold the B lymphocyte precursor frequency for LSK cells as determined by day 14 OP9-GFP limiting dilution co-culture analysis (Fig. 18B; Table 7). Taken together, these data demonstrate that prenatal TCDD exposure significantly decreases the number of fetal liver LSK cells with potential to produce T and B lymphocyte progeny.

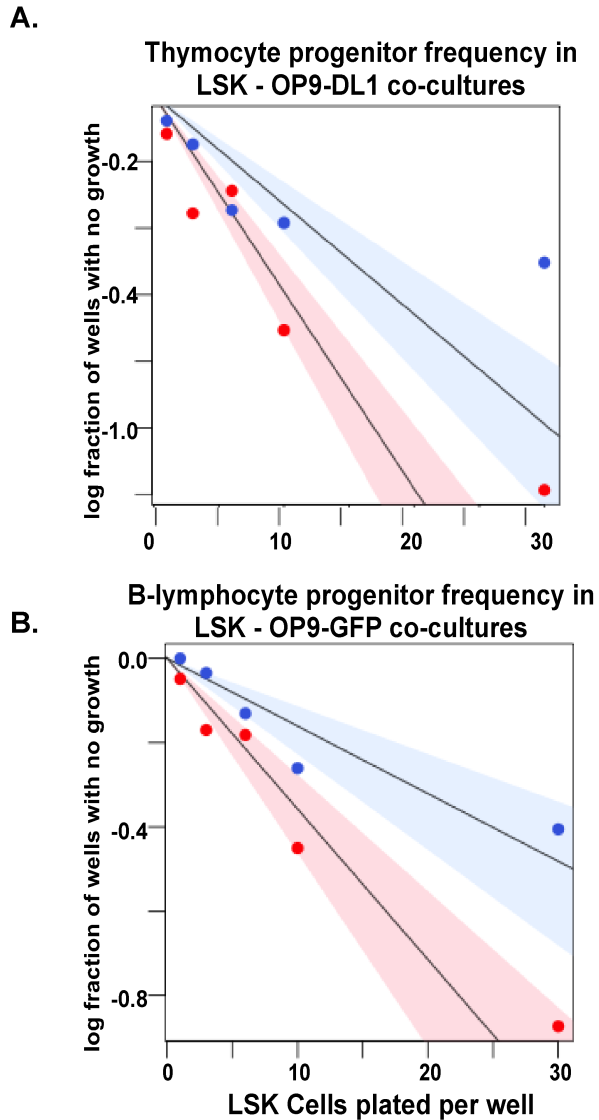


Figure 18: Developmental TCDD exposure decreases lymphocyte differentiation potential of murine fetal liver HSCs. Fetal liver LSK cells were isolated on GD14.5 as described. Exactly 1 to 30 LSK cells were directly sorted into individual wells of a 96 well culture plate containing a confluent layer of mitomycin C-treated OP9-DL1 or OP9-GFP cells and maintained for 12 (DL1) or 14 (GFP) days. Individual wells were analyzed for the generation of thymocytes (CD4+CD8+) or B cells (CD19+B220+). The number of positive responses for each cell concentration was entered into the *limdil* package in R Studio (RStudio, 2012) and plotted using ELDA software. A) Thymocyte wells from OP9-DL1 co-cultures were scored as a positive response if $\geq 2.8\%$ of total cells in the well were CD4+CD8+. B) B-lymphocyte wells from OP9-GFP co-cultures were scored as a positive response if $\geq 2.8\%$ of total cells in the well were CD19+B220+. Precursor frequency is reported in Tables 2 and 3. Vehicle samples are represented by red circles (3 replicates) and TCDD-exposed samples are represented by blue filled circles (3 replicates). 95% CI is shaded around each treatment (vehicle-pink; TCDD-light blue; $p \leq 0.001$).

Table 6. Thymocyte progenitor frequencies after developmental exposure^a

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

^aPregnant C57BL/6J mice were exposed on gestational days (GD) 0.5 and 7.5 to either 3µg/Kg of TCDD or vehicle; fetal liver LSK cells were isolated on GD 14.5 as described.

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

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

Table 7. B lymphocyte progenitor frequencies after developmental exposure^a

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

^aPregnant C57BL/6J mice were exposed on gestational days (GD) 0.5 and 7.5 to either 3µg/Kg of TCDD or vehicle; fetal liver LSK cells were isolated on GD 14.5 as described.

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

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

DISCUSSION

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

The observed increase in fetal LSK cells is consistent with studies showing similar alterations in adult bone marrow, however, the *in vitro* co-culture functional assay offers advantages compared with more traditional immunotoxicological hematopoiesis assays. Previous research has used stem cell transfer to determine the eventual differentiation ability of TCDD-exposed cells (Casado et al., 2011; Fine et al., 1989). However, these methods use an abundance of stem cells isolated from fetal livers and/or bone marrow. For example, when seeding *in vitro* OP9 cultures with 4000 LSK cells a sufficient number of LSK cells possessing T or B lymphocyte potential were present impacting the sensitivity necessary for determining if *in utero* exposure to TCDD impairs differentiation. It is possible that earlier LSK transfer experiments may have the same effect. To overcome the obstacle an excess of cells in an assay

may present, limiting dilution is used to determine frequencies of cells in a mixed population that have a particular function and therefore is commonly used for progenitor cell applications. The benefit of the *in vitro* limiting dilution co-culture differentiation system that we use is the ability to track the progression of a single HSC to its eventual progeny. Furthermore, numerous single cells from one sample of sorted HSCs can be tested to determine a numerical differentiation potential. This method allows focus not only on individual effects, but also on the earliest steps in differentiation. While the frequency of lymphocyte differentiation using the OP9 co-culture system has been determined using limiting dilution analysis from hematopoietic precursors in mice (Balciunaite et al., 2005) and in humans (De Smedt, Magda et al., 2011; Kyoizumi et al., 2013), this is the first time it has been employed to test the effect of a developmental toxicological exposure on a progenitor cell population's ability to differentiate.

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

mature and can be biased away from production of the common lymphoid precursors that directly produce lymphoid restricted cells. In support of this interpretation, we performed a test of heterogeneity for each limiting dilution experiment known as a log-dose slope test. We found that all treatment groups produced a log-dose slope of less than 1 (data not shown). A slope less than 1 demonstrates that the fetal liver LSK populations tested are a heterogeneous cell population (Hu and Smyth, 2009), with the TCDD-exposed fetuses producing a LSK cell population with significantly more heterogeneity than controls.

In support of the conclusion that developmental TCDD exposure changes the composition of the self-renewal potential of fetal LSKs, we observe higher Sca-1 expression on GD 14.5 (Fig. 12). Others have correlated high levels of Sca-1 expression with decreased differentiation in the erythroid lineage (Azalea-Romero et al., 2012) and decreased ability to form myeloid colonies (Bradfute et al., 2005) as well as patterns of decreased T cell lymphopoiesis in constitutively-expressing Sca-1 transgenic mice (Bamezai et al., 1995). Furthermore, in a mouse model for the autoimmune disease lupus, up-regulation of Sca-1 correlated with a more severe disease state (Kumar et al., 2005). Taken together, this supports Sca-1 as a potential target identifying loss of multi-lineage differentiation due to developmental exposure to TCDD. The higher mean fluorescence, and thus protein expression of Sca-1 could predict an increased risk of hematopoiesis defects over the life-course.

In summary, we have for the first time demonstrated that prenatal exposure to the environmental toxicant and AHR agonist TCDD significantly

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

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CHAPTER 4

**Developmental Arylhydrocarbon Receptor Activation in the Fetus
Decreases T Lymphocyte Differentiation Ability**

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ABSTRACT

Environmental exposures during gestation can profoundly affect future disease development. Immune cells, along with all other blood cells, descend from hematopoietic stem cells (HSCs), which are formed *in utero*. Exposure to 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD) *in utero* has previously been shown to decrease the potential of fetal liver HSCs to differentiate into mature lymphocytes and to shift the balance of adaptive and innate immune response in adult offspring. However, it is not known whether these effects are due to fetal or maternal factors. We hypothesized that persistent developmental aryl hydrocarbon receptor (AHR) activation in the fetal HSCs was responsible for the immune dysfunction, as TCDD is a potent AHR agonist. HSCs were harvested on GD 14.5 from gene-targeted mutant AHR mice developmentally exposed to 3 µg/kg TCDD or vehicle control. HSCs were quantified and placed into T lymphocyte differentiation cultures using a limiting dilution approach. We found the ability for HSCs to differentiate into mature thymocytes is mediated by the AHR in the individual fetuses. Specifically, the T cell differentiation potential of HSCs from TCDD-exposed fetuses lacking an AHR is similar to that of HSCs from vehicle-exposed wild type fetuses. Conversely, the TCDD-exposed siblings who had an AHR produced HSCs with a diminished T cell precursor potential. These data suggest that developmental AHR activation in HSCs reprograms the balance between self-renewal and differentiation, potentially affecting immune system development and function later in life.

INTRODUCTION

Hematopoietic stem cells (HSCs) are the foundational cells of the blood system, responsible for maintenance of all cells in the blood and immune system by balancing the processes of self-renewal and lineage differentiation to produce mature effector cells. The process of hematopoiesis maintains these immune cells throughout a person's lifetime. Consequently, there is a growing recognition that factors that disrupt the processes of HSC self-renewal or differentiation can have profound effects on long-term immunity (Seita and Weissman, 2010; Yamada et al., 2013). For example, transplacental exposure to the ubiquitous environmental contaminant 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD) leads to numerous immunological deficits later in life.

TCDD, which is produced as a combustion by-product, mediates its immunotoxicity in adult mice by binding to and activating the aryl hydrocarbon receptor (AHR), an important regulator of immune system development and function. AHR, a basic helix-loop-helix protein and member of the PAS family of transcription factors, is a principle player in the detoxification pathway of many environmental contaminants in addition to dioxins such as TCDD, specifically the halogenated aromatic hydrocarbons, including polychlorinated biphenyls (PCBs), aromatic amines, and polycyclic aromatic hydrocarbons (PAHs). The AHR is evolutionarily conserved, and can be activated by either endogenous or exogenous ligands (Smith et al., 2013).

AHR is known to be implicated in regulation of immune processes including inflammation (Hanieh, 2014; Nguyen et al., 2013) and autoimmunity

(Apetoh et al., 2010; Nguyen et al., 2013). The AHR is essential for immune development, including regulating hematopoiesis (Casado et al., 2010) and inducing regulatory T cells (N. P. Singh et al., 2011; Wu et al., 2011). However, most immunological research using TCDD as an AHR agonist uses adult animals or cell culture lines (Beedanagari et al., 2010b; Benson and Shepherd, 2011; Casado et al., 2011; Kerkvliet, 2009; Laiosa et al., 2002; Takamura et al., 2010). Almost all studies involving developmental TCDD exposures have focused only on adult endpoints (Lew et al., 2009; B A Vorderstrasse et al., 2004) and not directly on the HSCs. Understanding specifically the mechanisms by which AHR activation during development causes immune system malfunction and where it impacts hematopoiesis remains a significant knowledge gap.

The AHR is found in embryonic stem cells (Ko et al., 2014) and HSCs (Singh et al., 2009), and we have found that it is up-regulated in HSCs on GD11.5 following developmental exposure to 3 μ g/kg of TCDD (Ahrenhoerster et al., 2014). Our earlier research illustrates that developmental dioxin exposure decreases the ability of HSCs to differentiate into B or T lymphocytes (Ahrenhoerster et al., 2014). However, whether this is due to AHR activation in the fetus, the placenta, or the mother has yet to be elucidated. Therefore we set out to determine the mechanism by which the decreased differentiation occurs, hypothesizing that the AHR in fetal HSCs is directly affected by exposures during gestation.

In order to test this hypothesis, we co-cultured fetal liver HSCs, defined as Lin⁻, cKit⁺, Sca-1⁺ (LSK) cells, in an *in vitro* system designed to drive thymocyte

differentiation. LSKs were isolated from fetuses of AHR heterozygous (HZ; AHR^{+/-}) x AHR knockout (KO; AHR^{-/-}) or HZ x HZ crosses, thus producing siblings of different genotypes while holding all other variables related to development constant. Using limiting dilution analysis to examine the differentiation potential, we found a significant decrease in the thymocyte differentiation potential of HSCs developmentally exposed to TCDD and isolated from fetuses possessing AHR. HSCs isolated from fetuses from the same mother but lacking AHR did not exhibit a decreased differentiation potential. This model is especially robust as it allows us to focus on AHR activation in the fetus while providing a uniform uterine environment during gestation. These findings support the role of AHR in the fetus as a target of TCDD developmental immunotoxicity.

RESULTS

The question of whether AHR gene dosage impacts response has been considered by us and other researchers. Others have shown no response difference between AHR^{+/+} (wild type; WT) or AHR^{+/-} (HZ) mice, as both possess the AHR⁺ allele (K. P. Singh et al., 2011). We similarly found no significant difference in co-culture growth potential (unpublished data), so in our experiments, we combined AHR^{+/+} with AHR^{+/-} to represent AHR⁺ HSCs and compared them to AHR⁻ HSCs, consisting entirely of AHR^{-/-} (KO).

HSCs from AHR⁺ fetuses developmentally-exposed to TCDD showed a decreased potential to differentiate into mature thymocytes in comparison to HSCs from AHR⁻ fetuses developmentally-exposed to TCDD.

Developmental exposure to the potent AHR agonist TCDD in C57BL/6 (WT) mice decreases lymphocyte differentiation potential, and the AHR is expressed and transcriptionally active in fetal HSCs (Ahrenhoerster et al., 2014). We therefore hypothesized that the differentiation impairment following developmental TCDD exposure is due to activation of the AHR in the fetus, thus impacting HSCs at a very early timepoint in development.

Among TCDD-exposed siblings developing in the same uterine environment, the HSCs from AHR⁻ fetuses were over two-fold more likely to complete differentiation into mature thymocytes than HSCs from their AHR⁺ siblings (Fig 19A & C). This supports the hypothesis that the differentiation-limiting actions of TCDD are acting through the AHR in fetal HSCs and that the

absence of an AHR provides an element of protection to the HSCs exposed to TCDD during thymocyte commitment.

In order to further clarify our initial findings that the AHR must be present in order for TCDD to cause decreased differentiation of HSCs, we further hypothesized that HSCs isolated from either AHR+ or AHR- fetuses developmentally exposed to vehicle control throughout gestation would have comparable thymocyte differentiation potential. Using limiting dilution analysis, we measured the differentiation potential of HSCs isolated from sibling AHR+ and AHR- fetuses developmentally exposed to vehicle control. We found that HSCs from vehicle-exposed AHR+ and AHR- fetuses did not differ in thymocyte differentiation potential (Fig. 19B & 19C).

