

IMMUNOREGULATION OF LIVER REGENERATION BY THE
ARYL HYDROCARBON RECEPTOR: ROLE OF LYMPHOCYTES AND
INTERFERON-GAMMA

by

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ABSTRACT

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Christopher John Horras

Boise State University, 2011

Under the Supervision of Dr. Kristen A. Mitchell, Ph.D.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a persistent environmental contaminant that elicits toxicity by activating the aryl hydrocarbon receptor. The toxic effects associated with TCDD exposure include immunotoxicity and dysregulated cell cycle control, although the mechanisms are poorly understood. A previous report indicates that exposure to TCDD suppresses hepatocyte proliferation in a mouse model of liver regeneration induced by 70% partial hepatectomy (PH). Based on reports that liver regeneration is negatively regulated by interferon (IFN)- γ produced by activated natural killer (NK) cells, along with the well-established immunotoxic effects of TCDD in other model systems, we hypothesized that TCDD treatment attenuates liver regeneration by enhancing NK cell activation and IFN- γ production in the regenerating liver. We also considered the effects of TCDD on natural killer (NK) T and T cells, which are capable of producing IFN- γ as well. Mice were treated with TCDD (20 μ g/kg) one day prior to

surgical PH. Hepatocyte proliferation in the remnant liver was measured based on the incorporation of a thymidine analog, bromodeoxyuridine. Lymphocytes were collected from the spleen and remnant liver and analyzed by flow cytometry. IFN- γ was measured by intracellular staining followed by flow cytometry. Consistent with other reports, we found that TCDD treatment suppressed hepatocyte proliferation in the regenerating liver by 50-80%. However, contrary to our hypothesis, exposure to TCDD had no effect on the number of lymphocytes in the spleen or liver after PH, nor did it increase IFN- γ^+ production by lymphocytes in the regenerating liver. Moreover, exposure to TCDD did not increase the number of NK and NKT cells expressing CD69, an early activation marker. To determine the relevance of NK cells to the TCDD-mediated suppression of liver regeneration, mice were treated with an α -asialo-GM-1 antibody to deplete NK cells prior to TCDD administration and PH. Depletion of NK cells did not restore hepatocyte proliferation in TCDD-treated mice, indicating that NK cells are not required for the attenuation of liver regeneration by TCDD. Hence, exposure to TCDD suppresses *in vivo* hepatocyte proliferation by a mechanism that does not involve NK cells or enhanced production of IFN- γ .

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CHAPTER I: INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a chemical in the family of halogenated aromatic hydrocarbons (HAHs), which are widespread and persistent environmental contaminants (Figure 1.1). These compounds are structurally related, share a common mechanism, and are toxic to different degrees (Poland & Knutson, 1982). The structure of toxic HAHs generally includes two benzene rings and substituted halogen atoms at three or four ring positions, resulting in a lipophilic disposition (Poland & Knutson, 1982). The mechanism of toxicity for these compounds involves binding to the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that modulates gene expression. The relative toxicity of HAHs depends on their binding affinity for the AhR (Schechter & Gasiewicz, 2003). TCDD is considered to be the prototypical HAH, due to its high binding affinity for the AhR and resulting potency. Because of these properties, TCDD is used in research studies of HAH toxicity.

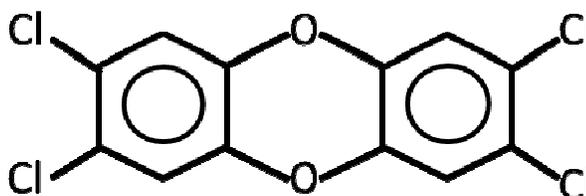


Figure 1.1 Structure of TCDD

TCDD was produced as an unintentional contaminant of herbicides starting in the 1940s. During the chemical manufacture of various herbicides, heating of chlorine-

containing compounds resulted in the formation of TCDD (Young, Thalken, & Harrison, 1979). These herbicides were then used to destroy unwanted vegetation in agricultural operations throughout the world. Such widespread use resulted in the persistent presence of TCDD throughout the environment, where the half-life ranges from months to years, depending upon the medium of contamination (Mukerjee, 1998). Although herbicides are now regulated to avoid further TCDD contamination, the persistence of TCDD, due to its resistance to biodegradation, has led to a long-term and potentially permanent presence of this pollutant in the environment (Kao, Chen, Liu, & Wu, 2001).

Several infamous environmental disasters culminated in the release of large concentrations of TCDD into the environment in a relatively short period of time (Freeman, Hileman, & Schroy, 1986; Lakshman, Campbell, Chirtel, & Ekarohita, 1985; Reggiani, 1978). Of these disasters, the use of the defoliant, Agent Orange, in the Vietnam War is the most well known. Agent Orange, like other defoliating herbicides, was unintentionally contaminated with TCDD during the manufacturing process. The United States military used Agent Orange to clear foliage from the jungle landscape in Vietnam as a tactical maneuver. Throughout this period, the United States military deposited large amounts of TCDD into the Vietnamese environment, resulting in the exposure of multitudes of U.S. and Vietnamese soldiers, along with Vietnamese civilians, directly to TCDD (Lakshman et al., 1985).

Currently, TCDD is primarily produced as a result of industrial processes that involve chlorine-containing compounds, such as the bleaching of pulp in paper production and the incineration of municipal waste (Cummings, Korb, & Evans, 1987; Lebel, Williams, & Benoit, 1992). A significant source of TCDD production in the U.S.

is the incineration of medical refuse (Thornton, McCally, Orris, & Weinberg, 1996). This process results in the production of TCDD as various chlorine-containing plastics are heated. The TCDD is then released into the atmosphere when combustion fumes are emitted. Atmospheric TCDD then settles onto various terrestrial, aquatic, and marine environments and bioaccumulates in the food chain. While acute, high-level human exposure to TCDD has resulted from environmental disasters, chronic low-level human exposure results from the consumption of contaminated food products. Because it is lipophilic, TCDD can be stored long-term in fat tissue, and can be released as a result of fat metabolism (Travis & Hattemerfrey, 1991).

Information about TCDD toxicity in humans is primarily derived from epidemiological studies of human exposure during industrial and environmental disasters; however, these studies are inherently limited due to a lack of appropriate controls and a multitude of confounding variables. Consequently, the toxic endpoints of TCDD exposure in humans, while well documented, remain contentious (White & Birnbaum, 2009). Hence, *in vitro* and rodent models have been used extensively to study TCDD toxicity. In rodents, TCDD treatment elicits a multitude of toxic effects including modulation of cell cycle progression, immunotoxicity, developmental toxicity, reproductive toxicity, cachexia (wasting syndrome), and cancer promotion (Abbott & Birnbaum, 1991; Chastain & Pazdernik, 1985; Gottlicher & Wiebel, 1991; Hanberg, Hakansson, & Ahlborg, 1989; Huff, 1992; Neubert, Krowke, Chahoud, & Franz, 1987; Rozman, 1984; Wolfle, Marotzki, Dartsch, Schafer, & Marquardt, 2000). Interestingly, TCDD has also been shown to exert protective effects against certain types of cancer (Biegel & Safe, 1990). These effects are diverse, tissue specific, and have not been

explained by a common mechanism of TCDD toxicity to this point. As a result, work remains to be done to uncover the risks to human health posed by TCDD exposure.

The Aryl Hydrocarbon Receptor

The toxicity of TCDD is mediated by the aryl hydrocarbon receptor (AhR), which has been well characterized (Safe, 2001). This protein is highly conserved and is ubiquitously expressed in vertebrate cells (Mandal, 2005). The AhR is a soluble protein that, while inactive, resides in the cytosol and belongs to the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) superfamily of proteins (Figure 1.2) (Fukunaga, Probst, Reiszporszasz, & Hankinson, 1995). Along with the other proteins in this superfamily, the AhR functions as a transcription factor (Henry & Gasiewicz, 1993); however, it is one of only two proteins in this group that also contains a ligand-binding domain, resulting in its properties as a conditionally activated receptor.

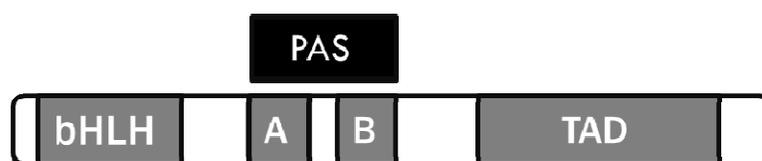


Figure 1.2 Structure of the AhR. The AhR is a member of the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) superfamily of proteins. It is comprised of a bHLH domain responsible for protein-protein interactions and DNA binding, two PAS domains (A and B) that facilitate binding with other bHLH/PAS proteins and ligand binding, and a glutamine-rich transactivation domain (TAD) involved in coactivator recruitment and transactivation (Fukunaga et al., 1995).

The AhR is sometimes referred to as an orphan receptor because a *bona fide* endogenous agonist has not been identified. Furthermore, the endogenous, physiological function of the AhR is unknown. Despite this fact, the AhR has been implicated in certain physiological processes, including fetal development and cell cycle progression (Bunger et al., 2008; Bunger et al., 2003; Harstad, Guite, Thomae, & Bradfield, 2006; Ma &

Whitlock, 1996). In particular, the vascular development in AhR-null mice is retarded, resulting in anatomical malformations (Schmidt, Su, Reddy, Simon, & Bradfield, 1996). This is the case in the developing liver where absence of the AhR results in a patent ductus venosus, an incomplete closure of the liver vasculature. The resulting liver is 50% smaller than wild type livers and displays a spectrum of defects (Schmidt et al., 1996). While AhR null mice are surprisingly viable, it is apparent that the AhR is necessary for proper development of the liver.

While the endogenous ligand and physiological function of the AhR remain a mystery, this receptor displays promiscuity in binding to xenobiotic ligands (Savouret, Berdeaux, & Casper, 2003). TCDD is the most potent of the xenobiotic AhR ligands due to its high binding affinity (K_d = approximately 5nM in humans), and the AhR is required for the toxic effects of TCDD to be manifested (Bunger et al., 2003; Harper, Prokipcak, Bush, Golas, & Okey, 1991; Vorderstrasse, Steppan, Silverstone, & Kerkvliet, 2001). It is well established that cells, as well as mice, that lack a functional AhR do not display the typical endpoints of TCDD toxicity (Fernandez-Salguero, Hilbert, Rudikoff, Ward, & Gonzalez, 1996; Schmidt et al., 1996).