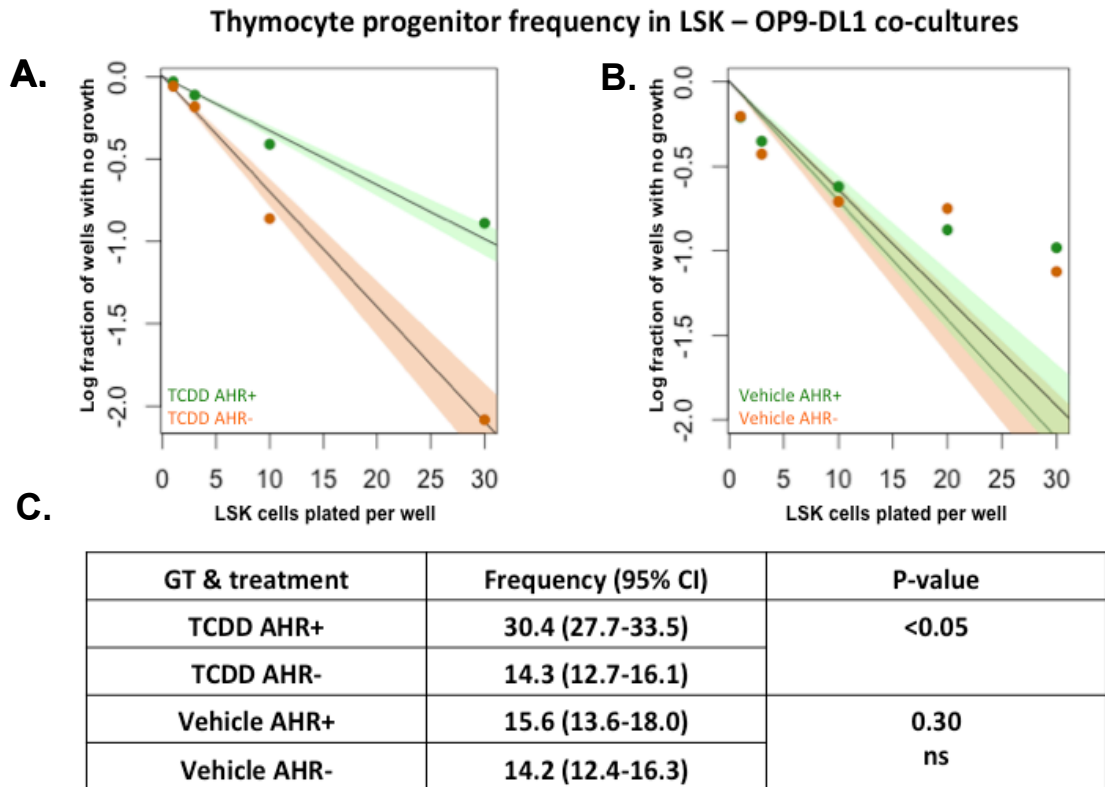


Figure 19. Presence of fetal AHR decreases lymphocyte differentiation potential of murine fetal liver HSCs exposed developmentally to TCDD, while vehicle-exposed HSCs show no difference in differentiation potential regardless of AHR presence. Fetal liver LSK cells were isolated on GD14.5 as described. Exactly 1 to 30 LSK cells were directly sorted into individual wells of a 96 well culture plate containing a confluent layer of mitomycin C-treated OP9-DL1 cells and maintained for 12 days. Individual wells were analyzed for the generation of thymocytes (CD4+CD8+). The number of positive responses for each cell concentration was entered into the *limdil* package in R Studio (RStudio, 2012) and plotted using ELDA software (Hu and Smyth, 2009). Thymocyte wells from OP9-DL1 co-cultures were scored as a positive response if $\geq 2.8\%$ of total cells in the well were CD4+CD8+. A) HSCs isolated from fetal mice developmentally exposed to 3 $\mu\text{g}/\text{kg}$ of TCDD throughout gestation (pooled data from 5 separate experiments); B) HSCs isolated from fetal mice developmentally exposed to vehicle control throughout gestation (pooled data from 3 separate experiments); C) Calculated precursor frequency; AHR- samples represented by orange and AHR+ samples represented by green; 95% CI is indicated by a color-matched shaded area around each treatment.

AHR knock-out fetuses are slightly less likely to survive to parturition.

Through the duration of limiting dilution experiments with AHR transgenic mice, the number of KO fetuses was anecdotally observed to be smaller than the number of either WT or HZ fetuses. We recorded the genotypes of all AHR variant fetuses as genotyped during all repeats of this experiment and observed genotype ratios were compared to expected genotype ratios using Chi square analysis (Table 8). We found that on average there were fewer KO fetuses than expected based on predicted Mendelian inheritance ratios, although none of the values reached levels of significance at the $p \leq 0.05$ value.

Table 8
Genotype results of offspring from AHR transgenic matings

Paternal GT ¹	treatment	#WT	#Het	#KO	p-value ²
HZ	vehicle	40	68	33	0.65
HZ	TCDD	23	28	14	0.15
KO	vehicle	-	21	11	0.11
KO	TCDD	-	12	8	0.50
HZ	vehicle + TCDD combined	63	96	47	0.18
KO	vehicle + TCDD combined	-	33	19	0.07

¹Maternal GT in all cases was HZ

²p-value calculated by Chi-square analysis, comparing observed to expected genotype ratios

Litters fathered by AHR KO mice are significantly less likely to survive until parturition.

We observed in the AHR transgenic mice, anecdotally, a high number of fetal reabsorptions or spontaneous abortions, determined by masses of tissue in the uterus which are significantly smaller than viable fetuses. It was noted that in these experiments, a larger percentage of fetal reabsorptions were observed

when the father was a KO male than in uteri of exclusively WT x WT vehicle mice, reported as $9.69\% \pm 11.6\%$ in the literature (Kusakabe et al., 2008), and similarly observed as $9.12\% \pm 11.7\%$ in our laboratory (unpublished observation).

We attempted to genotype the tissue masses that were in the process of being reabsorbed, but the placenta is a mixture of fetal and maternal tissue which we were unable to separate. Given that all mothers were HZ, all electrophoresis gel genotype results showed double bands, representative of the HZ genotype. However, the mean number of fetal reabsorptions per litter with a HZ father was 1.08, while with a KO father, it was 2.56 ($p \leq 0.05$). Additionally, while the *percentage* of fetal reabsorptions from a HZ father was not significantly different from WT x WT matings (Z-test; $p = 0.39$), it was significantly higher when the father was a KO (Table 9; Z-test; $p \leq 0.05$). Interestingly, adding the number of reabsorbed fetuses to the number of KO fetuses brings the genotype ratio closer to the expected 50% with KO father and 25% with HZ father (Tables 8 and 9).

Table 9
Reabsorbed fetuses (spontaneous abortions) by parental genotype

Maternal GT	Paternal GT	# reabsorbed	% reabsorbed	p-value*
HZ	HZ	19	12.3%	0.20
HZ	KO	22	19.4%	0.01
WT	WT	36	7.6%	0.14

*p-values based on z-test comparing percent reabsorptions to published vehicle WT reabsorption percentages (Kusakabe et al., 2008)

Genotype does not affect size of maternal and fetal sections of placentae.

Because we saw an increase in the number of fetal reabsorptions when the father was AHR- and because the number of viable fetuses trended toward AHR+, we hypothesized that a higher percentage of AHR- fetuses were not surviving gestation. Perturbation of cell death or proliferation-related proteins might deter the growth and maintenance of the placentae, driving subsequent fetal reabsorption. Furthermore, placental development has been used as a measure of genetic or epigenetic defect (Susiarjo et al., 2013), potentially resulting in a failed placenta followed by fetal death and reabsorption. We collected, preserved, and stained placenta samples from each AHR genotype of fetuses at GD14.5 and then measured the fetal and maternal regions of the placenta using the methods previously described (Susiarjo et al., 2013) (Fig. 20A & B). We did not find a significant difference between the various genotypes (Fig. 20C, $p=0.37$) at GD14.5. However, in consultation with Dr. Martha Susiarjo (Susiarjo, 2014), and also with placental pathologist Dr. Sara Szabo (Szabo, 2014), we determined that GD14.5 is too late developmentally to witness breakdown of the placenta. GD 11.5 or earlier coincides with early placental failure and histological identification of abnormality that may lead to fetal death and reabsorption. Additionally, one of the most accurate means of determining placental insufficiency is by comparing mass of placenta in each experimental group--a procedure we had not followed. Our lab's future developmental work with mice will include addition of earlier harvest timepoints and placental weights (Fig 21).

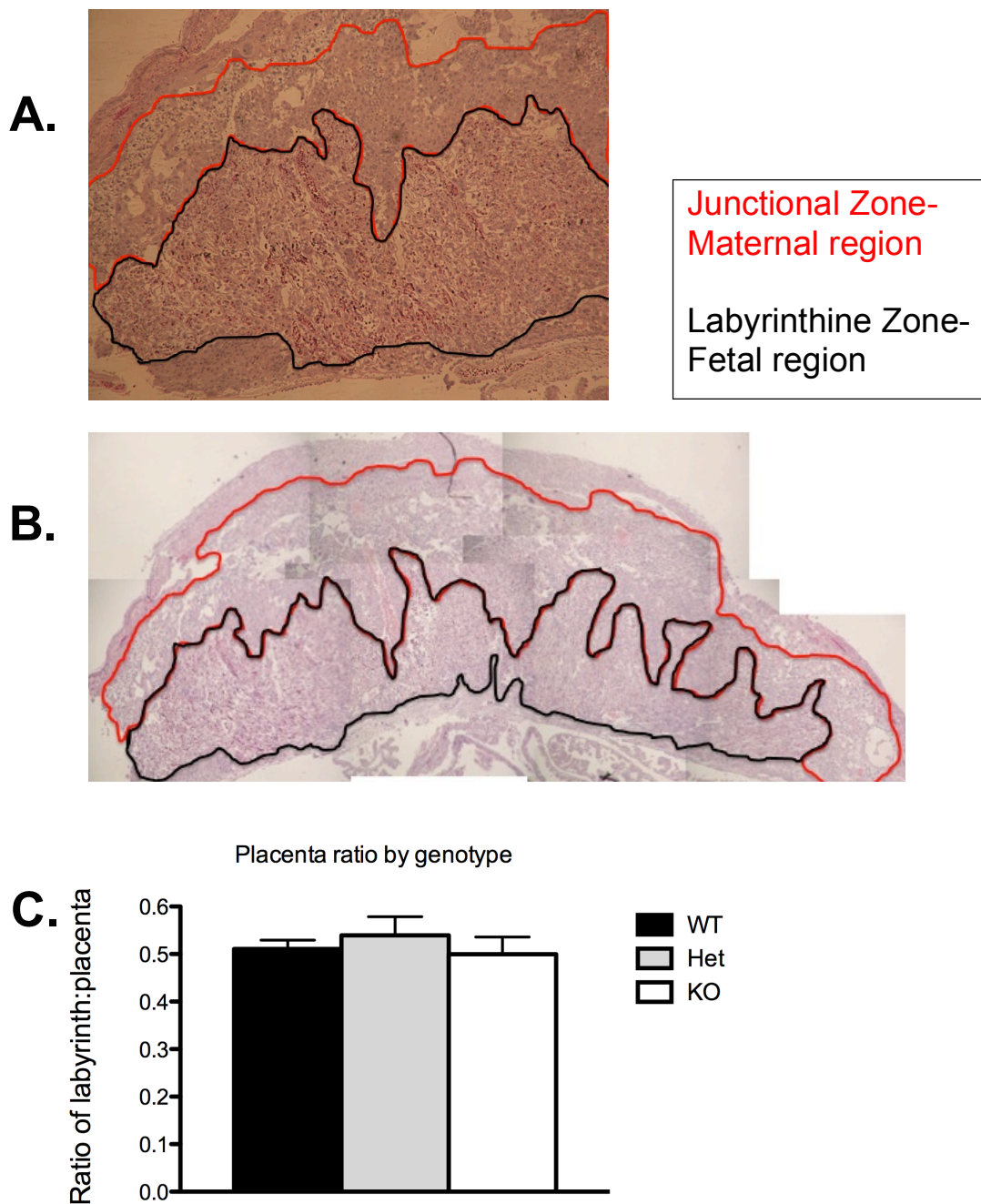


Figure 20. Labyrinth to placenta area ratio does not differ by genotype on GD14.5. Labyrinth is fetal portion of placental, as identified by presence of nucleated red blood cells. “Placenta” measurements include both the fetal and maternal portions illustrated in A and B. A) Representative section of a placenta harvested on GD14.5 viewed at the magnification necessary to differentiate maternal and fetal sections (40x); B) Cross-section of full placenta, piecing together numerous sections at lower magnification to illustrate placement on a full placenta (100x). No actual measurements were made at the lower magnification. C) Mean ratio of labyrinth (fetal) to full placenta (fetal + maternal regions) ($p=0.37$; $n=3$ of each genotype).

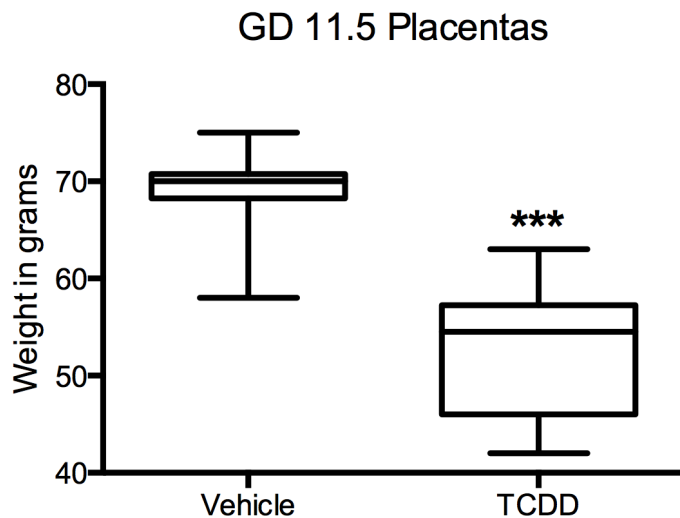


Figure 21. Mean placental weight differs between mice developmentally exposed to TCDD or vehicle control. Data from one sample experiment, illustrating the varied masses of placentae harvested on GD11.5 from WT mice developmentally exposed to either 3 μ g/kg TCDD or vehicle control. (n=8 vehicle; n= 22 TCDD; $p \leq 0.01$)

DISCUSSION

In the present study, we demonstrate that prenatal exposure to environmentally-relevant levels of TCDD acts through the fetal AHR to cause an alteration in lymphocyte differentiation ability. We show that a lack of AHR provides protection against the effects of developmental TCDD exposure, and that HSCs isolated from TCDD-exposed AHR- fetuses are able to complete T lymphocyte commitment at a frequency similar to HSCs developmentally exposed to vehicle control. After developmental TCDD exposure, HSCs from AHR+ mice, gestated in the same maternal environment as AHR- mice, exhibited a roughly two-fold decreased ability to commit as T lymphocytes, thus supporting the role of the fetal AHR in lymphocyte differentiation.