The classical mechanism of AhR activation by TCDD is widely accepted (Figure 1.3) (Fuji-Kuriyama & Kawajiri, 2010; Mitchell & Elferink, 2009). TCDD enters cells by simple diffusion due to its lipophilic nature. Prior to ligand binding, the AhR resides in the cytosol accompanied by the following chaperone proteins: two molecules of heat-shock protein 90 (hsp90), an immunophilin analogue protein (AIP), and a c-Src protein kinase (Carver & Bradfield, 1997; Chen & Perdew, 1994; Enan & Matsumura, 1996). Once in the cytosol, TCDD binds to the AhR, and the chaperone proteins dissociate from

the complex. The AhR then translocates to the nucleus, where it is joined by the AhR nuclear translocator (Arnt) (Elferink, Gasiewicz, & Whitlock, 1990; Gasiewicz, Elferink, & Henry, 1991; Ikuta, Eguchi, Tachibana, Yoneda, & Kawajiri, 1998). This complex is transcriptionally active and binds to DNA at dioxin response elements (DREs) (Elferink et al., 1990). DREs are stretches of DNA containing the core sequence 5'-GCGTG-3' within the consensus sequence 5'-T/GNGCGTGA/CG/CA-3' (Lusska, Shen, & Whitlock, 1993; E. S. Shen & Whitlock, 1992). Genes containing DREs include various xenobiotic-metabolizing enzymes, the archetype of which is *Cyp1a1*, which encodes the enzyme, cytochrome P4501A1 (Whitlock, 1999). The *Cyp1a1* gene contains six DREs (Ma, 2001) and is regulated exclusively by the AhR, which makes it a sensitive and reproducible hallmark of AhR activation (Fukunaga et al., 1995). While many exogenous AhR ligands are metabolized by cytochrome P4501A1, TCDD is an exception, as it is not a substrate for this enzyme. As a result, TCDD is not metabolically depleted and remains bound to the AhR for a sustained period of time (Fuji-Kuriyama & Kawajiri, 2010). In fact, the half-life of TCDD in mice is estimated at 10 days (Miniero, De Felip, Ferri, & di Domenico, 2001). The phenomenon of sustained AhR activation is thought to contribute to the toxicity of TCDD.

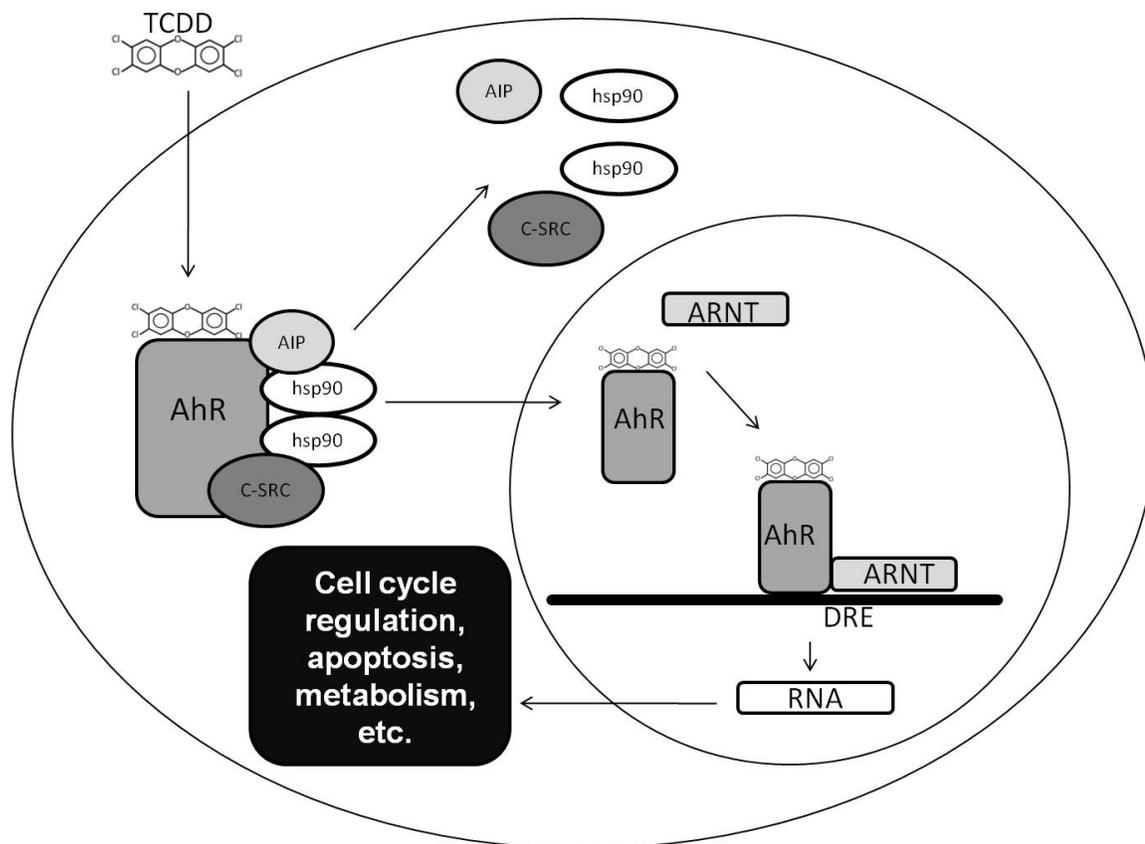


Figure 1.3 Classical Mechanism of AhR Activation. TCDD diffuses across the cell membrane and binds to the AhR in the cytosol. The AhR is bound to chaperone proteins hsp90, immunophilin analogue protein (AIP), and c-Src prior to ligand binding (Whitlock, 1999). Binding of TCDD to the AhR displaces the chaperone proteins, and the AhR translocates to the nucleus, where it binds to the AhR nuclear translocator (Arnt). This heterodimer is a transcriptionally active complex that binds to dioxin response elements (DREs) and modulates the transcription of target genes, resulting in changes in cell cycle regulation, apoptosis, metabolism, along with other processes.

Cell Cycle Regulation by the AhR

Increasing evidence demonstrates that the AhR regulates cell cycle progression in several types of cells, including hepatocytes (Gottlicher & Wiebel, 1991), lymphocytes (Laiosa et al., 2003), neuronal cells (Jin, Jung, Lee, & Kim, 2004), and keratinocytes (Ray & Swanson, 2003). The cell cycle is comprised of the following stages: G1, S, G2, and M (Alberts, 2010). Cells in G1 have recently divided and are growing for another

division or for entry into quiescence. If the proper signals are present, a cell will progress from G1 into S-phase. In S-phase, the cell replicates its DNA in preparation for division. Following further signaling, the cell passes into G2, where it duplicates its organelles and produces components necessary for mitosis, the next stage. The cell then progresses through the stages of mitosis, and ultimately completes division into two identical cells in the final step called cytokinesis (Alberts, 2010).

In rat 5L hepatoma cells, endogenous AhR activity is particularly important for transition through the G1/S-phase boundary, as AhR-deficient cells elicit a G1 arrest that is rectified upon reintroduction of a functional receptor (Ma & Whitlock, 1996). A similar cell cycle arrest is observed in 5L hepatoma cells treated with TCDD, which raises the intriguing possibility that transient, endogenous AhR activation promotes cell cycle progression. In contrast, sustained AhR activation by TCDD, a poorly metabolized exogenous agonist, impedes cell cycle progression (Barhoover, Hall, Greenlee, & Thomas, 2010; Gottlicher & Wiebel, 1991; Reiners, Clift, & Mathieu, 1999). Several studies demonstrate that the mechanism by which TCDD elicits an *in vitro* G1 arrest involves the AhR-mediated induction of p27Kip1 (Kolluri, Weiss, Koff, & Gottlicher, 1999). p27Kip1, and the related protein p21Cip1, inhibit S-phase progression by blocking the activity of cyclin-dependent kinases that otherwise phosphorylate and inactivate the tumor suppressor, retinoblastoma protein (Rb) (Albrecht et al., 1998). Using a mouse model of liver regeneration induced by 70% partial hepatectomy (PH), it was found that TCDD treatment elicits a similar G1 arrest in hepatocytes in the regenerating liver. However, no overt changes in p27Kip1 or p21Cip1 expression were observed, raising the

possibility that the mechanisms by which TCDD elicits a G1 arrest *in vivo* are distinct from those mechanisms identified *in vitro* (Mitchell, Lockhart, Huang, & Elferink, 2006).

Physiology of Liver Regeneration

The PH model system is well established for studying mechanisms of *in vivo* cell cycle control (Fausto, Campbell, & Riehle, 2006). Surgical removal of 70% of the liver results in coordinated proliferation of the remaining hepatocytes. While the precise mechanisms of liver regeneration are poorly understood (Michalopoulos, 2010), a working model divides the process into four phases: priming, progression, cell cycle, and termination (Michalopoulos, 2007). The priming phase occurs within the first 2-6 hours after PH and involves changes in the extracellular matrix and production of tumor necrosis factor (TNF)- α and interleukin (IL)-6, which leads to the STAT3-mediated expression of immediate early genes needed for hepatocytes to transition from G0 (quiescence) to G1 phase of the cell cycle. The progression phase occurs as hepatocytes move into G1 of the cell cycle and is marked by increased expression of hepatocyte growth factor, epidermal growth factor, transforming growth factor, and inducible nitric oxide synthase (iNOS). The next step is the cell cycle phase, in which molecular signals permit the transition of hepatocytes from G1 to S phase. Cells subsequently progress through the cell cycle and proliferate continuously until the original liver mass is restored. Termination of hepatocyte proliferation presumably depends on an unidentified "stop" signal (Michalopoulos, 2010).

Although the precise mechanisms that terminate hepatocyte proliferation remain unclear (Michalopoulos, 2007), one potential mechanism is the production of interferon (IFN)- γ by natural killer (NK) cells, which increase in number following PH (Vujanovic

et al., 1995). Presumably, resident NK cells in the liver are activated, proliferate, and produce IFN- γ , resulting in a hepatocyte cell cycle arrest. In addition, activated NK cells could potentially be recruited to the liver from the spleen (Figure 1.4).

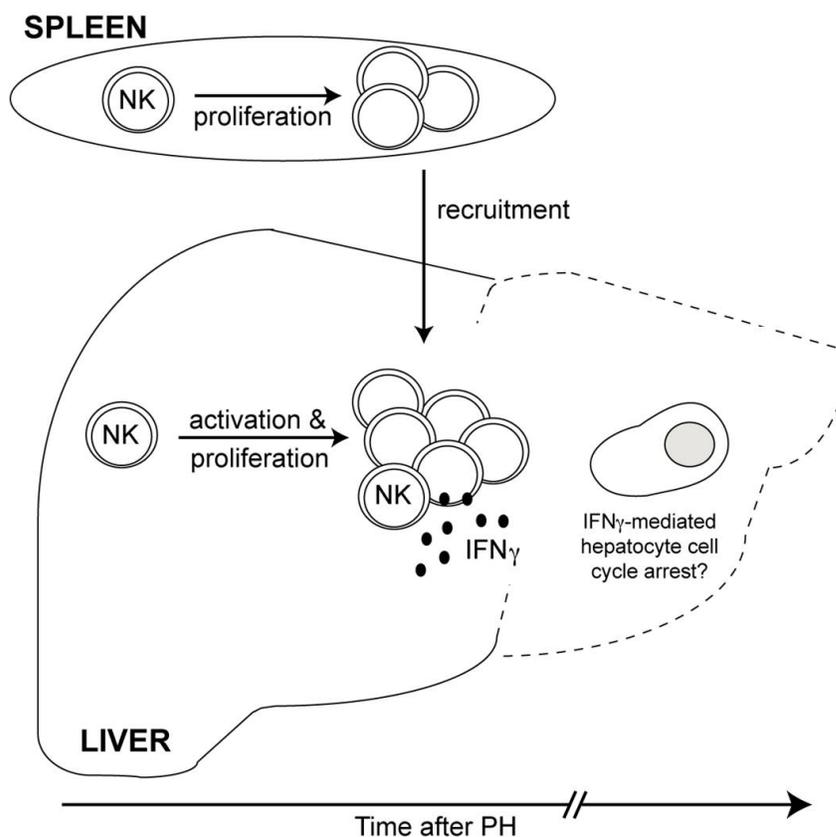


Figure 1.4 Potential Role of NK cells and IFN- γ in Attenuating Liver Regeneration. Immediately after PH, NK cells in the liver become activated, proliferate and produce IFN- γ . IFN- γ binds to the IFN- γ receptor on hepatocytes (not shown), activating Stat1, leading to cell cycle arrest. Furthermore, NK cells may directly kill regenerating hepatocytes. Finally, NK cells in the spleen may also become activated after PH and migrate to the liver (Ochi et al., 2004; Sun & Gao, 2004; Sun et al., 2006).

NK Cells and IFN- γ

The immune system utilizes antigen-specific (adaptive) and non-specific (innate) defenses that function together to defend an organism from invading pathogens and the progression of cancer. The adaptive branch of the immune system includes cells that demonstrate specificity in their defense functions, including the use of variable receptors

and antibodies for the elimination of antigens. The innate branch of the immune system includes cells and soluble factors that lack specificity for antigens. NK cells are one component of the innate immune system. They develop in the bone marrow from the lymphoid lineage of cells and reside primarily in the blood, spleen, and liver. While NK cells make up a small percentage of lymphocytes in the blood and spleen (<5%), the liver contains a relatively large population of resident NK cells, where they account for 10-25% of all intrahepatic lymphocytes (Gao, Jeong, & Tian, 2008). In fact, recent reports emphasize the immunological nature of the liver due to a plethora of resident immune cells found here (Crispe, 2009; Gao et al., 2008; Racanelli & Rehermann, 2006). NK cells are responsible for general clearance of virus-infected cells and cancer cells and are activated by an array of cytokines, including IL-2 and IL-12, both of which are produced by macrophages, another facet of the innate immune system. NK cells destroy cancerous and infected cells by FAS-dependent apoptosis signaling, by becoming directly cytotoxic, and by producing IFN- γ (Goldsby, Kindt, Osborne, & Kuby, 2000).

IFN- γ is an inflammatory cytokine recognized for its antiviral and immunomodulatory properties. Also known as type II interferon, IFN- γ exerts its actions by binding to the IFN- γ receptor (IFNGR), a heterodimeric transmembrane protein expressed on a variety of cell types, including regenerating hepatocytes (Billiau & Matthys, 2009; Nagao et al., 2000; Valente et al., 1992; Volpes et al., 1991). The IFNGR is comprised of a constitutively expressed alpha chain (IFNGR1), which contains a ligand-binding domain and an inducible, non-ligand-binding beta chain (IFNGR2) (Farrar & Schreiber, 1993). IFN- γ interacts with IFNGR1, which initiates the association of IFNGR1 with IFNGR2 and subsequent phosphorylation of JAK1 and JAK2 kinases.

Activated JAK kinases phosphorylate the cytoplasmic tails of IFNGR1 subunits, providing a docking site for Stat1, a member of the signal transducer and activator of transcription (Stat) family of transcription factors. Stat1 becomes phosphorylated and dissociates from IFNGR1, forms homo- or heterodimers, and translocates to the nucleus to modulate transcription of IFN- γ -responsive genes, including interferon regulatory factor (IRF)-1 (Ihle et al., 1994). IRF-1 is a transcription factor that binds to interferon-sensitive response elements to drive the expression of an additional set of IFN- γ -responsive genes, including those involved in apoptosis and cell cycle regulation (Ihle et al., 1994; Tamura, Yanai, Savitsky, & Taniguchi, 2008).

Recent work has revealed a role for innate immunity in regulating liver regeneration (Dong, Wei, Sun, & Tian, 2007; K. Shen et al., 2008; Sun & Gao, 2004; Wei, Wei, Wang, Tian, & Sun, 2010). Studies indicate that NK cells increase in number and activity during liver regeneration following PH (Lai, Chen, & Chen, 1996). Additionally, the notion that IFN- γ , produced primarily by NK cells, negatively regulates regeneration is supported by observations that hepatocyte DNA synthesis is increased in the regenerating liver of mice that are nullizygous for the IFN- γ receptor, as well as in mice that were depleted of NK cells prior to surgery (Sun & Gao, 2004). IFN- γ may inhibit hepatocyte proliferation in the regenerating liver through the Stat1-mediated induction of IRF-1 and p21Cip1, as administration of IFN- γ induced the expression of these proteins, as well as phosphorylated Stat1, within 4 h after PH. Moreover, treatment with IFN- γ *in vitro* inhibited cell proliferation of wild-type mouse hepatocytes but not STAT1^{-/-}, IRF-1^{-/-}, or p21Cip1^{-/-} hepatocytes (Sun et al., 2006).

While previous reports have identified NK cells as the primary source of IFN- γ production during liver regeneration after PH (Sun & Gao, 2004), other lymphocyte types, including T cells and NKT cells, have also been reported to produce IFN- γ in other models (Dong, Zhang, Sun, Wei, & Tian, 2007; Komita et al., 2006; Miyagi et al., 2004; Otani et al., 1999). Murine NKT cells are a subset of T cells that possess an invariant T cell receptor (TCR), a transmembrane CD3 protein, and the NK cell marker, NK1.1. The NKT TCR recognizes CD1d antigen presentation molecules containing α -galactosylceramide, a glycolipid found in a variety of infectious bacteria. In contrast to NKT cells, murine NK cells lack a TCR and the CD3 transmembrane protein while expressing the NK1.1 surface marker (Bendelac, Savage, & Teyton, 2007). The liver contains a relatively high concentration of NKT cells compared with other lymphoid organs, and murine NKT cells have been shown to increase in propensity and activation following PH (Minagawa et al., 2000). Taken together, these data support the notion that NK cells, NKT cells, and T cells all represent possible sources of IFN- γ that could negatively regulate liver regeneration after PH.

TCDD Immunotoxicity

TCDD is one of the most immunotoxic compounds known and displays a wide array of effects on various aspects of the immune system (Kerkvliet, 2009). Generally speaking, exposure to TCDD results in suppression of adaptive immune responses. However, TCDD treatment has also been shown to augment the innate immune response, including IFN- γ production, in several model systems (Knutson, Neff-LaFord, & Lawrence, 2003; Neff-LaFord, Teske, Bushnell, & Lawrence, 2007; Sugita-Konishi, Kobayashi, Naito, Miura, & Suzuki, 2003). Conversely, TCDD has been shown to

decrease IFN- γ in other model systems (Benson & Shepherd, 2011; Prell, Oughton, & Kerkvliet, 1995). Reports of the consequences of TCDD treatment on NK cell activity are equally inconsistent (Funseth & Ilback, 1992; X. H. Wang, Zhou, Xu, Wang, & Lu, 2009) yet provide evidence that these cells may indeed be modulated by exposure to TCDD.

Hypothesis

It has been previously demonstrated that TCDD treatment attenuates liver regeneration (Mitchell et al., 2006). The current study aims to elucidate a possible mechanism for this effect. Evidence from previous studies implicate NK cells in negatively regulating liver regeneration via IFN- γ production (Sun & Gao, 2004). Furthermore, TCDD treatment has been shown to modulate the innate immune response, including NK cell activity and IFN- γ production in other model systems (Benson & Shepherd, 2011; Funseth & Ilback, 1992; Prell et al., 1995; X. H. Wang et al., 2009). Based on these observations, we hypothesized that TCDD treatment attenuates liver regeneration by increasing the number of NK cells and enhancing IFN- γ production in the regenerating liver. The aims of the study were as follows:

- Determine the effect of TCDD treatment on the number of NK, NKT, and T cells in the spleen and regenerating liver after PH.
- Determine the effect of TCDD treatment on the production of IFN- γ by NK, NKT, and T cells in the regenerating liver.
- Determine the effect of TCDD treatment on the number of activated NK, NKT, and T cells in the regenerating liver by assessing the expression of the early activation marker, CD69.

- Determine if TCDD-induced attenuation of liver regeneration requires the presence of NK cells.