While our original hypothesis was supported, we are less than fully confident in our results, due to several inconsistencies in the data. AHR KO mice have deficiencies in multiple systems (Fernandez-Salguero et al., 1997). Given that the AHR is located in almost all murine and human tissues, additional, additional non-hematological defects likely exist, which might explain our difficulties in achieving consistent results throughout our experimental repeats.

In our experiments, we crossed HZ dams with either HZ or KO males, resulting in both AHR+ (HZ and WT) and AHR- (KO) fetuses housed in AHR+ mothers, to decrease external effects and to enable us to focus on fetal effects alone, controlling for any potential maternal factors that might alter development and/or hematopoiesis. We relied heavily on male AHR KO mice in order to increase the likelihood of KO fetuses and provide greater power to our HSC

differentiation calculations. HZ x HZ crosses have published 1:2:1 ratios of WT:HZ:KO (Schmidt et al., 1996), but the AHR has recently been implicated for its role in embryonic development (Ko et al., 2014; Sartor et al., 2009). While research shows that activation of the AHR can cause defects in development, absence of the AHR might also affect development. The lower number of AHR-fetuses found on GD14.5 supports the likelihood that AHR KO males have multiple defects, including reproductive ones. AHR KO males do display defects in seminiferous tubule and sperm development and are less likely to fertilize eggs (D. A. Hansen et al., 2013). It is therefore possible that fewer eggs were successfully fertilized or that those which were fertilized had genetic defects that resulted in spontaneous abortion. In fact, our counts of fetus size support this, in that AHR litters are slightly smaller than C57BL/6 litters, though not at a statistically significant level (data not shown). The HZ mothers were able to carry litters to term, so the defect does not seem to be entirely dependent on them and any maternal effect would be constant throughout these experiments, regardless of paternal genotype. We are not the first to notice the variations in genotype ratio (Hirabayashi and Inoue, 2009), although others posit that the lower KO ratios are due to competitive fertilization by AHR+ sperm instead of the demise of KO fetuses.

In order to reach sufficient power in our limiting dilution experiments, the results from several experiments were combined, and in all but one case, we examined only vehicle-exposed or only TCDD-exposed cohorts on any given day. The TCDD-exposed cohorts each gave very similar results with little

variation. However, we completed over twice as many vehicle-exposed experiments in order to establish consistent repeats. As described in the “Materials and Methods” section, we eliminated experiments with insufficient HSC co-culture growth, as defined by a calculated differentiation potential in excess of the published 95% confidence interval for WT TCDD-exposed HSCs (Ahrenhoerster et al., 2014). We further eliminated experiments lacking sufficient harvest of LSKs to sort a minimum of twenty 30-count wells. Additionally, the data points for the vehicle-exposed mice, while overall consistent, did not fit the graph constructed by the ELDA program as tightly (Fig. 19B) as did the points on the TCDD-exposed graph (Fig. 19A).

It is possible that we saw less variation in experiments with the TCDD-exposed cohorts because the presence of the potent AHR agonist TCDD is a stronger decision-maker in terms of lineage commitment than other endogenous mechanisms. Therefore, without the presence of this strong AHR agonist, the whole-organism defects of the AHR KO model become the predominant lineage decision-maker. Our work with this particular whole-mouse AHR KO model has posed several new questions about determination of AHR’s effect on hematopoiesis in the fetus.

One possibility, recently illuminated by the NIH Revitalization Act (Clayton and Collins, 2014), is related to the gender effect. Perhaps developmental TCDD exposure or AHR activation has a different effect in females than in males. TCDD is a known estrogen disruptor (Safe et al., 1998), and all mothers were HZ, so AHR+. Our rapid AHR genotyping allowed us to pool fetal livers to increase yield

of HSCs, but we did not genotype for fetal gender, so some alterations in response might also be gender-dependent. Any differences observed in the TCDD-exposed fetuses may have been modified by endocrine responses. Furthermore, without knowing fetal gender, we don't know if one genotype group had more of a single gender, or, indeed, if the fetal survival is affected by gender. If TCDD, as an endocrine disruptor, biases the sex ratio within a litter, then the TCDD-exposed fetuses would have comprised a more uniform gender group without our knowledge. The lack of variability in the TCDD-exposed experiments supports this explanation. Similarly, it is possible that AHR is not *required* for gender determination, but its presence or absence may influence gender. Future work with these mice should incorporate fetal sex determination.

There is increasing understanding that the intrauterine environment has great impact on the developing fetus. This applies not only to exposure to maternal nutrition and exposure to environmental contaminants, but potentially also to factors such as placement in the uterus. Most definitively, it has been shown that testosterone-related gender effects appear in mammal fetuses based on the gender of neighboring fetuses. Indeed, uterine position affects gender-related anatomy such as anogenital distance (Ryan and Vandenberg, 2002), physiology including levels of testosterone and estrogen (Vandenberg and Huggett, 1994; vom Saal, 1989) and gender ratio of future offspring (Vandenberg and Huggett, 1994), and behavior including novelty-seeking behavior (Palanza et al., 2001) and aggression (Ryan and Vandenberg, 2002). Furthermore, in rats this effect is enhanced by a single gestational dose of TCDD

(Timms et al., 2002). This concept, known as the “womb-mate effect,” has been examined in other phenotypes as well. For example, murine fetal circulation is negatively affected by gestational location near a dead/reabsorbed fetus or a fetus with a different genotype (Raz et al., 2012). Because our litters were mixes of WT, HZ and KO fetuses, in some cases a KO might have been located between two WT or HZ fetuses in the uterus. Given the fact that AHR is involved in multiple developmental pathways, even though the KO fetus lacked any AHR alleles, its proximity to an AHR+ fetus (or a reabsorbed fetus) might have affected its development and therefore the response of its HSCs. Interestingly, our results from the limiting dilution analyses using HSCs from vehicle-exposed fetuses (Fig. 19B) did not fit the ELDA model as well as those from TCDD-exposed mice (Fig. 19A). It is possible that some of the intrauterine effects described here were responsible for the variability seen from experiment to experiment in vehicle-exposed mice. When developmental TCDD exposure was added into the mix, it became the predominant determinant of HSC fate, overriding lesser effects based on gender or uterine position.

Overall, the evolution of AHR appears to benefit longevity in multiple ways (Hirabayashi and Inoue, 2009), and therefore it should not be surprising that the AHR KO model that we used for our research had numerous complications. One way to overcome these difficulties would be to switch to a mouse model that is a conditional knock-out, which maintains the functionality of AHR for fertility, development, and general longevity, but that lacks the AHR in hematopoietic cells. This would allow our observations to focus on the hematopoietic effects,

and also decrease the number of mice required to reach significant power in our experiments.

The experiments outlined in this chapter provide the first steps in definitively showing that the decreased lymphocyte differentiation following developmental TCDD exposure that our lab previously observed (Ahrenhoerster et al., 2014) is brought about due to the presence and activation of AHR in the fetus. Many of these findings will drive future work in the lab in terms of understanding the limits of the AHR full KO model and can increase the usefulness of limiting dilution analysis in testing developmental immunotoxicity of other environmental contaminants.

CHAPTER 5

DEVELOPMENTAL EXPOSURE TO 2,3,7,8 TETRACHLORODIBENZO-*p*-
DIOXIN ATTENUATES LATER-LIFE NOTCH1-MEDIATED T CELL
DEVELOPMENT AND LEUKEMOGENESIS

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ABSTRACT

Over half of T-cell acute lymphoblastic leukemia (T-ALL) patients have activating mutations in the Notch gene. Moreover, the contaminant 2,3,7,8 Tetrachlorodibenzo-*p*-dioxin (TCDD) is a known carcinogen that mediates its toxicity through the aryl hydrocarbon receptor (AHR), and crosstalk between activated AHR and Notch signaling pathways has previously been observed. Given the importance of Notch signaling in thymocyte development and T-ALL disease progression, we hypothesized that the activated AHR potentiates disease initiation and progression in an *in vivo* model of Notch1-induced thymoma. This hypothesis was tested utilizing adult and developmental exposure paradigms to TCDD in mice expressing a constitutively active Notch1 transgene (Notch^{ICN-TG}). Following exposure of adult Notch^{ICN-TG} mice to a single high dose of TCDD, we observed a significant increase in the efficiency of CD8 thymocyte generation. We next exposed pregnant mice to 3µg/kg of TCDD throughout gestation and lactation to elucidate effects of developmental AHR activation on later-life T cell development and T-ALL-like thymoma susceptibility induced by Notch1. We found that the vehicle-exposed Notch^{ICN-TG} offspring have a peripheral T-cell pool heavily biased toward the CD4 lineage, while TCDD-exposed Notch^{ICN-TG} offspring were biased toward the CD8 lineage. Furthermore, while the vehicle-exposed Notch^{ICN-TG} mice showed increased splenomegaly and B to T cell ratios indicative of disease, mice developmentally exposed to

TCDD were largely protected from disease. These studies support a model where developmental AHR activation attenuates later-life Notch1-dependent impacts on thymocyte development and disease progression.

INTRODUCTION

In humans, rates of chronic immune diseases and disorders have progressively increased over the last forty years (Bach, 2002; Dietert et al., 2010; Howlader et al., 2013; Jacobson et al., 1997). Some have suggested the increase in immunodeficiencies is due to the interaction of the environment with genetic susceptibility factors leading to altered immune state or immune cell development (Fazekas de St Groth, 2007; Perl, 2010). Environmentally initiated alterations in developmental pathways may lead to latent disease risk that can emerge at any time later in life following secondary exposures (Barker, 2007; Gluckman, Peter D. et al., 2005; Schug et al., 2012). Therefore, children are more vulnerable than adults to such exposures, as they have the entire life course ahead of them, resulting in more opportunities to impact sensitive time points (Perera and Herbstman, 2011). Environmental factors that are present during early life developmental stages may have particularly long-ranging impact by developmentally reprogramming progenitor cells through epigenetic changes at the chromatin level that potentially impact gene expression later in life. The impacts of progenitor cell reprogramming on later-life disease susceptibility are dependent on the interaction between the maternal intrauterine environment, the timing of exposure to potential insults, and the genetic susceptibility of the child. For example, early mutations in hematopoietic stem cells can increase the risk for development of leukemia (Corces-Zimmerman and Majeti, 2014). Moreover, 60% of all cases of T cell acute lymphoblastic leukemia (T-ALL) are associated with activating genetic mutations in Notch1 (Van Vlierberghe and Ferrando,

2012; Weng et al., 2004), a transmembrane receptor required for T cell development and function and regulation of CD4 vs. CD8 lineage commitment (Fowlkes and Robey, 2002).

Pinpointing environmental exposures as risk factors linked directly to disease outcomes is often complicated because of the latency associated with chronic, low level exposures. However, developmental exposure to the ubiquitous, persistent contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), acting through the aryl hydrocarbon receptor (AHR), has been connected to immune dysfunction later in life (Dietert, 2009; Vorderstrasse et al., 2004), and epidemiological studies that have followed the population in Seveso, Italy accidentally exposed to TCDD in 1976, show an increase in both lymphatic and hematological cancers in adults (Consonni et al., 2008). Furthermore, children whose parents had elevated TCDD in their serum exhibited a decrease in thyroid function (Eskenazi et al., 2010). These results suggest that the adverse effects of TCDD can occur following both acute adult exposure and chronic low level developmental exposure and that the developing immune system is a sensitive target of TCDD in humans.

A potential connection between the AHR and Notch was elucidated in human acute lymphoblastic leukemia (ALL) where hypermethylation of the AHR promoter region was observed (Mulero-Navarro et al., 2006). Additionally, AHR responsive elements (AHREs) have been found in Notch promoters (Bock, 2013), and Notch signaling induces stimulation of the AHR in activated T cells (Alam et al., 2010). However, whether Notch1 and developmental AHR

activation interact to promote disease has yet to be determined. This potential of developmental Notch1-AHR crosstalk during hematopoietic system disease initiation warrants further consideration given the genetic link to Notch1 mutations in T-ALL and continued production and persistence of TCDD and other environmental combustion by-products that act on the AHR, particularly in many developing countries undergoing rapid industrialization (Tanabe and Minh, 2010; Terauchi et al., 2009; Tue et al., 2013).

Previously we have demonstrated that developmental exposure to TCDD decreases the ability of hematopoietic progenitor cells to differentiate into mature lymphocytes (Ahrenhoerster et al., 2014). To determine if this phenomenon could have later-life disease implications, we examined the effect of prenatal AHR activation by transplacental exposure to TCDD on development and progression of a Notch1-induced thymoma in mice. We utilized a Notch1 transgenic mouse prone to developing T cell thymoma in order to probe a potential Notch1-AHR gene-environment interaction in disease. We hypothesized that developmental activation of AHR would produce a more severe form of Notch1- influenced thymoma in transgenic mice prone to disease than in unexposed control transgenic mice. To test this hypothesis, timed pregnant dams were exposed to 3 μ g/kg TCDD or vehicle control throughout pregnancy and at two days past parturition (PPD2). We found that while there was no significant difference in disease onset, severity or incidence between the groups, there was a significant difference in the ratios of circulating T-cells in the adult mice developmentally exposed to TCDD, ultimately impacting the lineage of the

thymomas identified in the peripheral immune organs. Specifically, whereas unexposed Notch1 transgenic mice had a higher proportion of circulating CD4⁺ cells in the blood, Notch1 transgenic mice exposed to TCDD *in utero* had an increased proportion of circulating CD8⁺ cells as adults. This T cell lineage switch suggests an AHR-dependent reprogramming of a hematopoietic precursor during development that impacts the later-life intrinsic Notch signal transduction occurring in the CD4 versus CD8 T-cell lineage choice. These data have implications for disease susceptibility in vulnerable populations that may possess genetic instability in the Notch locus and/or have been exposed to environmental AHR agonists developmentally.

RESULTS

Notch1^{ICN-TG}-dependent maturation of CD8+ thymocytes is potentiated by TCDD activation of the AHR.

In order to elucidate the potential interaction between Notch1 and the AHR during thymocyte development, we exposed four-week old Notch1^{ICN-TG} and C57BL/6 mice to a single dose of 10 or 30µg/kg TCDD or vehicle control, and analyzed thymus size, cell number, and composition 10 days after exposure. The Notch1^{ICN-TG} transgenic mice utilized for these studies expresses the constitutively active intracellular Notch1 under control of the *lck*-proximal promoter, restricting its expression to developing thymocytes. These mice have previously been shown to produce a larger percentage of CD8+ thymocytes than CD4+ thymocytes, in contrast to proportions found in wild type C57BL/6 mice (Robey et al., 1996). This increased population of CD8+ cells found in the Notch1^{ICN-TG} thymus is similar to observations made in mice exposed to the AHR ligand TCDD (Gill et al., 2008; Kremer et al., 1995). Following exposure to TCDD, both C57BL/6 and Notch1^{ICN-TG} mice showed a significant decrease in thymic weight and cellularity (Fig. 22A-B). Notably, we found that vehicle-exposed adult Notch1^{ICN-TG} mice had larger thymuses by weight (Fig. 22A) but lower cellularity (Fig. 22B) than C57BL/6 mice. Examination of flow cytometry FSC-A histograms for cell size distribution show a bi-modal peak in Notch1^{ICN-TG} mice compared to the C57BL/6 indicating there is a greater population of large blast-like thymocytes (Fig. 23).