Experimental Design

Figure 1.5 outlines the experimental design used to investigate the role of NK cells in the suppression of liver regeneration in TCDD-treated mice. Mice were treated by oral gavage with 20 $\mu\text{g}/\text{kg}$ TCDD or peanut oil vehicle. After 24 h, mice were anesthetized by continuous isoflurane inhalation, and three of the five liver lobes were surgically removed. To measure hepatocyte proliferation, mice were injected with bromodeoxyuridine (BrdU) two hours before euthanasia. BrdU is a thymidine analog and is incorporated into newly synthesized DNA. Mice were euthanized 0, 12, 24, 36, and 42 h after PH. Liver, spleen, and plasma were collected. Lymphocytes were isolated and analyzed by flow cytometry. Hepatocyte proliferation was measured using immunohistochemistry. In NK cell depletion studies, mice were treated with α -Asialo GM-1 (α ASGM-1) antibody or PBS 24 h prior to TCDD or vehicle treatment.

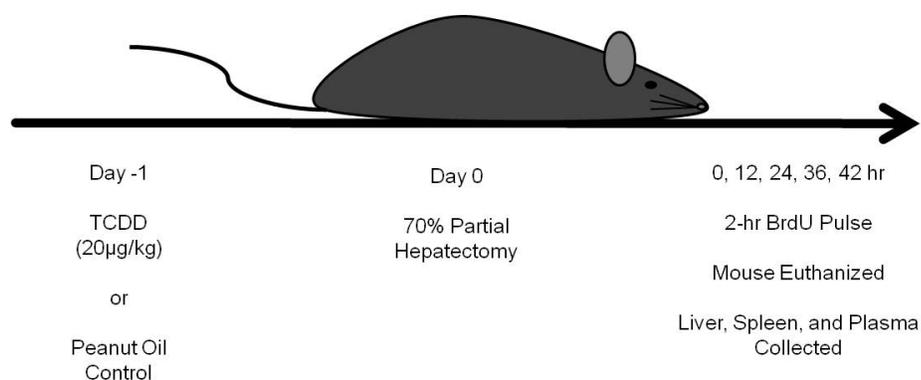


Figure 1.5 Experimental Design

CHAPTER II: MATERIALS AND METHODS

Animal Treatment

Female C57Bl/6 mice (7-8 weeks old; The Jackson Laboratory, Bar Harbor, ME) were housed in microisolators, maintained on a 12-h light cycle, and provided with free access to food and water. TCDD (Cambridge Isotope Laboratories, Andover, MA) was dissolved in anisole, diluted in peanut oil, and administered by oral gavage at 20 $\mu\text{g}/\text{kg}$ bodyweight. Control mice received peanut oil that contained an equivalent amount of anisole. This dose of TCDD is not overly toxic (Pohjanvirta, Unkila, & Tuomisto, 1994) and represents the lowest dose at which consistent, reproducible suppression of liver regeneration was observed. The next day, mice were anesthetized with inhaled isoflurane, and PH was performed by surgically resecting 70% of the liver, as described elsewhere (Mitchell et al., 2006). To measure hepatocyte proliferation, mice were injected i.p. with 50 mg/kg bromodeoxyuridine (BrdU; Sigma-Aldrich, St. Louis, MO) 2 h before euthanasia. For experiments in which NK cells were depleted, lyophilized rabbit α -ASGM-1 antibody (Wako Pure Chemical Industries, Osaka, Japan) was resuspended in 2 ml water and injected i.p. at 80 $\mu\text{l}/\text{mouse}$ 24 h prior to administration of TCDD or vehicle. This volume of antibody was selected based on the manufacturer's recommendations. Based on FACS analysis using NK1.1 and CD3 antibodies, this treatment regimen effectively depleted NK cells for at least four days. All experiments were performed in compliance with the standards in place at the Boise VA Medical Center.

Immunohistochemistry

Remnant liver tissue was fixed in Ultralight Zinc Formalin Fixative (Bi-Biomics, Nampa, ID) prior to being paraffin embedded and processed for immunohistochemical staining with a BrdU staining kit (Invitrogen, Carlsbad, CA). Briefly, 5- μ m sections were incubated with a biotinylated BrdU antibody followed by avidin-conjugated horseradish peroxidase and the substrate 3,3-diaminobenzidine (DAB); tissue was counterstained with hematoxylin. To quantify hepatocyte proliferation, brown-stained (BrdU⁺) nuclei were counted in four separate fields (400X magnification) per animal and expressed as a percentage of the total number of nuclei.

Splenocyte Isolation

Spleens were harvested into ice-cold RPMI 1640 with 2.5% FBS and kept on ice throughout processing. Spleens were pressed between frosted microscope slides to prepare single-cell suspensions, and red blood cells were removed by hypotonic lysis. Cells were centrifuged at 350xg for 10 minutes at 4°C, and the supernatant was discarded. Cells were resuspended in fresh media, and debris was settled and removed. Cells were then enumerated and stained for flow cytometry.

Intrahepatic Lymphocyte Isolation

Livers were perfused with ice-cold Hank's Buffered Salt Solution (HBSS) containing 50 mM EDTA. The portal vein was cannulated, and the inferior vena cava was severed below the liver to allow blood outflow. Livers were then transferred to ice-cold RPMI 1640 with 2.5% FBS, minced into a slurry, and poured into a nylon cell strainer atop a 50-ml conical tube. The slurry was pressed against the cell strainer using the plunger end of a 10-cc syringe and washed repeatedly. Samples were centrifuged at 60xg,

1 minute, at room temperature with no brake; pellets containing hepatocytes were discarded. Samples were then centrifuged at 500xg, 10 minutes, at room temperature with high brake. Resulting pellets were resuspended in 10 ml of 37.5% isotonic Percoll in RPMI 1640 without FBS and centrifuged at 850xg, 30 minutes, at room temperature with no brake. Pellets were depleted of red blood cells by hypotonic lysis. Remaining lymphocytes were washed and resuspended in PBS containing 1% fetal bovine serum.

Flow Cytometry

Freshly isolated splenocytes and intrahepatic lymphocytes were incubated with Fc receptor block (BD Biosciences, San Jose, CA) for 10 minutes before staining with the following fluorochrome-conjugated antibodies purchased from BD Biosciences: NK1.1 (PK136), CD3 (145-2C11), and CD69 (H1.2F3). Control cells were incubated with isotype-matched, fluorochrome-conjugated antibodies. To measure intracellular IFN- γ , cells were stained with antibodies against surface antigens, incubated with eBioscience Intracellular Fixation Buffer (eBioscience, San Diego, CA) for 30 minutes on ice then incubated with eBioscience Permeabilization Buffer for 5 minutes at room temperature. Cells were washed and incubated with an APC-conjugated α -IFN- γ antibody (XMG1.2, BD Biosciences) diluted in permeabilization buffer. Stained cells were analyzed on an Accuri C6 flow cytometer (Ann Arbor, MI). At least 50,000 events were collected from unpooled samples and analyzed using Accuri CFlow Plus software.

Statistical Analysis

Statistical analyses were performed using Prism (version 4.0a; GraphPad Software, San Diego, CA). Data were evaluated by two-way analysis of variance followed by a Bonferroni post hoc test to evaluate differences between TCDD- and

vehicle-treated groups. One-way analysis of variance was used to evaluate differences among time points within a single treatment group. Data were considered significantly different at $p \leq 0.05$.

Cell Percentage Graphs and Cell Number Graphs

Throughout this thesis, changes in lymphocyte populations are depicted by graphs for both percentage and total number of cells. Absolute lymphocyte numbers were calculated from FACS data, and percentages of specific cell phenotypes were multiplied by lymphocyte numbers to yield the total number of cells. As a consequence, the trends in percentage graphs differ from the trends in graphs depicting the total number of cells. The total number of cells was considered the mechanistically relevant metric since the number of hepatocytes per liver did not vary widely, and the number of lymphocytes available to exert actions on hepatocytes was considered the most crucial factor.

CHAPTER III: RESULTS

Exposure to TCDD Suppresses Hepatocyte Proliferation during Liver Regeneration

To confirm the finding that exposure to TCDD elicits a G1 cell cycle arrest in hepatocytes in the regenerating liver, hepatocyte proliferation was measured based on incorporation of the thymidine analog, bromodeoxyuridine (BrdU), which is incorporated into newly synthesized DNA during S phase. In vehicle-treated mice, approximately 40% of hepatocyte nuclei were BrdU⁺ 36 and 42 h after PH (Figure 2.1A, B), which is when peak DNA synthesis occurs in the regenerating mouse liver (Mangnall, Bird, & Majeed, 2003). In contrast, TCDD treatment reduced the percentage of BrdU⁺ nuclei by two- to five-fold, indicating that fewer cells in the regenerating liver had entered S-phase in these mice.