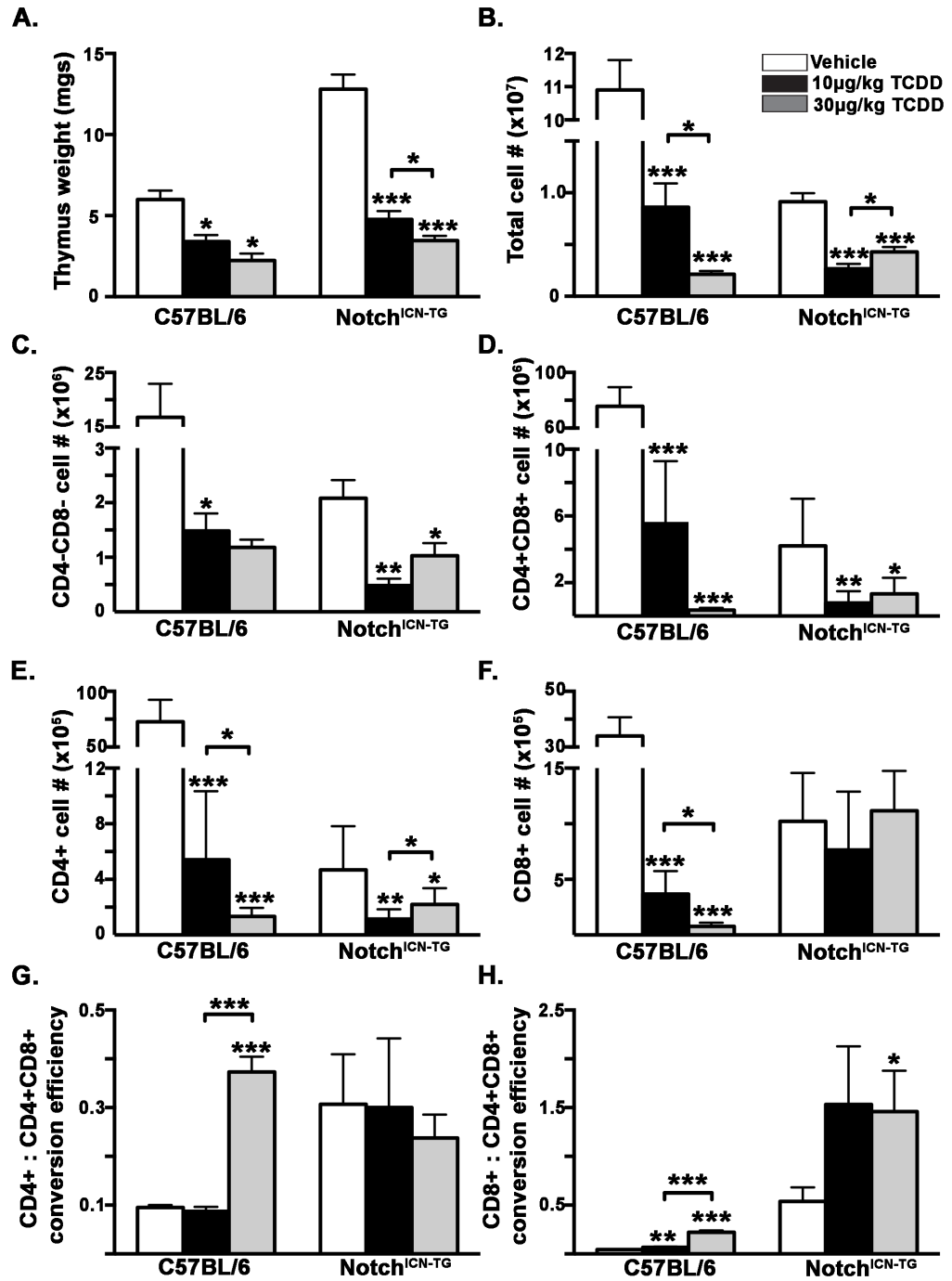


Figure 22: Thymus cellularity and efficiency of thymocyte conversion in adult male C57BL/6 or Notch1^{ICN-TG} mice after administration of TCDD or vehicle control. Wild type C57BL/6 or Notch1^{ICN-TG} mice were administered 10µg/kg TCDD, 30µg/kg TCDD or vehicle control via oral gavage at 4 weeks of age. Ten days later, mice were euthanized, thymuses removed, single cell suspensions made, and cells stained with fluorescent-conjugated antibodies directed against CD4 and CD8. Cells were acquired on a BD FACS Aria III flow cytometer and data analyzed in FlowJo. A) Thymus weight ± SEM; B) Thymocyte cellularity ± SEM; C-F) Absolute cell number (mean ± SEM) in each thymocyte cell population; G-H) Efficiency of SP (CD4+ or CD8+) generation, calculated by dividing the number of CD4+ or CD8+ thymocytes in by the number of CD4+CD8+ precursors; Vehicle mice are represented with white bars, 10µg/kg TCDD mice with black bars, and 30µg/kg TCDD-exposed mice with gray bars. A single * indicates p≤0.05; ** indicates p≤0.01; *** indicates p≤0.001. All compared to vehicle control *within genotype* unless otherwise indicated by bracket. (n=at least 4 B6 mice in each group; n=at least 8 Notch1^{ICN-TG} mice in each group)

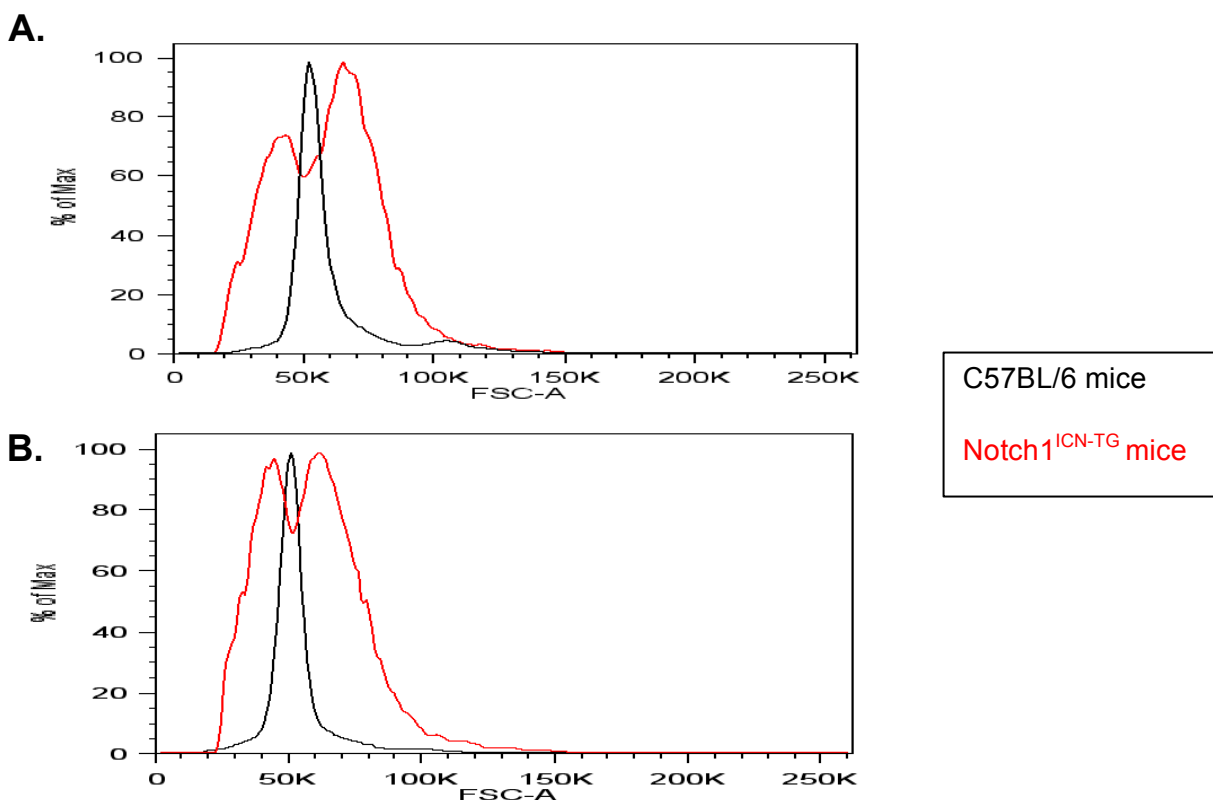


Figure 23. Thymocytes from Notch1^{ICN-TG} mice exhibit a bimodal forward scatter histogram. Thymocytes were isolated from Notch1^{ICN-TG} and C57BL/6 mice, then analyzed on a BD FACS Aria III flow cytometer for forward scatter, a measure of cell size A) representative plot of thymocytes in female mice B) representative plot of thymocytes in male mice; Black histogram represents C57BL/6 mice and red histogram represents Notch1^{ICN-TG} mice. *Figure originally included as supplementary for Chapter 5 publication.*

Analysis of thymocyte sub-populations revealed effects dependent on either Notch1^{ICN-TG}, TCDD activation of the AHR, or on the combination of Notch1^{ICN-TG} and TCDD activation of the AHR. Specifically, we found that in both C57BL/6 and Notch1^{ICN-TG} mice, TCDD-activation of the AHR led to a significant decrease in the absolute number of the most immature population of thymocytes identified by the absence of both CD4 and CD8 (Fig 22C). The largest population of thymocytes by cell number, the CD4+CD8+, is reduced in number by more than 10 fold in the C57BL/6 mice after exposure to TCDD as previously reported (Fig 22D; $p \leq 0.001$; Laiosa et al., 2003, 2002). Moreover, CD4+CD8+ thymocyte numbers in Notch1^{ICN-TG} are also reduced by approximately 10 fold ($p \leq 0.05$), however, the number of CD4+CD8+ thymocytes in the vehicle-exposed Notch1^{ICN-TG} is 10 fold lower than vehicle-exposed C57BL/6, suggesting that the intracellular Notch1 transgene alters the basal thymocyte cell number. In comparison to the reduction observed in CD4+CD8+ thymocytes, the production of CD4+ and CD8+ progeny that result from positive selection are differentially sensitive to the combined effects of Notch1^{ICN-TG} and the TCDD-activated AHR. Specifically, while the number of CD4 lineage thymocytes is significantly reduced by exposure to TCDD in both C57BL/6 and Notch1^{ICN-TG} mice (Fig 22E), CD8+ cells are present in equal number in the vehicle- and TCDD-exposed Notch1^{ICN-TG} mice (Fig 22F). Given the differences in thymocyte cell number between C57BL/6 and Notch1^{ICN-TG} mice, we used a ratio of single positive CD4+ or CD8+ to CD4+CD8+ as a way to normalize the efficiency of thymocyte conversion. Typically, 95-99% of CD4+CD8+ thymocytes fail selection, which is reflected by

ratios of less than 0.2 in the C57BL/6 mice. In comparison, the thymocyte conversion efficiency in Notch1^{ICN-TG} mice is 3 to 5 fold higher in the CD4 and CD8 lineages, respectively. Moreover, 30µg/kg TCDD produces an excess of CD8 cells compared to DP cells thus yielding a ratio of CD8+ to CD4+CD8+ cells that is greater than one, indicating an increase in CD8 conversion efficiency in Notch1^{ICN-TG} mice (Fig 22H; p≤0.05). These results suggest a potential additive interaction between the thymocyte-restricted constitutively active intracellular Notch1 and the ligand-activated AHR during thymocyte development.

Developmental Exposure to TCDD Does Not Affect Survival Into Adulthood of Notch1^{ICN-TG} transgenic mice.

Based on our recent findings linking developmental TCDD exposure to attenuation of Notch1-dependent lymphocyte differentiation (Ahrenhoerster et al., 2014), and previous work demonstrating that developmental AHR activation adversely impacts later life immunity (Hogaboam et al., 2007), we hypothesized that developmental AHR activation by TCDD reprograms hematopoietic progenitor cells such that susceptibility to later-life genetically-based T-cell leukemia-like disease is increased. We tested this hypothesis in T-cell thymoma prone Notch^{ICN-TG} mice by initially following the survival of Notch1^{ICN-TG} mice exposed to either vehicle or 3µg/kg of TCDD throughout gestation (GD 0.5, 7.5, 14.5 and PPD 2.5). The *in utero* and lactationally-exposed pups were followed until death or 12 weeks of age. Although approximately 40% of Notch1^{ICN-TG} mice died during the experiment, there was no significant difference in overall survival between TCDD- or vehicle-exposed Notch1^{ICN-TG} mice (Fig. 24).