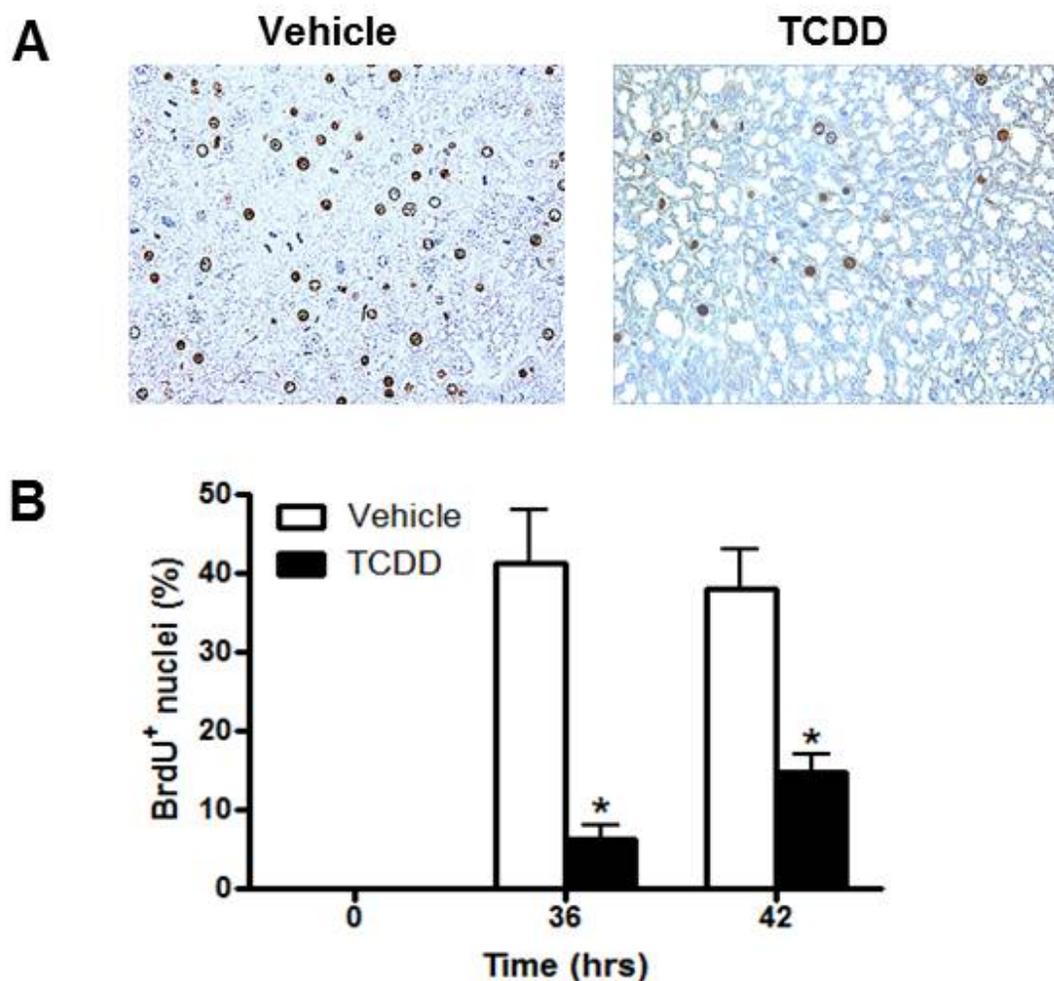


Figure 3.1 TCDD Treatment Suppresses Hepatocyte Proliferation after PH. Mice were treated with vehicle or TCDD 24 h before PH. Mice were pulsed with BrdU and euthanized at the indicated times. A) Representative BrdU staining in the regenerating liver of vehicle- or TCDD-treated mice 36 hr after PH. BrdU⁺ cells are identified by the dark brown (3,3'-diaminobenzidine-stained) nuclei (400X). B) Average percentage of BrdU⁺ nuclei (\pm SEM) in liver tissue from (n=3-6). *Significantly different from vehicle-treated group at same time point ($p \leq 0.05$).

TCDD Treatment Does Not Alter Lymphocyte Populations in the Spleen during Liver Regeneration

The spleen represents an extrahepatic source of immune cells, such as NK cells, which may become activated to produce inflammatory cytokines or migrate to the regenerating liver (J. Wang, Xu, Zhang, Wei, & Tian, 2005). Hence, we investigated the consequences of TCDD on the size and cellular composition of the spleen during liver regeneration. Partial hepatectomy caused an increase in splenocyte number at 36 h (Figure 2.2A), and TCDD treatment did not alter this trend. Although NK cells comprise a small portion of splenocytes (Figure 2.2B, C), the number of NK cells in the spleen increased 36 h after PH (Figure 2.2D). TCDD had no effect on the number of splenic NK cells at any time point after PH.

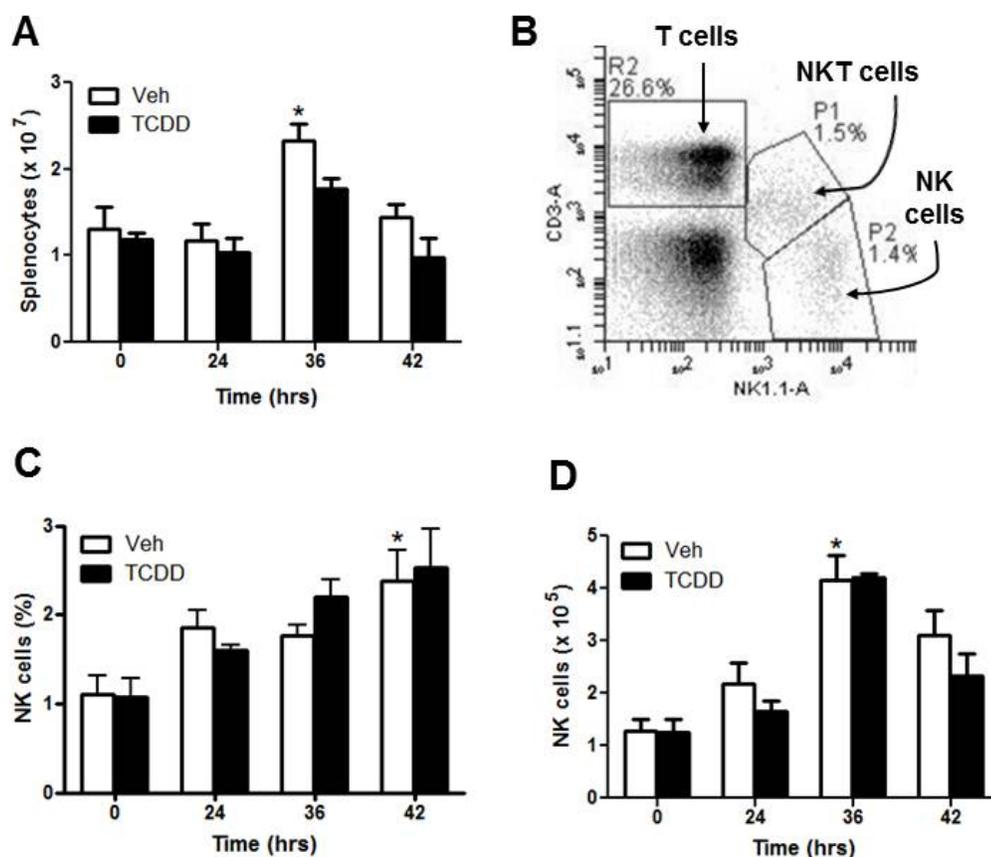


Figure 3.2 TCDD Treatment Does Not Alter Lymphocyte Populations in the Spleen during Liver Regeneration. Mice were treated with vehicle or TCDD 24 h prior to PH and euthanized postoperatively at the indicated times. A) Average number of splenocytes (\pm SEM) from 3-9 mice per treatment group at each time point. B) Representative FACS histogram depicting populations of T cells, NKT cells and NK cells based on dual staining of splenocytes with NK1.1 and CD3 antibodies. C, D) Average percentage and number of splenic NK cells (\pm SEM) from 3-9 mice per treatment group at each time point. Results are representative of three separate experiments. *Significantly different from 0 h vehicle-treated mice ($p \leq 0.05$).

Exposure to TCDD Does Not Increase the Number of Lymphocytes in the Regenerating Liver

Based on reports that the number of NK cells in the liver increases following PH (Lai et al., 1996), we investigated the ramifications of TCDD exposure on lymphocyte populations in the regenerating liver. The total number of intrahepatic lymphocytes doubled 42 h after PH in both vehicle- and TCDD-treated mice (Figure 2.3A). NK cells, NKT cells, and CD3⁺ T cells were identified in the regenerating liver based on staining with anti-NK1.1 and -CD3 antibodies (Figure 2.3B). Numbers of intrahepatic NK and NKT cells increased after PH (Figure 2.3C). TCDD treatment did not alter the percent or number of NK cells in the liver at any time point tested but did suppress the expansion of NKT cells in the liver 42 h after PH. Exposure to TCDD had no appreciable effect on the prevalence of CD3⁺ T cells in the liver (Figure 2.3C).