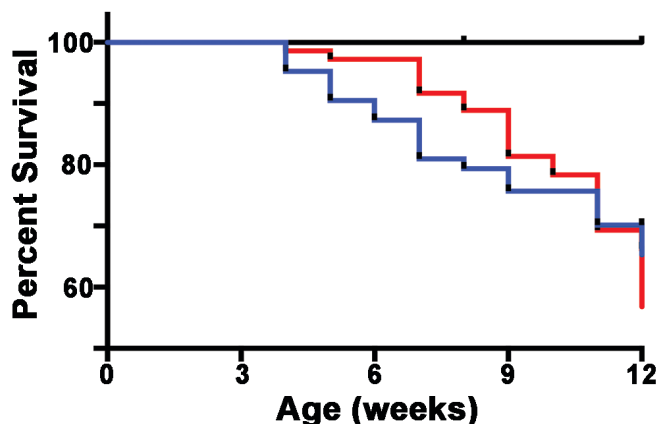


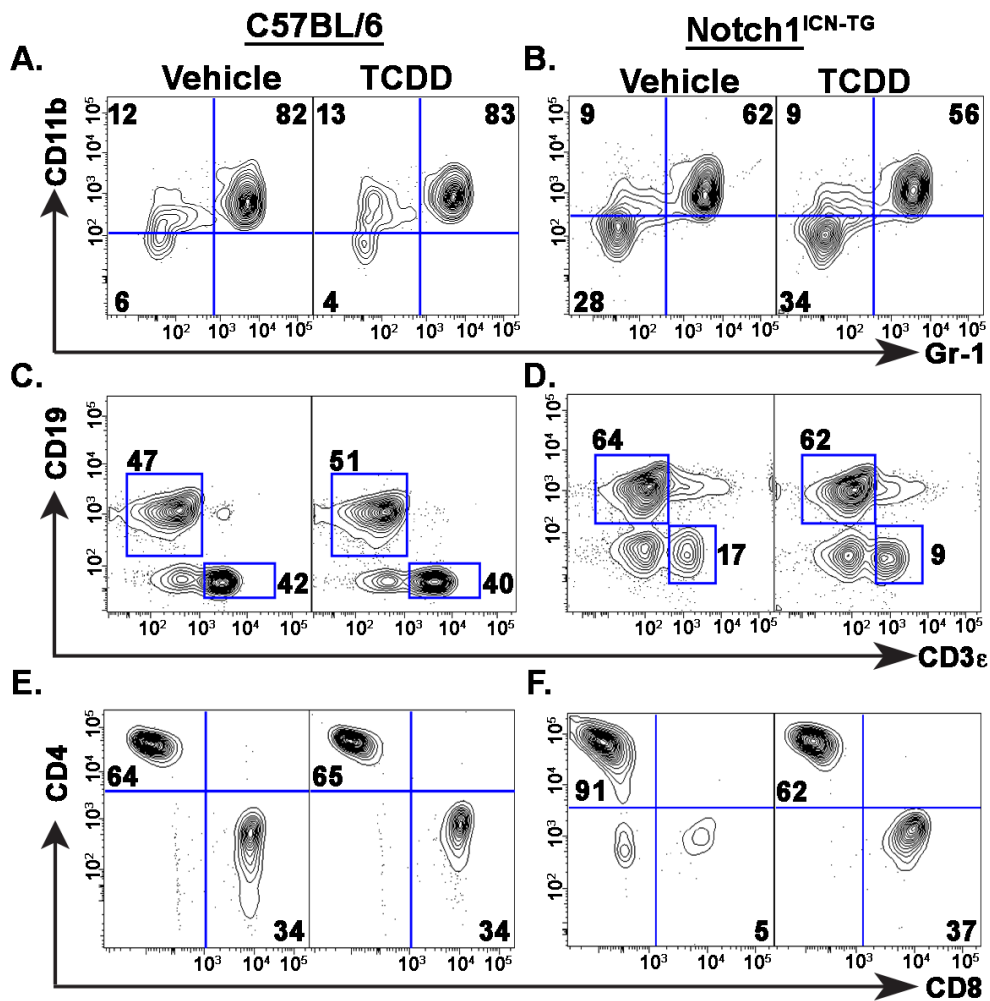
Figure 24: Survival of C57BL/6 or Notch1^{ICN-TG} mice following developmental exposure to 3 μ g/kg TCDD or vehicle control. Pregnant C57BL/6 dams (paired with either C57BL/6 or Notch1^{ICN-TG} males) were exposed to 3 μ g/kg TCDD or vehicle control throughout gestation (GD0.5, GD7.5, GD14.5, PPD2.5). Resulting offspring were followed through twelve weeks of age to determine age of death. C57BL/6 mice are represented with a black line (vehicle and TCDD; n=35). Notch1^{ICN-TG} mice are represented with a red line (vehicle; n=72) or blue line (TCDD; n=63). Survival fractions were calculated using Kaplan-Meier analysis, and survival curves were compared using the log-rank (Mantel-Cox) test (C57BL/6 p=1.00; Notch1^{ICN-TG} p=0.62).

Developmental TCDD exposure in Notch1^{ICN-TG} mice skews blood B cell to T cell and CD4 to CD8 ratios in adult mice.

It is well established that Notch1^{ICN-TG} mice have an altered ratio of CD4+ to CD8+ T cells compared to non-transgenic mice (Fowlkes and Robey, 2002; Robey et al., 1996), but the combined effect of developmental exposure to AHR agonists such as TCDD had not been examined in these mice. In order to more specifically quantify differences in thymoma initiation and progression following developmental exposure to TCDD in Notch1^{ICN-TG} mice, we collected blood

samples each week between 5 and 12 weeks of age, and quantified the proportions of circulating lymphocytes (Fig. 25A-C). No significant difference was found between treatments at any time point in either C57BL/6 or Notch1^{ICN-TG} mice in the percentage of macrophages and granulocytes, as evidenced by percent of CD11b⁺ or Gr1⁺CD11b⁺ cells, respectively (Fig. 25A, B & G). Similarly, we found no significant difference between vehicle or TCDD exposure in the ratio of B cells to T cells or in CD4⁺ T cells to CD8⁺ T cells in the C57BL/6 mice (Fig. 25C & H). However, in Notch1^{ICN-TG} mice, we saw a significantly lower ratio of B cells to T cells following developmental exposure to TCDD (Fig. 25D & H). Additionally, we saw a significantly lower ratio of CD4⁺ T cells to CD8⁺ T cells in mice developmentally exposed to TCDD (Fig 25F & I). Notably, in Notch1^{ICN-TG} mice, the ratio of B cells to T cells, and the ratio of CD4⁺ to CD8⁺ T cells in mice developmentally exposed to TCDD decreased almost to the level found in C57BL/6 mice. These data suggest that developmental exposure to TCDD provides protection from the alterations caused by a constitutively active Notch1 in thymocytes.

To determine if the change in the CD4⁺ to CD8⁺ ratio following developmental TCDD exposure could be explained by preferential survival of the CD8⁺ cells, we used the TUNEL assay to compare apoptosis in the thymus of 8 week old mice. We found no significant difference in the survival of CD4⁺ cells, CD8⁺ cells, or CD4⁺CD8⁺ cells following developmental TCDD exposure in either gender of mice (Fig. 26).



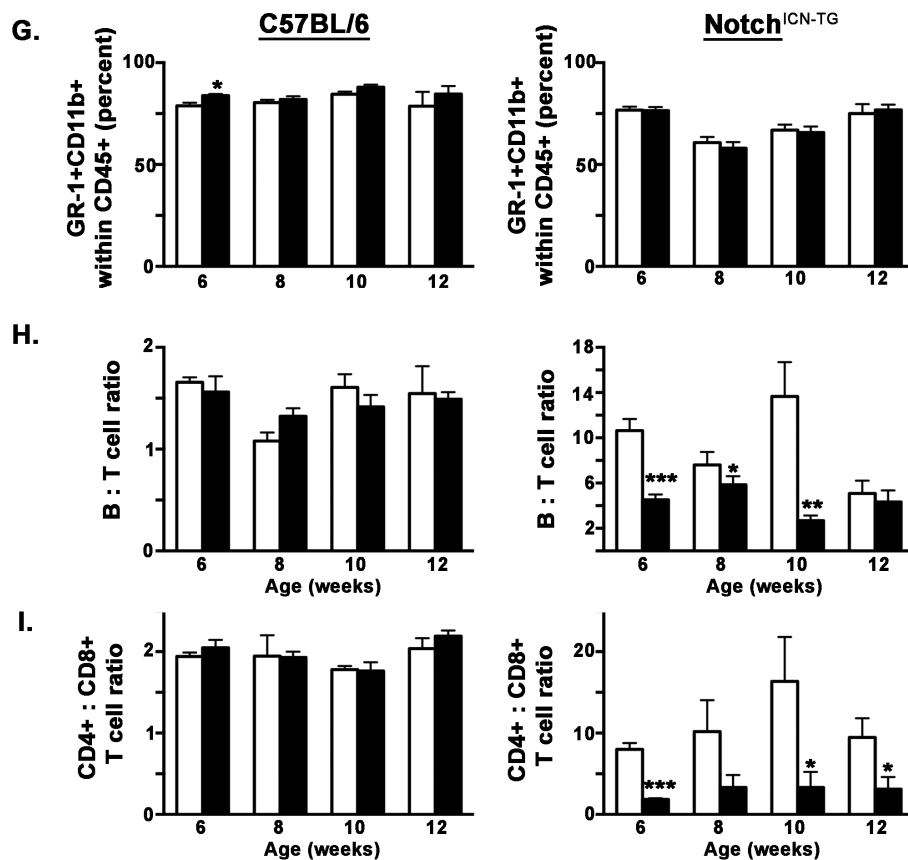


Figure 25: Proportion circulating lymphocytes in the blood following developmental exposure to 3µg/kg TCDD or vehicle control. Pregnant C57BL/6 dams (after pairing with either C57BL/6 or Notch1^{ICN-TG} males) were exposed to 3µg/kg TCDD or vehicle control throughout gestation (GD0.5, GD7.5, GD14.5, PPD2.5). A-F) Representative flow cytometry plots from week 8 blood samples, illustrating distribution and analysis of CD11b and Gr-1 positive cells (A), CD3+ T cells and CD19+B cells (C & D), and CD8+ and CD4+ T cells, gated on CD3+ (E & F). Frequency of Gr1+CD11b+ blood cells (G), the ratio of B cells to T cells in blood (H), or the ratio of CD4+ T cells to CD8+ T cells in blood (I) in both C57BL/6 mice (graphs on left) and Notch1^{ICN-TG} (graphs on right). Vehicle treated mice are represented by a white bar and mice treated with TCDD are represented by a filled bar in G-I. Bars graph mean with SE. A single * indicates p≤0.05; ** indicates p≤0.01; *** indicates p≤0.001.

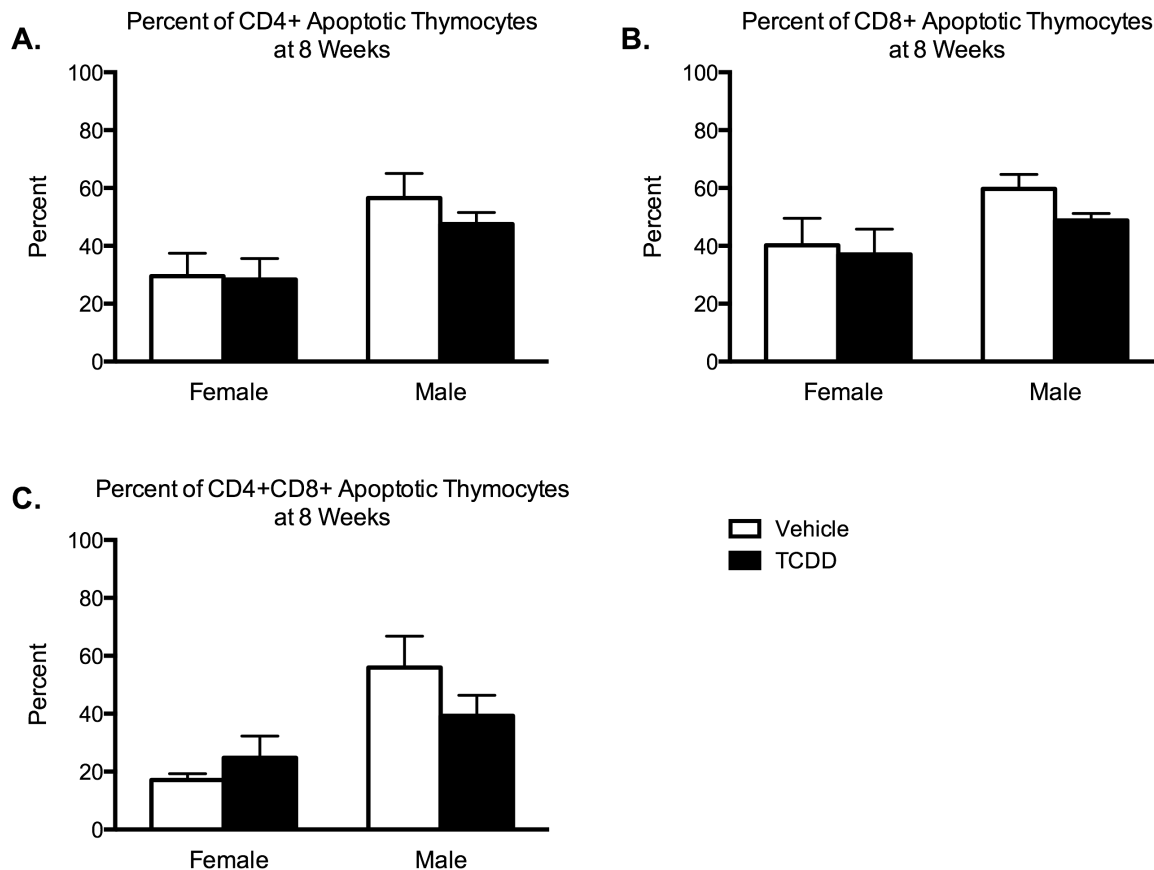


Figure 26. Percentage of apoptotic thymocytes in $Notch1^{ICN-TG}$ mice are not significantly different following developmental exposure to vehicle control or 3 μ g/kg TCDD. $Notch1^{ICN-TG}$ mice were exposed to 3 μ g/kg TCDD or vehicle control on GD 0.5, 7.5, 14.5 and PPD 2.5. Thymuses were harvested from 8 week old mice and a TUNEL assay was used to determine percentage of cells undergoing apoptosis. A) CD4+ thymocytes; B) CD8+ thymocytes; C) Double positive (CD4+CD8+) thymocytes. Figure originally included as supplementary for Chapter 5 publication.

Peripheral thymomas in $Notch1^{ICN-TG}$ mice following developmental TCDD exposure exhibit altered B cell:T cell ratios and differential expression of CD5.

The spleen is almost always involved in murine leukemia and neoplasms (Yang et al., 2013) thus we further examined splenic tissue to search for metastatic thymomas. We observed alterations in the B cell to T cell ratio in spleens (Fig. 28), similar to that found in blood (Fig. 25). Specifically, TCDD-

exposed C57BL/6 mice showed a significantly lower B cell to T cell ratio difference at 8 weeks (Fig. 28A & E; $p \leq 0.01$). Notch1^{ICN-TG} mice also showed a significant decrease in the ratio of B cells to T cells following developmental exposure at both 8 weeks ($p \leq 0.001$) and 12 weeks ($p \leq 0.05$) of age (Fig. 28B, D & E). Although both week 8 C57BL/6 and weeks 8 and 12 Notch1^{ICN-TG} mice showed significant differences in B cell to T cell ratios, the magnitude of difference in Notch1^{ICN-TG} mice was much greater. Whereas in C57BL/6 mice, the ratio decreased by 30 percent (2.7 in vehicle-exposed to 1.9 in TCDD-exposed), in Notch1^{ICN-TG} mice, the ratio decreased over 50 percent at week 8 (23.6 in vehicle-exposed to 11.4 in TCDD-exposed) and over 60 percent at week 12 (9.7 in vehicle-exposed to 3.3 in TCDD-exposed).

We further measured CD5 expression in CD4+ and CD8+ splenic T cells as a proxy for T cell activation (Fig. 28F). We found the mean fluorescence intensity (MFI), an indication of the density of CD5 on the cell surface, was nearly two-fold lower in the CD8+ population. However, in both the CD4+ and CD8+ populations, cells from mice developmentally exposed to vehicle control had greatly decreased MFI. Additionally, in both cases, MFI in cells isolated from spleens of Notch1^{ICN-TG} mice developmentally exposed to TCDD returned to near the MFI levels seen in C57BL/6 mice. These data show that Notch1^{ICN-TG} mice developmentally exposed to TCDD approach a B cell to T cell ratio and a CD5 MFI similar to the wild type C57BL/6 vehicle-exposed mice.