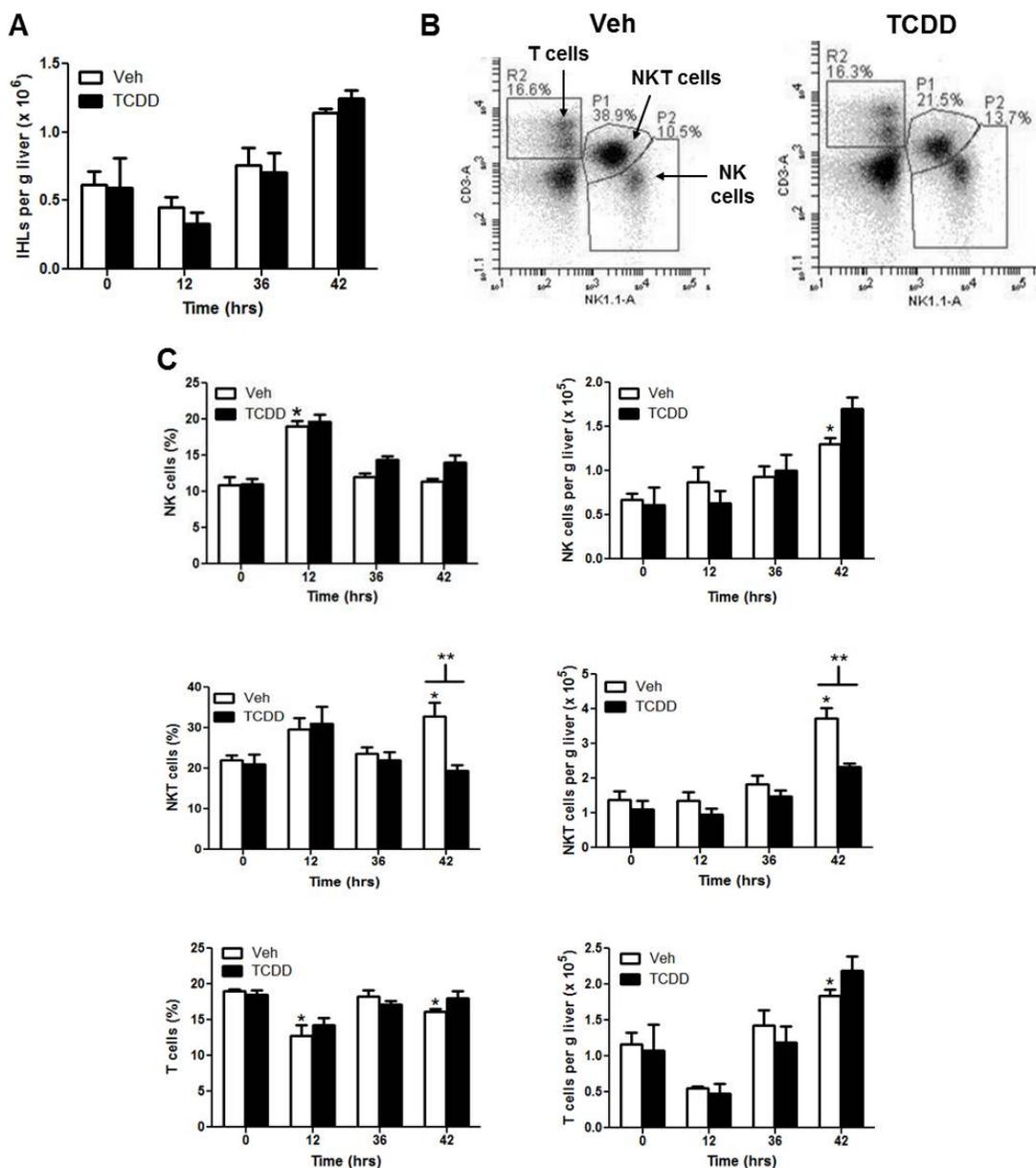


Figure 3.3 Exposure to TCDD Does Not Increase the Number of Lymphocytes in the Regenerating Liver. Mice were treated with vehicle or TCDD 24 h prior to PH and sacrificed postoperatively at the indicated times. A) Average number of intrahepatic lymphocytes (IHLs) per gram liver (\pm SEM). B) Representative FACS histogram depicting lymphocyte subpopulations in the liver of vehicle- and TCDD-treated mice 42 hr after PH. Freshly isolated IHLs were dual-stained with NK1.1 and CD3 antibodies. C) Average percentage and number NK cells, NKT cells, and T cells in the regenerating liver of vehicle- and TCDD-treated mice (\pm SEM). Three to six mice were used per treatment group at each time point; results are representative of three separate experiments. *Significantly different from 0 h vehicle-treated mice ($p \leq 0.05$). **Significant difference between vehicle- and TCDD-treated mice at same time point.

TCDD Treatment Does Not Increase the Activation of Intrahepatic Lymphocytes

Activated NK cells and NKT cells are implicated in the inhibition of liver regeneration, and these lymphocytes have been shown to increase expression of the activation marker CD69 during regeneration (Dong et al., 2007; Dugan, Fullerton, Roth, & Ganey, 2011). We found that CD69 was expressed on a greater number of lymphocytes in the regenerating liver after PH (Figure 2.4A-C). This was observed at 42 h for NK cells, 36 and 42 h for NKT cells, and at 42 h for CD3⁺ T cells. While TCDD treatment altered the percentage of CD69⁺ NK and NKT cells in the liver after PH, it did not alter the number of CD69⁺ NK, NKT, or T cells in the regenerating liver at any time point after PH.

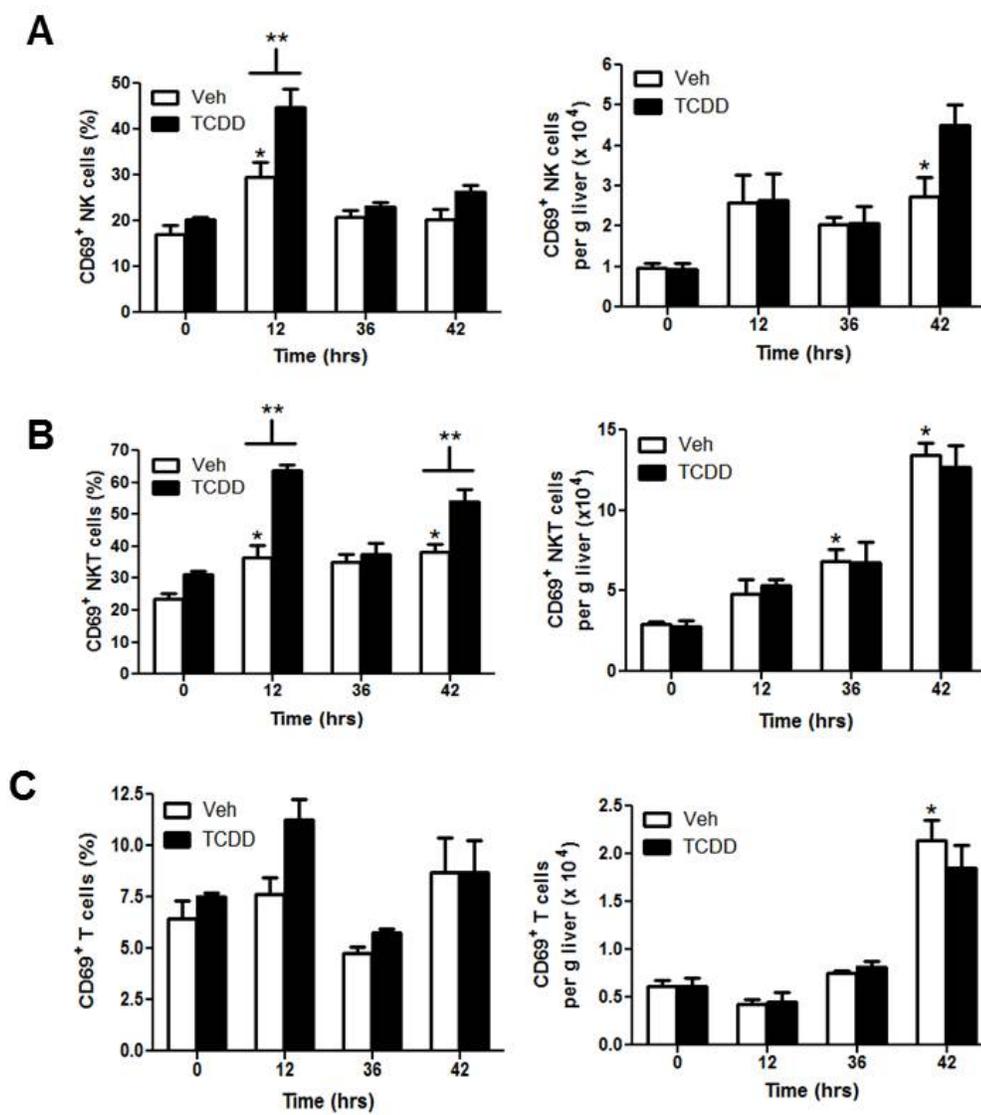


Figure 3.4 TCDD Treatment Does Not Increase the Activation of Lymphocytes in the Regenerating Liver. Mice were treated with vehicle or TCDD 24 h prior to PH and sacrificed postoperatively at the indicated times. Data represent average percentage and number of IHLs (A), NK cells (B), NKT cells (C), and CD3⁺ T cells (D) that express high levels of CD69. Data represent the average percentage and number (\pm SEM) of CD69⁺ IHLs (A), NK cells (B), NKT cells (C), and CD3⁺ T cell (D). Three to six mice were used per time point in each treatment group. *Significantly different from 0 h vehicle-treated mice ($p \leq 0.05$). **Significant difference between vehicle- and TCDD-treated mice at same time point.

TCDD Treatment Does Not Increase IFN- γ Production by Lymphocytes in the Regenerating Liver

Based on the well-established inhibitory effects of IFN- γ on hepatocyte proliferation (K. Shen et al., 2008; Sun & Gao, 2004; Sun et al., 2006; Wei et al., 2010), we tested the hypothesis that TCDD treatment increased IFN- γ production by lymphocytes in the regenerating liver. Flow cytometry was used to quantify IFN- γ -producing lymphocytes in the regenerating liver of vehicle- and TCDD-treated mice (Figure 2.5A). Within 36 h after PH, the number of IFN- γ -producing lymphocytes in the liver increased 3-5 fold (Figure 2.5B). Intracellular expression of IFN- γ was detected not only in NK cells (Figure 2.5C), but also in NKT and CD3⁺ T cells (Figure 2.5D and E); however, TCDD treatment did not enhance IFN- γ expression in any of these lymphocyte subpopulations.

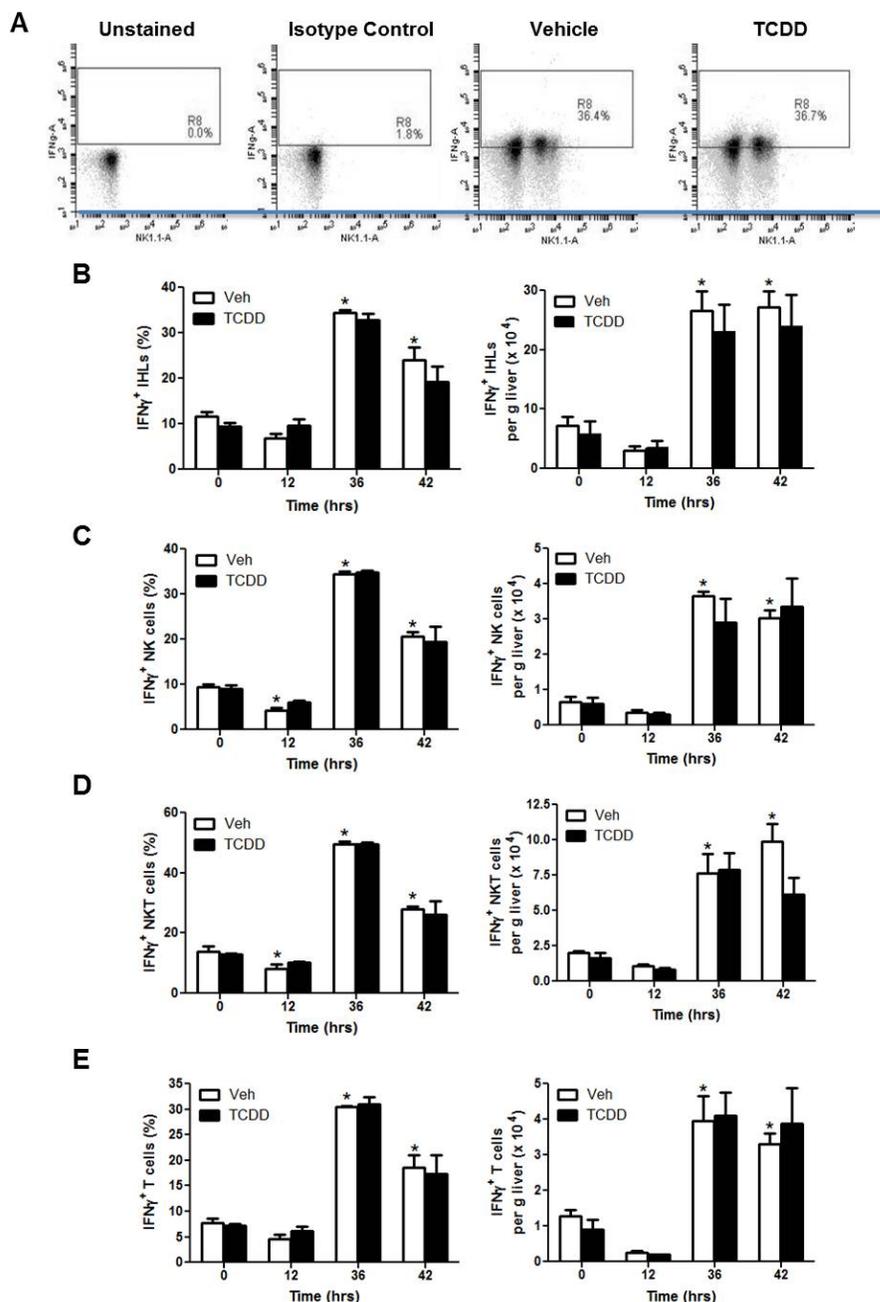


Figure 3.5 TCDD Treatment Does Not Increase IFN- γ Production by Lymphocytes in the Regenerating Liver. Mice were treated with vehicle or TCDD 24 h prior to PH and sacrificed postoperatively at the indicated times. A) Representative FACS histograms depicting IFN γ^+ staining profile in pooled cells left unstained or stained with an isotypic control antibody, as well as representative staining in IHLs isolated from vehicle- and TCDD-treated mice 36 h after PH. Graphs represent average percentage and number (\pm SEM) of IFN- γ^+ IHLs (B), NK cells (C), NKT cells (D), and CD3 $^+$ T cells (E). *Significantly different from 0 h vehicle-treated mice ($p \leq 0.05$). **Significant difference between vehicle- and TCDD-treated mice at same time point.

Depletion of NK Cells Does Not Ablate TCDD-Induced Attenuation of Liver Regeneration

Previous studies have demonstrated that depletion of NK cells enhances liver regeneration (Sun & Gao, 2004; Sun et al., 2006), which underscores the potential for these cells to curtail hepatocyte proliferation in the regenerating liver. Hence, we surmised that if NK cells were essential to the suppression of liver regeneration by TCDD, then depletion of NK cells should abrogate the suppressive effects of TCDD on hepatocyte proliferation during regeneration. To this end, mice were depleted of NK cells by administration of α -ASGM-1 antibody prior to TCDD treatment and surgical PH. Treatment with α -ASGM-1 did not impact NKT or CD3⁺ T cell populations (Figure 2.6A); however, it effectively depleted NK1.1⁺ cells for at least 42 h after PH (Figure 2.6A and B). Detection of BrdU incorporation in regenerating hepatocytes revealed fewer BrdU⁺ nuclei in TCDD-treated mice that were administered either PBS or α -ASGM-1 (Figure 2.6C). Levels of hepatocyte proliferation were similar in vehicle-treated mice injected with either PBS or α -ASGM-1 (Figure 2.6D). Moreover, TCDD treatment markedly reduced hepatocyte proliferation in both PBS- and α -ASGM-1-treated mice, which indicates that NK cells are dispensable for the TCDD-mediated suppression of liver regeneration.

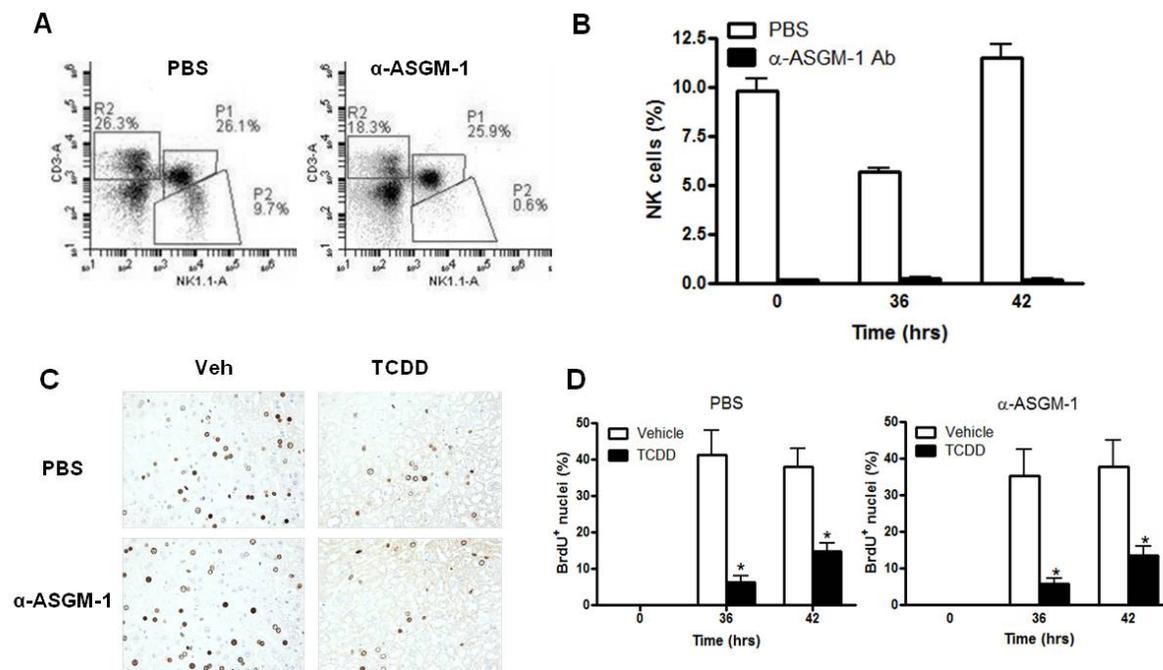


Figure 3.6 TCDD Treatment Suppresses Liver Regeneration in Mice Depleted of NK Cells. To deplete NK cells, mice were treated with PBS or α -ASGM-1 antibody prior to administration of TCDD and surgical PH. A) Representative FACS histograms showing the effective depletion of intrahepatic NK cells (region "P2") in mice treated with α -ASGM-1 compared to PBS-treated mice. Treatment with α -ASGM-1 did not alter the percentage of CD3⁺ T cells (region "R2") or NKT cells (region "P1"). B) Average percentage of intrahepatic NK cells (\pm SEM) in PBS- and α -ASGM-1-treated mice (n=3-6). C) Representative photomicrographs (400X) of BrdU⁺ nuclei in liver tissue 42 h post-PH. D) Average percentage of BrdU⁺ nuclei (\pm SEM) in liver tissue (n=3-6). *Significantly different compared to vehicle-treated mice at same time point ($p \leq 0.05$).

CHAPTER IV: DISCUSSION

Increasing evidence demonstrates a physiological role for the AhR in regulating hepatocyte cell cycle progression (Marlowe & Puga, 2005). The murine model of PH-induced liver regeneration provides a means to investigate AhR-mediated cell cycle control in non-transformed hepatocytes *in vivo*. During liver regeneration, hepatocyte proliferation is tightly regulated by the coordinated expression of cytokines and growth factors (Taub, 2004). Production of these soluble mediators represents a confounding variable in understanding how AhR activity regulates *in vivo* cell cycle progression, as TCDD treatment may suppress proliferation through direct effects on hepatocytes, as well as by modulating cytokine production by other cells in the liver. In this thesis, we investigated the ramifications of TCDD exposure on one of these soluble mediators, IFN- γ , to determine its involvement in AhR-mediated cell cycle control during liver regeneration.

The possibility that IFN- γ could contribute to the TCDD-induced cell cycle arrest in regenerating hepatocytes is compelling because this cytokine inhibits DNA synthesis in freshly isolated hepatocytes and in several hepatoma-derived cell lines (Detjen et al., 2003; Kano, Watanabe, Takeda, Aizawa, & Akaike, 1997). Furthermore, during PH-induced liver regeneration, hepatocyte proliferation is enhanced in IFN- γ and IFNGR knockout mice (Sun & Gao, 2004). Our results indicate that intrahepatic lymphocytes increase IFN- γ production 36 and 42 h after PH, which is consistent with other reports (Sun & Gao, 2004; Sun et al., 2006).

Results from this thesis suggest that IFN- γ is produced not only by NK cells in the liver, but also by NKT and CD3⁺ T cells (Fig 3.5). Whereas, NK cells have been reported elsewhere to comprise nearly all of the IFN- γ ⁺ cells in the liver at this same time after PH (Sun & Gao, 2004). If NKT and CD3⁺ T cells are major sources of IFN- γ , then this could explain why depletion of NK cells failed to increase hepatocyte proliferation as we had expected (Figure 3.6). NKT cells are indeed capable of producing IFN- γ and may regulate liver regeneration under certain conditions, such as infection with hepatitis B virus (HBV). For instance, in HBV-tg mice, impaired liver regeneration coincided with an accumulation of activated, IFN- γ -producing NKT cells in the liver and was rectified when NKT cells were depleted (Dong et al., 2007). Hence, while NK cells are an important source of IFN- γ during PH-induced regeneration, data from this thesis suggest that the contribution of IFN- γ -producing NKT and CD3⁺ T cells may warrant further investigation.

Contrary to our hypothesis, exposure to TCDD did not alter the magnitude or duration of IFN- γ production by NK cells in the regenerating liver, nor did it modulate IFN- γ production by NKT cells or T cells. Exposure to TCDD has been shown in other model systems to modulate IFN- γ production. For example, during murine infection with influenza virus, TCDD treatment increased IFN- γ levels in the lung (Knutson et al., 2003). While this coincided with enhanced numbers of NK cells in the lung, increased IFN- γ levels were attributed to macrophage and neutrophil populations, rather than NK cells (Neff-LaFord et al., 2007). In contrast to several other cytokine genes, the murine gene encoding IFN- γ does not contain a canonical DRE, which supports speculation that changes in IFN- γ expression may result from dysregulated leukocyte activation and are

probably not the direct result of AhR-mediated changes in IFN- γ gene transcription (Kerkvliet, 2009).