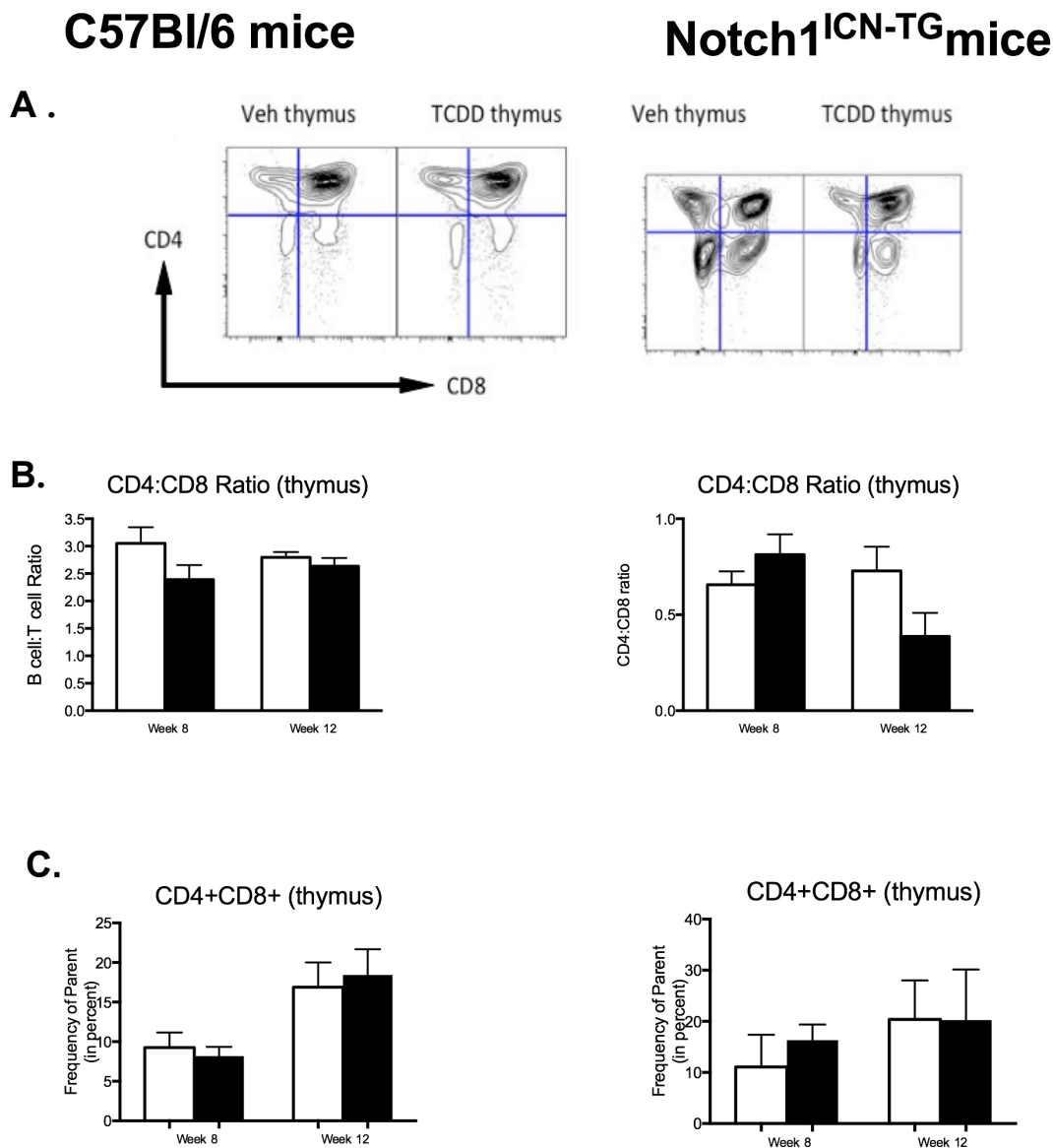


Figure 27: CD4 and CD8 cell proportions in thymus are not significantly different following developmental exposure to 3 μ g/kg TCDD or vehicle control. Pregnant C57BL/6 dams (paired with either C57BL/6 or Notch1^{ICN-TG} males) were exposed to 3 μ g/kg TCDD or vehicle control throughout gestation (GD0.5, GD7.5, GD14.5, PPD2.5). A) Representative flow cytometry plots from week 8 thymus samples; B) Ratios of B cells to T cells at 8 weeks and 12 weeks, with C57BL/6 mice on the left and Notch1^{ICN-TG} mice on the right; C) Frequency of viable cells (defined by absence of sytox blue stain) in double positive (CD4+CD8+) quadrant of flow cytometry plots. Vehicle treated mice are represented by a white bar and mice treated with TCDD are represented by a filled bar in B-C. Bars graph mean with SE. (n=3 C57BL/6 vehicle; n=3 C57BL/6 TCDD; n=8 Notch1^{ICN-TG} vehicle; n=7 Notch1^{ICN-TG} TCDD at week 8; n=4 C57BL/6 vehicle; n=5 C57BL/6 TCDD; n=6 Notch1^{ICN-TG} vehicle; n=8 Notch1^{ICN-TG} TCDD at week 12); C57BL/6 mice are pictured on the left. Notch1^{ICN-TG} mice are pictured on the right. *Figure originally included as supplementary for Chapter 5 publication.*

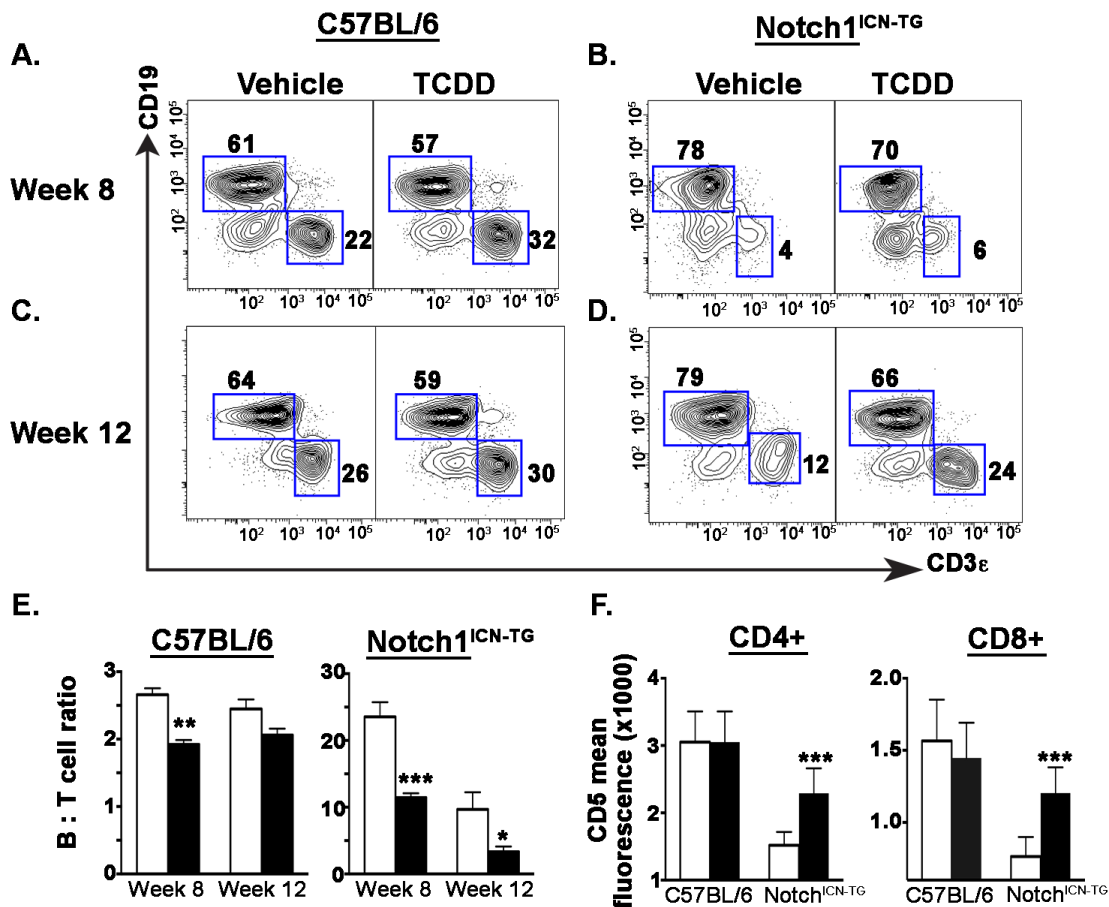


Figure 28: Cell proportions in spleen are altered following developmental exposure to 3µg/kg TCDD or vehicle control. Pregnant C57BL/6 dams (paired with either C57BL/6 or Notch1^{ICN-TG} males) were exposed to 3µg/kg TCDD or vehicle control throughout gestation (GD0.5, GD7.5, GD14.5, PPD2.5). A and C) Representative flow cytometry plots delineating B and T cells from weeks 8 and 12 C57BL/6 spleen samples; B and D) Representative splenic B versus T cell flow cytometry plots from weeks 8 and 12 in Notch1^{ICN-TG}; E) Ratios of B cells to T cells at 8 weeks and 12 weeks, with C57BL/6 mice on the left and Notch1^{ICN-TG} mice on the right; F) Mean fluorescence intensity of CD5 in T-cells from 6-8 week old mice, within the CD4 population on the left and the CD8 population on the right; Vehicle treated mice are represented by a white bar and mice treated with TCDD are represented by a filled bar in D-E. Bars graph mean with SE. A single * indicates $p \leq 0.05$; ** indicates $p \leq 0.01$; *** indicates $p \leq 0.001$. (n=3 C57BL/6 vehicle; n=3 C57BL/6 TCDD; n=8 Notch1^{ICN-TG} vehicle; n=7 Notch1^{ICN-TG} TCDD at week 8; n=4 C57BL/6 vehicle; n=5 C57BL/6 TCDD; n=6 Notch1^{ICN-TG} vehicle; n=8 Notch1^{ICN-TG} TCDD at week 12).

Spleen size was significantly smaller in Notch1^{ICN-TG} mice following developmental TCDD exposure than in vehicle controls.

Upon necropsy, many of the vehicle control Notch1^{ICN-TG} mice had enlarged spleens--a sign of leukemic infiltration (Zhang et al., 2013) and this enlargement was much more common and severe in vehicle mice than in developmentally TCDD-exposed Notch1^{ICN-TG} mice. Specifically, we found a statistically significant difference in spleen weight, size and length between the exposed and control groups ($p \leq 0.01$; Fig. 29A-C). Furthermore, we used a numerical grading system in order to insure that spleens exhibiting severe splenomegaly did not bias the mean values shown in Fig. 29A-C. As previously published (Duggan et al., 2012), the grading system categorized spleens into grades 1-4 based on the following weights: normal (< 0.2 g; Grade 1), moderate enlargement (≥ 0.2 - < 0.5 g; Grade 2), severe enlargement (≥ 0.05 - < 1.0 g; Grade 3), or morbid enlargement (≥ 1.0 g; Grade 4). Whereas over 80% of spleens in the gender-combined TCDD-treated group fell into the "normal spleen" range, less than half of those in the vehicle group could be categorized as normal (Fig. 29D). Furthermore, this effect was even more pronounced in the female Notch1^{ICN-TG} mice. None of the C57BL/6 wild type mice used in this experiment exhibited any spleen enlargement, supporting presence of metastatic thymomas as the cause of splenomegaly.

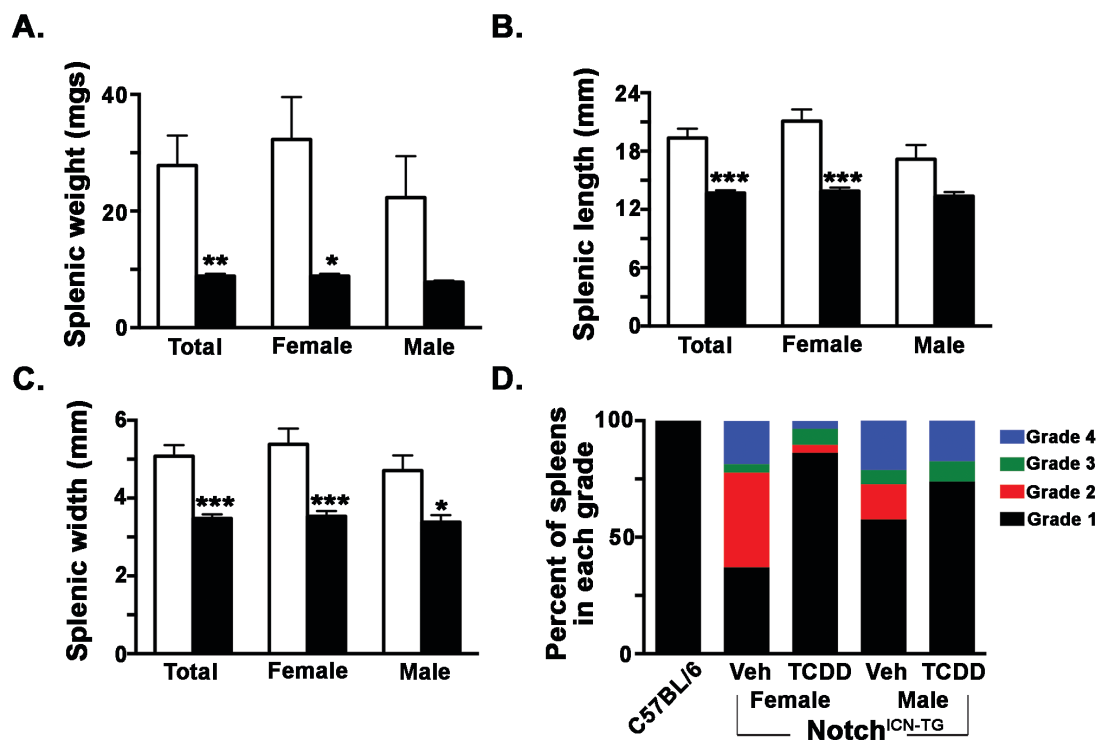


Figure 29: TCDD-exposed Notch1^{ICN-TG} mice fail to develop Notch1-mediated splenomegaly. Developmental exposure to TCDD in Notch1^{ICN-TG} mice results in significantly smaller adult spleen size. Pregnant C57BL/6 dams were paired with Notch1^{ICN-TG} males and exposed to 3 μ g/kg TCDD or vehicle control throughout gestation (GD0.5, GD7.5, GD14.5, PPD2.5). Vehicle treated mice are represented by a white bar and mice treated with TCDD are represented by a filled bar. Bars graph mean with SE. A single * indicates $p \leq 0.05$; ** indicates $p \leq 0.01$; *** indicates $p \leq 0.001$. Comparisons of spleen weight (A), length (B), and width (C) were all made on 8 week old mice; spleen grading was done across all mice between 8-12 weeks of age at any age of necropsy. (n= 38 vehicle and 23 TCDD mice in A-C; n=61 vehicle and 52 TCDD mice in D)

DISCUSSION

In the present study, we demonstrate that developmental exposure to the environmental contaminant and AHR agonist TCDD during development in Notch1-dependent thymoma-prone mice reprograms long-term HSCs such that the adult thymic progeny of these cells have an attenuated response to the Notch1-dependent CD4 versus CD8 lineage decision. Specifically, the unusually large CD4:CD8 ratio observed in Notch1^{ICN-TG} mice is restored to nearly C57BL/6 wild-type levels in the Notch1^{ICN-TG} TCDD-exposed offspring. These data are therefore consistent with the conclusion that developmental AHR activation provides a means of disease protection to mice possessing a Notch1-mediated propensity to develop leukemic thymomas.