Generally speaking, the consequences of TCDD treatment on NK cell activation are inconsistent, with reports that TCDD can suppress (X. H. Wang et al., 2009) or enhance (Funseth & Ilback, 1992) splenic NK cell activity. Both splenic and hepatic NK cells reportedly display cytotoxicity against regenerating hepatocytes (Itoh, Abo, Sugawara, Kanno, & Kumagai, 1988; Ohnishi et al., 1993). However, we did not measure cytotoxicity as an endpoint of NK cell activation because it was previously reported that TCDD treatment did not induce appreciable levels of apoptosis in the regenerating liver (Mitchell et al., 2006). Nevertheless, we did measure the expression of the early activation marker, CD69, on lymphocytes in the regenerating liver (Figure 3.4). While the number of CD69⁺ intrahepatic lymphocytes increased after PH, exposure to TCDD did not alter this trend. Hence, TCDD does not appear to increase the activation of lymphocytes in the regenerating liver.

Exposure to TCDD reduced the number of NKT cells in the regenerating liver 42 h after PH. Mechanisms by which NKT cells, as well as NK cells, accumulate in the liver are poorly understood (Gao, Radaeva, & Park, 2009). NKT cells accumulate in the liver during numerous stress-related conditions, including PH-induced liver regeneration (Minagawa et al., 2000). However, NKT cell numbers in the liver decrease under other conditions, such as during bacterial infection and when NKT cells are activated by Concanavalin A or α -galactosylceramide (Biburger & Tiegs, 2005; Kim et al., 2008; Takeda et al., 2000). The liver has been shown to regulate the depletion and repopulation of NKT cells through different mechanisms depending on the conditions under which

NKT cells are activated (Subleski, Wiltrout, & Weiss, 2009). Understanding how the liver regulates the fate of activated NKT cells may shed light on why numbers of these lymphocytes are diminished in TCDD-treated mice. However, it is worth mentioning that reduced numbers of NKT cells were detected in TCDD-exposed mice 42 h after PH, whereas hepatocyte proliferation in these mice was reduced as early as 36 h after PH. Hence, we question the mechanistic relevance of reduced NKT cell numbers to the suppression of hepatocyte proliferation in TCDD-treated mice. An alternative explanation is that, as a result of diminished regeneration, the liver in TCDD-treated mice enhances NKT cell depletion in order to restore proliferation rates in the remnant liver. Indeed, previous reports indicate that activated NKT cells suppress liver regeneration (Gao et al., 2009); hence, depleting these cells from the liver of TCDD-treated mice may represent a compensatory response to restore optimal hepatocyte proliferation.

Results from this thesis indicate that spleen cellularity increases after PH. TCDD treatment induced a notable trend toward decreased spleen cellularity in TCDD-treated mice, although it was not statistically significant. TCDD treatment correlates with decreased spleen cellularity in other model systems, where it likely reflects the suppressive effects of TCDD on the clonal expansion of splenic lymphocytes (Kerkvliet, 2002). An alternative explanation for decreased spleen cellularity during liver regeneration in TCDD-treated mice is the accelerated evacuation of lymphocytes that could migrate to the liver (Twilley, Mason, Talmadge, & Wiltrout, 1987). However, this possibility is unlikely because we found that TCDD treatment failed to enhance recruitment of lymphocytes to the regenerating liver.

In conclusion, results from this study indicate that the TCDD-mediated suppression of hepatocyte proliferation during liver regeneration does not require NK cells and most likely does not involve changes in IFN- γ production. It remains feasible that exposure to TCDD modulates the production of other soluble mediators that initiate or terminate liver regeneration. Future considerations should include distinguishing between direct effects of TCDD on regenerating hepatocytes and indirect effects on non-parenchymal cells that produce regulatory cytokines and growth factors.

CHAPTER V: SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

Summary

The overarching goal of the studies in this thesis was to identify a mechanism for the attenuation of liver regeneration resulting from TCDD treatment. We showed that TCDD attenuates liver regeneration at 36 and 42 h after PH, corroborating previous findings (Mitchell et al., 2006). NK cells and IFN- γ reportedly act as negative regulators of hepatocyte proliferation during liver regeneration (Sun & Gao, 2004). Additionally, T cells and NKT cells may regulate liver regeneration as they have been shown to produce IFN- γ in other models of liver injury (Otani et al., 1999). In fact, NKT cells have been reported to increase in propensity and activity during liver regeneration after PH (Minagawa et al., 2000). Hence, multiple components of the innate immune system may contribute to the immunoregulation of liver regeneration. Given that TCDD modulates a wide array of immune functions, including the production of IFN- γ , in other model systems (Kerkvliet, 2009), we sought to determine if alterations in the innate immune system could explain the suppression of liver regeneration observed in TCDD-exposed mice. We hypothesized that TCDD treatment attenuates liver regeneration by increasing the number and activation of NK cells while also enhancing IFN- γ production in the regenerating liver. Furthermore, we considered the possibility that modulation of NKT and T cells could contribute to the TCDD-dependent inhibition of liver regeneration.

TCDD Treatment Does Not Increase the Number of Lymphocytes in the Spleen or Liver after PH

To determine if TCDD treatment modulates the number of lymphocytes during liver regeneration, we analyzed NK, NKT, and T cells from the spleen and liver at various time points following PH. According to our analysis, while numbers of lymphocyte subpopulations increased after PH, TCDD generally did not alter the number of NK, NKT, or T cells in the liver or spleen during liver regeneration. The one exception to this trend was at 42 h after PH, when TCDD treatment significantly decreased the number of NKT cells in the liver. However, it is unlikely that this decrease contributes to the TCDD-induced attenuation of liver regeneration because attenuation begins at 36 h after PH, before any changes in NKT cell numbers are detected. Furthermore, decreased numbers of NKT cells would be expected to accelerate liver regeneration, which was not observed 42 h after PH in TCDD-treated mice.

TCDD Treatment Does Not Increase the Number of Activated Lymphocytes in the Liver after PH

To determine if TCDD treatment increases the activation of lymphocytes in the regenerating liver, we considered the expression of the early activation marker, CD69, on NK, NKT, and T cells in the liver after PH. According to our analysis, while the number of CD69⁺ lymphocytes in the liver increased after PH, TCDD treatment did not alter this trend. Thus, TCDD does not appear to increase the activation of NK, NKT, or T cells in the regenerating liver.

TCDD Treatment Does Not Augment the Production of IFN- γ in the Liver after PH

To determine if TCDD treatment augments the production of IFN- γ in the regenerating liver, we analyzed changes in the number of IFN- γ^+ NK, NKT, and T cells in the liver at various time points following PH. According to our analysis, the number of IFN- γ^+ lymphocytes increased after PH. However, TCDD treatment generally did not alter the number of IFN- γ^+ NK, NKT, or T cells in the regenerating liver. The one exception to this trend was again at 42 h after PH, when TCDD treatment decreased the number of IFN- γ^+ NKT cells in the liver. However, this is again contrary to our hypothesis, as we would expect that decreased numbers of IFN- γ -producing cells would result in accelerated liver regeneration, which was not observed 42 h after PH in TCDD-treated mice.

Depletion of NK Cells Has No Effect on TCDD-Induced Attenuation of Liver

Regeneration

To determine if NK cells are required for TCDD-induced attenuation of liver regeneration, we depleted NK cells in mice using an α -ASGM-1 antibody and carried out a typical time course experiment with TCDD and vehicle treatments followed by PH. A BrdU pulse was administered two hours prior to euthanasia, and liver tissue was processed for immunohistochemistry and stained for BrdU $^+$ nuclei. Using this technique, we discovered that TCDD attenuated liver regeneration at 36 and 42 h after PH despite the absence of NK cells. Thus, NK cells do not play a vital role in TCDD-induced attenuation of liver regeneration.

Conclusions and Future Directions

Contrary to our hypothesis, TCDD treatment does not exert its anti-proliferative effects on hepatocytes during liver regeneration by enhancing NK cell activation or increasing IFN- γ production. There are other possible targets of TCDD toxicity that may be involved in inhibiting hepatocyte proliferation after PH. In particular, one recent report indicates that the AhR interacts with cell cycle regulatory proteins CDK4 and cyclin D1 to promote the progression of cells from G1 to S phase of the cell cycle, and that this interaction is abolished by TCDD treatment (Barhoover et al., 2010). It is possible that TCDD treatment renders the AhR inaccessible as a binding partner to CDK4 and cyclin D1. This could conceivably result in a G1 cell cycle arrest, halting proliferation. Furthermore, the AhR has been shown to interact with other cell cycle regulatory proteins, such as retinoblastoma protein (Ge & Elferink, 1998). This raises the distinct possibility that the anti-proliferative effects of TCDD during liver regeneration are due to direct effects of TCD on hepatocytes, rather than on cells in the immune system. Future studies should consider the potential interaction of the AhR with cell cycle regulatory proteins to determine if TCDD elicits a G1 cell cycle arrest by disrupting protein-protein interactions that are essential for optimal cell cycle progression.

Our original hypothesis was predicated upon studies that identified NK cells as the primary producers of IFN- γ in the regenerating liver (Sun & Gao, 2004). Moreover, these reports also asserted that IFN- γ is responsible for negatively regulating liver regeneration (Sun et al., 2006). These studies reported that livers of partially hepatectomized mice displayed augmented hepatocyte proliferation following depletion of NK cells or knockdown of the IFN- γ receptor (Sun & Gao, 2004). However, we found

that NK, NKT, and T cells, along with other lymphocytes, all produce significant amounts of IFN- γ in the liver after PH. Moreover, we found that NKT cells, not NK cells, represent the highest percentage of IFN- γ ⁺ lymphocytes in the regenerating liver. This finding aligns with other recent studies that emphasize the importance of NKT cells in negatively regulating liver regeneration (Minagawa et al., 2000). We found that depletion of NK cells did not alter the proliferation of hepatocytes in the regenerating liver. Finally, although we attempted to measure changes in IFN- γ levels in liver tissue and plasma during liver regeneration by employing a variety of methods (Western blot, RT-PCR, immunofluorescence, ELISA, and cytokine bead array), IFN- γ detection proved to be elusive. These observations indicate that circulating and hepatic IFN- γ levels remain extremely low after PH and cause us to reconsider the physiological relevance of IFN- γ as a negative regulator of hepatocyte proliferation in the regenerating liver. Taken together, these findings indicate that future studies should aim to reconsider the source and importance of IFN- γ in the liver after PH, while also continuing to hone in on the overall role of the innate immune system in the regenerating liver.

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