We have previously shown that developmental exposures to TCDD reprogram long-term reconstituting HSCs, impacting their ability to complete lymphocyte lineage commitment (Ahrenhoerster et al., 2014). These studies extend the impact of AHR-induced HSC reprogramming by demonstrating a long-term attenuated response to Notch1-mediated T-cell differentiation evidenced by the CD4 to CD8 lineage switch observed in peripheral T-cells from offspring exposed to TCDD during development. Taken together, these data are consistent with the conclusion that in a progenitor cell population in the fetus there are epigenetic or stable changes to the chromatin influenced by AHR activation that have later-life impacts on gene expression and cellular differentiation. Notably, the impact of this reprogramming on hematopoietic

differentiation is not readily apparent in wild type C57BL/6 mice. Rather, for the later-life alterations to produce a measurable change in cellular function, the progeny of the long-term progenitor cells need to differentiate and be challenged by a secondary stressor such as the constitutively active intracellular Notch transgene in the thymus.

The acute exposure experiment demonstrated an increase in CD8 conversion efficiency following TCDD exposure in both C57BL/6 and Notch1^{ICN-TG} adult mice in the thymus, though not in the periphery (data not shown), providing initial evidence of potential AHR-Notch crosstalk. However, the substantial thymic atrophy resulting from adult TCDD exposure complicated interpretation of the mechanism for the increase in CD8+ cell conversion efficiency. Thus the switch to a developmental exposure model combined with a 10-fold decrease in dose throughout gestation allowed us to more clearly elucidate the effect that AHR activation had on Notch signal transduction throughout the lifecourse. Using this developmental model, any TCDD-induced thymic atrophy that occurred in fetal or neonatal mice was resolved by adulthood as indicated by similar thymic CD4+ and CD8+ populations as adults. Thus, the long-term developmental studies identified more sensitive and longer lasting outcomes than the adult exposure experiments. Specifically, developmental TCDD-exposure affected peripheral T-cell homeostasis demonstrated by a significant change in the efficiency of generating post-selection CD8+ T cells.

One interpretation of this study could be that early life AHR activation by TCDD is actually beneficial for Notch-mediated hematopoietic disease. However,

Notch signaling is crucial for fetal HSC development and proliferation, for T lineage choice (Bigas and Espinosa, 2012), and for normal T cell development in both the fetus and adult (Osborne and Minter, 2007; Schmitt and Zúñiga-Pflücker, 2002). Therefore, impairment of physiological Notch signaling in HSCs and T cells has the potential to alter normal development and the consequences of this attenuation could impact a spectrum of hematological-based diseases.

Physiological Notch1-dependent signal transduction is characterized by engagement of the extracellular portion of Notch with Jagged or Delta-like ligands on a neighboring cell. This protein-protein engagement results in the gamma secretase-mediated cleavage of the intracellular Notch1 (ICN) domain. The ICN translocates into the nucleus and associates with the CBF1/Su(H)/Lag-1 (CSL) transcription factor complex where it acts as a co-activator driving transcription of a broad array of genes, including those responsible for T cell lineage commitment. A potential consequence of ICN over-expression in Notch1^{ICN-TG} mice could be that the transgene stoichiometrically saturates the CSL binding site in the Notch signaling pathway leading to aberrant gene regulation attenuating physiological Notch activity (Beres et al., 2006). The importance of the transgenic ICN saturation of the CSL binding site in our studies follows the knowledge that physiological Notch1 is an AHR-regulated gene (Stevens et al., 2009). Though it is not known whether Notch transcription is directly controlled by the AHR (Kiss and Diefenbach, 2012), our data suggests that AHR activation during development leads to a change in responsiveness to ICN-mediated T cell development that persists throughout the life course.

Despite equivalent survival rates between Notch1^{ICN-TG} mice developmentally exposed to TCDD or vehicle (p=0.62), the fatality rate in vehicle-exposed Notch1^{ICN-TG} mice was higher than the 20% tumor rate previously reported at 5 months (Beverly and Capobianco, 2003). It is unknown whether the propensity for thymoma development increased in our sub-strain of Notch1^{ICN-TG} mice prior to arriving in our vivarium, or was selected for inadvertently from our original breeder pair. However, this increased spontaneous thymoma rate made it difficult to form a conclusion about the original hypothesis that developmental TCDD exposure would increase later-life disease susceptibility. Nevertheless, the altered T cell ratios in the blood and splenomegaly differences are consistent with the conclusion that developmental TCDD exposure impacts Notch1-dependent disease initiation.

In terms of the splenomegaly, the expected result that tumor-resistant C57BL/6 mice survived for the duration of the 12-week experiment without splenomegaly, indicates that the enlarged spleens are unique to the transgenic strain we used and not due to the developmental TCDD exposure. The moderate protection against splenomegaly by developmental TCDD is similar to autoimmune and inflammatory amelioration following AHR activation (Busbee et al., 2013; Di Meglio et al., 2014; Kerkvliet, 2009). These data raise the intriguing possibility that AHR modulators could have efficacy as chemopreventatives or chemotherapeutics in certain types of leukemia, particularly if combined with existing treatments allowing for the decreasing of current doses. For example, gamma-secretase inhibitors (GSIs) that block Notch activation are a promising

recent treatment for T-ALL, however, the negative side-effects include dose-limiting diarrhea (Hernandez Tejada et al., 2014; Takebe et al., 2014).

In addition to the CD4 to CD8 ratio changes mediated by developmental TCDD exposure, we also observed alterations in the CD5 MFI in both CD4+ and CD8+ splenic T cells. Specifically, CD5 levels, as determined by MFI, were significantly decreased in vehicle-exposed Notch1^{ICN-TG} mice when compared to C57BL/6 mice (Fig. 5E). However, following developmental exposure to TCDD, the MFI of CD5 in both CD4+ and CD8+ T cells is partially restored to C57BL/6 levels. The consequences of the lower CD5 expression is consistent with a model of higher TCR signal transduction in T cells from Notch1^{ICN-TG} mice (Germain, 2002), leading to a potential increase in activation-induced cell death in the periphery. While we were not able to detect any increases in apoptosis *in vivo* (supplemental figure 3), we and others have previously reported the difficulty in measuring apoptosis *in vivo* due to rapid clearance by macrophages (Laiosa et al, 2007). Thus we relied on indirect observations, such as splenomegaly to support the hypothesis of increased activation-induced cell death given that spleen enlargement is consistent with the removal of dead and dying cells. Moreover, elevated removal of dead and dying cells as occurs in a number of hematological disorders, including leukemia and lymphoma, is a hallmark of disease. Taken together, the developmental TCDD-dependent increase in CD5 expression in T cells from Notch1^{ICN-TG} mice and the reduction in splenomegaly in TCDD-exposed Notch1^{ICN-TG} mice supports a model of inhibition of T cell activation later in life.

While developmental AHR activation offers protection from later-life CD4+ to CD8+ T cell ratio imbalance and splenomegaly caused by constitutively-active ICN, the increase in CD8+ T cells that restores the ratio may not be beneficial in a more physiological model. Specifically, a decrease in the ratio of CD4+ T cells to CD8+ T cells in blood has been linked to poorer disease outcomes following diagnosis and treatment in several immunological diseases, including childhood leukemia (Lustfeld et al., 2014), hepatitis (Ikeda et al., 2012), and HIV (Serrano-Villar et al., 2014). We found that Notch1^{ICN-TG} mice developmentally exposed to TCDD showed a persistently lower CD4+ to CD8+ ratio in peripheral blood, suggesting a less favorable response to disease treatment.

In summary, our results indicate that prenatal exposure to the environmental toxicant and AHR agonist TCDD reprograms HSCs and significantly changing the course of disease in a Notch1-induced murine thymoma model, as measured by ratios of B cells to T cells, and CD4+ T cells to CD8+ T cells. Future research on epigenetic changes influenced by exogenous stimulation of the AHR during development may provide insight into the spectrum of secondary exposures vulnerable populations are burdened with that increase their propensity for later-life hematological and other immune deficiencies.

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CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

It has long been known that TCDD is a ubiquitous environmental contaminant and carcinogen linked to disease in multiple systems. TCDD and related contaminants exert their toxicity through the AHR, a transcription factor which contributes to regulation of hematopoiesis, among its many functions.

Hematopoiesis, the process by which all blood and immune cells are formed from HSCs, is a delicate balance between self-renewal and differentiation of HSCs. Given that HSCs are formed *in utero*, any alteration during gestation can have long-term effects on health. Development depends on numerous factors present (or absent) in the intrauterine environment, and there is growing knowledge that exposures *in utero* and soon after birth may have persistent, detrimental effects on health and disease which may not manifest until later in life (Fig. 30).

In the research described here, we have shown that exposure to an environmentally-relevant level of TCDD *in utero* has effects on the developing immune system. In mice, the HSCs formed following developmental exposure to TCDD have a greatly diminished capacity to differentiate into T and B effector cells. Furthermore, we have shown that developmental TCDD exposure exerts this effect through the AHR in the fetus.

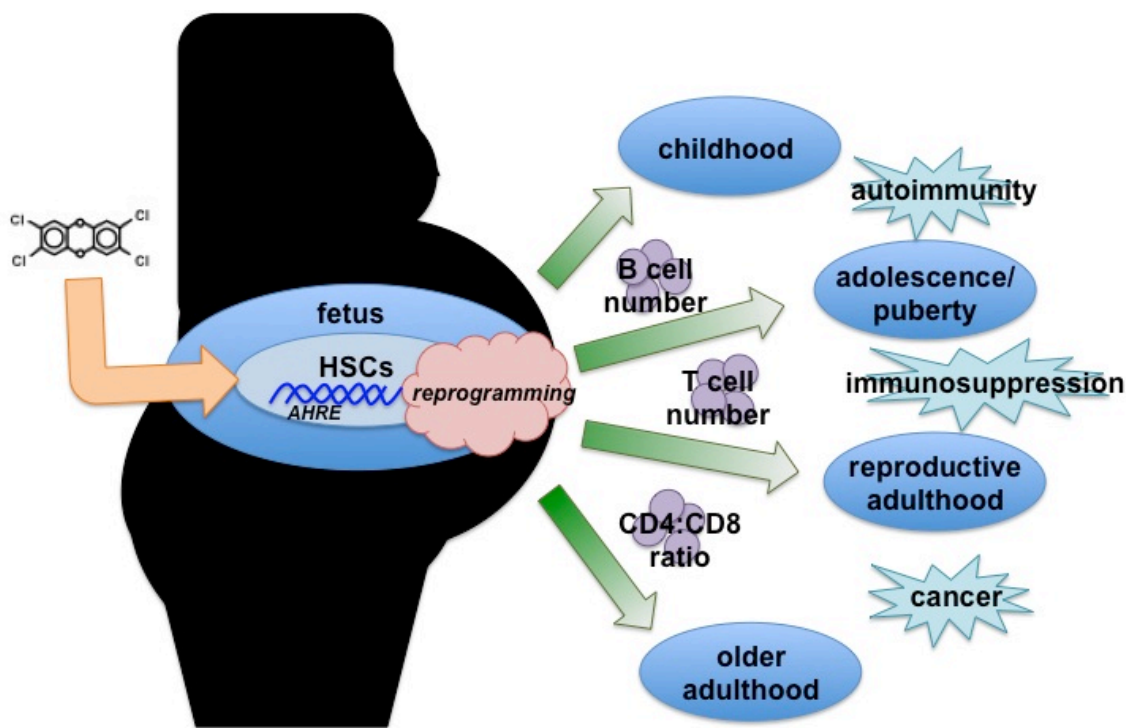


Figure 30. Exposure to TCDD *in utero* potentially affects offspring at particularly sensitive periods of development. This model illustrates the possible impact of developmental exposure to AHR-activating environmental contaminants, such as TCDD. TCDD exposure in the pregnant mother activates the AHR in the fetus, leading to a reprogramming of HSCs which we have shown attenuates the number of mature B cells, number of mature T cells, and ratio of CD4+ to CD8+ T cells. As the exposed fetus matures, at particularly sensitive timepoints such as childhood, puberty, during the reproductive years, and into older adulthood, the immune system formed from reprogrammed HSCs *may* result in an increase in the incidence of hematological diseases including, but not limited to, autoimmunity, immunosuppression, and cancer.

While determining HSC differentiation potential, an interesting observation was made: although the ability of the HSCs isolated from exposed mice to differentiate into lymphocytes was significantly decreased, the number of HSCs isolated per fetus was increased. Interestingly, both alterations were approximately two and a half-fold, thus potentially canceling out the effect of either alone. Dysfunction in cell-cycle regulation should be explored to provide a clearer picture of the true long-term consequence of developmental exposures by determining the net quantity of functional effector cells produced.

Throughout this work, we have used the terms “LSK” and “HSC” somewhat interchangeably, but they are not technically the same. The cell surface markers that we used to identify HSCs (as LSKs) resulted in a heterogeneous mixture of ST- and LT-HSCs, potentially at different stages of lineage commitment when isolated. Additional cell surface markers, including the SLAM family (CD48, CD150, CD229, CD244) are known to identify HSCs earlier in the hierarchy of stem cell-ness and therefore with greater pluripotency (Oguro et al., 2013). Determining the differentiation potential of LT-HSCs isolated from fetal livers and comparing to the LSK potentials would help pinpoint the level at which TCDD-induced HSC re-programming is occurring. Impact on the myeloid lineage could also be examined to more closely focus the level of HSC hierarchy at which re-programming appears.

Complete understanding of fetal HSC reprogramming must include all sites of hematopoiesis as well as all maturation states. The yolk sac and AGM are the site of hematopoiesis before the fetal liver is, and the placenta has also been suggested as a potential embryonic site of hematopoiesis (Golub and Cumano, 2013). Testing the differentiation potential of HSCs isolated from these alternate embryonic sites of hematopoiesis, in conjunction with gene expression data, would expand our knowledge of the mechanism by which HSCs are reprogrammed during development.

As a follow-up to our experiments showing decreased differentiation potential of HSCs following developmental AHR activation, we also have shown that, in a mouse model genetically prone to thymoma, environmental exposure to

TCDD radically changes the progression of disease as measured by CD4+ and CD8+ T lymphocyte levels in the blood and spleen. Individual genetic variation in addition to epigenetic programming can interact with effects caused by environmental exposures during development. Polymorphisms or epigenetic reprogramming act in conjunction with developmental AHR activation and maternal health to produce the variability seen in disease development.

Forming a functional immune system requires integration of self renewal, proliferation, differentiation, and activation of HSCs. The limiting dilution experiments that we used only determined *lymphocyte differentiation potential* of the HSCs isolated from developmentally-exposed mice. Although it was not determined that the decreased differentiation potential has a functional effect, we would expect that it would. While we observed changes in the course of disease following developmental exposure in Notch transgenic mice, we saw no significant impact in the wild type mice. However, functionality of the lymphocytes that develop from HSCs developmentally exposed to TCDD could be tested in culture or after isolation from adult mice. Comparing cytokine baseline levels or levels after an immune response, or measuring changes in gene expression in either case may provide support for true functional impact.

Though we only determined HSC differentiation potential, these findings still suggest considerable potential consequences on lifelong immunity. Because the adult immune system develops from HSCs, we would expect that developmental exposure also affects such immunological health outcomes as development of cancer or autoimmune diseases. Epidemiologically, rates of

immune diseases have risen in the last forty to fifty years, which coincides with industrialization, especially in developing countries. Others have shown that mice developmentally exposed to TCDD show a weakened immune response to influenza challenge (B A Vorderstrasse et al., 2004), which lends support to there being long-term consequences following early alterations in lymphocyte differentiation potential.

Our research uses TCDD as an AHR agonist, but many other environmental contaminants, including PCBs, PAHs, and dibenzofurans, are also AHR ligands. Additionally, many of these substances exist environmentally as mixtures. Though non-TCDD ligands are more readily metabolized and the resulting AHR activation is not as persistent, the possibility exists that developmental exposure to a combination of AHR ligands may cause similar or altered effects on T cell development or function. An important next step would be to examine consequences of developmental exposure to a mixture of AHR ligands.

In addition to exploring additional AHR ligands, it is important to investigate the impact of developmental TCDD exposure on additional body systems. Because the AHR is involved in a large number of developmental pathways, we would expect that gestational exposure to an AHR agonist would impact not only the hematopoietic system, but other systems as well. Exposure to an AHR agonist such as TCDD *in utero* might also cause long-term impact on the nervous system. In fact, migration of one type of stem cell, neural crest cells, has been shown to be altered by cigarette smoke acting through the AHR (Sanbe

et al., 2009). Additionally, neurons derived from umbilical cord blood stem cells express the highest levels of AHR and related genes early in development (A. K. Singh et al., 2013) and embryonic stem cells express AHR, which drives their differentiation into cardiomyocytes (Wang et al., 2013). The potential for AHR-related effects following a developmental exposure are extensive and provide opportunities to expand the findings reported here for the immune system.

Although we controlled for multiple variables, one of the variables that we did not take into account in this research was the gender effect. Research has, by convention, more often used male organisms in research studies (Clayton and Collins, 2014), and we initially followed this convention with the adult mice used. However, TCDD has endocrine disruption potential, and therefore could have potentially affected results by gender. Results might be biased in either direction, as, for example, developmental exposure to TCDD has been shown to feminize male rats (Ikeda et al., 2005), and to alter expression of more genes in ovaries than in testes (Magre et al., 2012). We did not genotype fetuses when harvesting HSCs from GD 14.5 livers, so each experiment contains HSCs from a mix of male and female. Future research might determine that the differences we found are more extreme when categorized by gender.

Another variable which might benefit from tighter control is timing, or more specifically, circadian rhythm. Though our samples were all harvested within a two hour window, even within that window circadian differences might emerge. For timed pregnancies, mating may have occurred at any time within a 15 hour time period, and implantation might have varied as well. Finding a means to more

accurately time pregnancies, and keeping HSC harvest especially within a small timeframe might increase the strength of differences seen.

Finally, we observed the greatest inconsistencies in results when working with AHR transgenic mice. After initial testing to determine response similarity between AHR^{+/+} (WT) and AHR^{+/-} (HZ) mice, we eliminated WT mice from our experiments. Although HZ mice, by virtue of having an AHR allele, should respond to AHR activation in a manner similar to WT mice, there is a possibility that unknown factors may be present within the heterozygous population. Assaying all three genotypes—WT, HZ and KO—might illuminate any subtle variations between the genotypes.

In conclusion, this research provides evidence for the developmental origin of health and disease as it pertains to the immune system. We have shown that developmental exposure to the ubiquitous environmental contaminant TCDD causes a reprogramming of HSCs such that they are two and a half fold less likely to differentiate into mature, committed lymphocytes. We have shown that this effect is mediated through the AHR in fetuses, as fetal mice lacking an AHR were not affected by the developmental TCDD exposure. Finally, we have demonstrated that developmental exposure to TCDD, in crosstalk with Notch1 overexpressed in a transgenic mouse, alters the progression of a T cell specific disease, manifested by development of thymomas. Taken together, they provide support for the life-long impact of an early life exposure, and offer a potential explanation for the increasing rates of immune disease over the last fifty years. This research illustrates the importance of using scientific evidence to drive

public health practices such as prenatal supplementation and newborn screening, as well as public health policy such as environmental regulations.

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Lori S. Ahrenhoerster

EDUCATION

- Ph.D. Environmental and Occupational Health
December 2014
Joseph J. Zilber School of Public Health
University of Wisconsin – Milwaukee, Milwaukee, WI
Dissertation: Impact of Gestational Exposure to 2,3,7,8-Tetrachlorodibenzo-p-dioxin on T Lymphocytes
- B.S. Major: Secondary Broadfield Science
Minors: Biology and Chemistry
December 1992 with honors
University of Wisconsin – La Crosse, La Crosse, WI

PUBLICATIONS

- Ahrenhoerster, L.S., Tate, E.R., Lakatos, P.A., Wang, X., Laiosa, M.D., 2014.
Developmental exposure to 2,3,7,8 tetrachlorodibenzo-p-dioxin attenuates capacity of hematopoietic stem cells to undergo lymphocyte differentiation. *Toxicol. Appl. Pharmacol.* 277, 172–182. doi:10.1016/j.taap.2014.03.020
- Ahrenhoerster, L.S., Leuthner, T.C., Tate, E.R., Lakatos, P.A., Laiosa, M.D.
Developmental Exposure To 2,3,7,8 Tetrachlorodibenzo-P-Dioxin Attenuates Later-Life Notch1-Mediated T cell Development and Leukemogenesis (Manuscript in final review for *Toxicology and Applied Pharmacology*)

SELECTED PRESENTATIONS

- 2014 **L. Ahrenhoerster**, M. Peck, T. Salm Ward. “Fantastic space!”: Prioritizing components to create a warm and inviting lactation room.” Wisconsin Public Health Association Conference, Milwaukee, WI; May 13-15
- 2014 **L. Ahrenhoerster**, D. Almagro, P. Lakatos, T. Leuthner and M. Laiosa. Gene-Environment interactions influence leukemogenesis in mice developmentally exposed to dioxin. Joseph J. Zilber School of Public Health Student Research Symposium, Milwaukee, WI; January 31

- 2013 **L. Ahrenhoerster**, M. Wolter, C. Weirich, T. Achankunju. Home Grown Research: Current Environmental Health projects from Ph.D. students at Zilber School of Public Health. Wisconsin Public Health Association Conference, Wisconsin Dells, WI; May 21-23
- 2013 **L. Ahrenhoerster**, P. Lakatos, M. Laiosa. Developmental arylhydrocarbon receptor activation attenuates hematopoietic stem cell capacity to undergo lymphocyte differentiation. Abstract No. 398. 2013 Society of Toxicology Annual Meeting Itinerary. San Antonio, TX; Poster also selected for Visiting Undergraduate Poster Session; March 11
- 2013 M.D Laiosa, **L.S. Ahrenhoerster**, D. Almagro and P.A. Lakatos. Developmental exposure to 2,3,7,8 Tetrachlordibenzo-p-dioxin (TCDD) interacts with Notch signaling in the thymus later in life affecting leukemogenesis in tumor prone mice. Abstract No. 812. 2013 Society of Toxicology Annual Meeting Itinerary. San Antonio, TX; March 11
- 2012 **L. Ahrenhoerster**, P. Lakatos, X. Wang, M. Laiosa. Developmental dioxin exposure reprograms hematopoietic stem cell differentiation capacity: Implications for immune system function and health outcomes across the lifespan. Session No. 3085.0. American Public Health Association Annual Meeting and Conference, San Francisco, CA. October 29
- 2012 **L. Ahrenhoerster**, P. Lakatos, X. Wang, M. Laiosa. If you give a mouse dioxin: Implications for immune system development and future disease screening and prevention. Wisconsin Public Health Association Conference, Wisconsin Dells, WI. May 22-23
- 2011 **L. Ahrenhoerster**, P. Lakatos, M. Laiosa. Persistent developmental arylhydrocarbon receptor activation modulates Notch1 - dependent T - cell but not B - cell differentiation potential. Abstract No. 1126. 2011 Society of Toxicology Itinerary. Washington, D.C.; March 2
- 2010 **L. Ahrenhoerster**. The Milk of Human (Resources) Kindness: Multicultural Breastfeeding Support in the Workplace. Wisconsin Public Health Association Conference, Middleton, WI; May 26-27

AWARDS AND HONORS

- 2014 Best Oral Presentation
Joseph J. Zilber School of Public Health Student Research Symposium
- 2013 Interpretation of 2013 National Public Health Week Theme
Wisconsin Public Health Association
- 2013 Second Place, Best Presentation by a Graduate Student in Immunotoxicology
Society of Toxicology

2013	Second Place, Reproductive and Developmental Toxicology Graduate Student Award Society of Toxicology
2013	Graduate Student Travel Award Society of Toxicology
2012	Student Poster Award American Public Health Association, Environment Section
2013	Travel Award American Public Health Association, Environment Section
2012	Student Poster Presentation Award Wisconsin Public Health Association Conference
2011-2012	Chancellor's Graduate Student Award University of Wisconsin – Milwaukee
2011	Graduate School Travel Award University of Wisconsin – Milwaukee
2010-2011	Chancellor's Graduate Student Award University of Wisconsin – Milwaukee
2010	Student Poster Presentation Award Wisconsin Public Health Association Conference

RESEARCH EXPERIENCE

Research Assistant

Lab of Developmental Immunotoxicology, UW-Milwaukee; June 2010-present

- ◆ Mentor: Michael D. Laiosa, Ph.D.
- ◆ Conducting original research in an immunotoxicology lab setting
- ◆ Learning lab skills, mouse husbandry, flow cytometry, cell culture, qPCR

Project Assistant

Center for Urban Population Health, UW-Milwaukee, Project Health; January-June 2010

- ◆ Mentor: Paul Florsheim, Ph.D.
- ◆ Assisted Dr. Florsheim and his Ph.D. student with administration of surveys to High School students for the Project Health program

Project Assistant

Great Lakes WATER Institute, UW-Milwaukee; October 2009-January 2010

- ◆ Mentor: Michael J. Carvan, Ph.D.
- ◆ Assisted Dr. Carvan and his graduate students with projects, including zebrafish and trout developmental toxicology studies; assisted with fish husbandry

COLLEGE LEVEL TEACHING EXPERIENCE

University of Wisconsin--Milwaukee; Milwaukee, WI; September 2013-present

- ◆ Designed and taught Introduction to Public Health to undergraduate students
- ◆ Supervised Teaching Assistants

Carroll University, Waukesha, Wisconsin; September-December 2012

- ◆ Designed and taught Environmental and Occupational Health as a component of the undergraduate degree in Public Health

OTHER EDUCATION AND TRAINING

- ◆ Lactation Counselor Training and Certification (45 hours)
October 14-18, 2013; CLC expires 12/31/2016
Healthy Children's Center for Breastfeeding
- ◆ Leadership Training (12 hours total)
Magda Peck, Ph.D.; Founding Dean and Professor, Joseph J. Zilber School of Public Health, Milwaukee, WI
- ◆ Assorted training for teaching online (18 hours total)
Staff; Online & Blended Teaching Program, Learning and Technology Center, UW-Milwaukee, Milwaukee, WI
- ◆ Learner-Centered Course Design (8 hours total)
Connie Schroeder, Ph.D.; Center for Instructional and Professional Development, UW-Milwaukee, Milwaukee, WI
- ◆ "R" Statistical Software Programming Courses (18 hours total)
Dave Armstrong, Ph.D.; Center for Applied Behavioral Health Research, UW-Milwaukee, Milwaukee, WI
Todd R. Miller, Ph.D.; Joseph J. Zilber School of Public Health, Milwaukee, WI
- ◆ CITI Training in IRB—Biomedical and Social & Behavioral Combined Researchers – Basic Course (2014)

PROFESSIONAL MEMBERSHIPS

1999-present	MCBC—Milwaukee County Breastfeeding Coalition
2009-present	WPHA—Wisconsin Public Health Association
2011-present	SOT—Society of Toxicology
2011-present	APHA—American Public Health Association
2013-present	WEHN—Wisconsin Environmental Health Network
2014-present	NACCHO—National Association of County and City Health Officers

PROFESSIONAL SERVICE

- ◆ NACCHO-- Grant Application Reviewer for Reducing Disparities in Breastfeeding through Peer and Professional Lactation Support Project; 2014
- ◆ APHA SA—Student Assembly
Abstract Reviewer for 141st APHA Annual Meeting; 2013
Abstract Reviewer for 142nd APHA Annual Meeting; 2014
- ◆ APHA SA—Student Assembly
Student Liaison to Zilber School of Public Health; 2013-present
- ◆ Escape Fire—Nationwide Screening Day; September 17, 2013
Co-led and organized screening and panel discussion to follow;
Hosted by PHGSA at Zilber School of Public Health
- ◆ SOT Peer Mentor for Undergraduate Education Program
Committee on Diversity Initiatives
San Antonio, TX; March 9-11, 2013
- ◆ APHA—Breastfeeding Forum
Liaison to the Environment Section; 2012-present
- ◆ Lactation Room Committee; co-chair
Designed and developed budget for Lactation Room at Zilber School of Public Health Building; Grant funding obtained from private donor; 2012-2013
- ◆ MCBC—Milwaukee County Breastfeeding Coalition;
Content curator for Facebook page; 2013-present
- ◆ PHGSA—Public Health Graduate Student Association at UW-Milwaukee
Member 2012-present;
National Public Health Week content curator for Facebook page 2013-present
- ◆ GPC—Graduate Program Committee at UW-Milwaukee Zilber School of Public Health; Elected Ph.D. representative; 2012-2013
- ◆ Hiring committee—Lab of Developmental Immunotoxicology Research
Specialist; 2012