2,3,7,8-TCDD ALTERS THE DEVELOPMENT AND SEVERITY OF HEPATOTOXICITY IN A MOUSE MODEL OF IMMUNE-MEDIATED LIVER INJURY INDUCED BY CONCANAVALIN A.

By

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ABSTRACT

2,3,7,8-TCDD ALTERS THE DEVELOPMENT AND SEVERITY OF HEPATOTOXICITY IN A MOUSE MODEL OF IMMUNE-MEDIATED LIVER INJURY INDUCED BY CONCANAVALIN A.

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Liver diseases, including viral and autoimmune hepatitis, represent a major public health concern. Inflammatory immune cells play an important role in the development and progression of liver injury in these diseases. There is evidence that exposure to various xenobiotics enhances the response of immune cells to inflammatory stimuli, resulting in exacerbation of hepatic inflammation and development of liver injury. In particular, the persistent environmental pollutant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) represents a serious environmental health concern and has numerous adverse effects in humans and other mammals that include altered immune function. Information on the effects of TCDD on hepatic immune cells following inflammatory stimulus is limited, and the effects of TCDD on development of hepatic autoimmune disease are unknown. Administration of concanavalin A (Con A) to rodents is widely used as a model of immune-mediated liver injury with pathophysiology resembling that of autoimmune hepatitis in humans. In this dissertation I tested the hypothesis that TCDD pretreatment enhances the response of intrahepatic immune cells to the inflammatory stimulus Con A resulting in increased hepatotoxicity in a mouse model of autoimmune liver disease. Mice were pretreated with TCDD, and the development of liver injury after the administration of Con A was assessed. Mice pretreated with TCDD had an increased response to Con A, resulting in severe liver

injury mediated by inflammatory cytokines and hepatic innate immune cells. Interferon gamma (IFNγ) was critical to the development of TCDD/Con A-induced liver injury: pretreatment with TCDD increased the plasma concentration of IFNγ after Con A, and IFNγ knockout mice were protected from injury. In addition, pretreatment with TCDD induced activation of hepatic NK and NKT cells after Con A administration. In TCDD-pretreated mice, the percentage of NKT cells expressing Fas ligand was increased after Con A. Further investigation determined that Fas ligand-mediated killing of hepatic parenchymal cells was an important contributor to TCDD/Con A-induced liver injury and mice deficient in Fas ligand were protected from injury. NKT cell-deficient mice were protected against injury from Con A alone, whereas liver injury from TCDD/Con A treatment was only partially reduced suggesting the involvement of other immune cell populations in the exacerbation of Con A-mediated liver injury. Neither hepatic macrophages nor infiltrating neutrophils were involved in the increased sensitivity to Con A-induced liver injury; however, increased activation of NK cells was an important contributor. NK cell depletion prevented the development of liver injury and decreased plasma concentration of IFNγ after TCDD/Con A treatment. These results indicate that TCDD alters the severity and pathogenesis of Con A-mediated liver injury and raise the possibility that exposure to environmental toxicants like TCDD might contribute to the development of hepatic autoimmune disease in humans.

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KEY TO SYMBOLS AND ABBREVIATIONS

Actb	beta-actin
AhR	aryl-hydrocarbon receptor
AhRR	aryl hydrocarbon receptor repressor
AIH	autoimmune hepatitis
ALT	alanine aminotransferase
ANOVA	analysis of variance
APC	antigen presenting cell
ARA	aryl hydrocarbon receptor associated
Arnt	aryl hydrocarbon receptor nuclear translocator
bHLH	basic helix-loop-helix
Con A	concanavalin A
CD	cluster of differentiation
Cyp	cytochrome p450
DRE	dioxin response element
FasL	fas ligand
FBS	fetal bovine serum
FICZ	formylindolo[3,2-b]carbazole
Gapdh	glyceraldehyde-3-phosphate dehydrogenase
GI	gastrointestinal
HAH	halogenated aromatic hydrocarbons
HLA	human leukocyte antigens
Hprt	hypoxanthine guanine phosphoribosyl transferase

HSP	heat shock protein
Icam	intracellular adhesion molecule
IFNγ	interferon gamma
IL	interleukin
iNKT	invariant natural killer T
KC	keratinocyte chemoattractant
KO	knock out
LFA	lymphocyte function-associated antigen
LPS	lipopolysaccharide
Mac	macrophage
MCP	monocyte chemotactic protein
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
NIOSH	National Institute for Occupational Safety and Health
NK	natural killer
NKT	natural killer T
PAH	polycyclic aromatic hydrocarbons
PAMP	pathogen-associated molecular pattern
PAS	Per-Arnt-Sim
PBC	primary biliary cirrhosis
PBS	phosphate buffered saline
PCB	polychlorinated biphenyls
PCDD	polychlorinated dibenzo-p-dioxins

PCR	polymerase chain reaction
PRR	pattern recognition receptor
PSC	primary sclerosing cholangitis
RAG	recombination activating gene
SEC	sinusoidal endothelial cell
SRBC	sheep red blood cells
T cell	thymus cell
TCDD	2,3,7,8 tetrachlorodibenzo-p-dioxin
TCR	T cell receptor
TEF	toxic equivalency factor
TEQ	toxic equivalency
TGFβ	transforming growth factor beta
Th	T helper
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
TRAIL	TNFα related apoptosis inducing ligand
Treg	T regulatory cell
UDP	uridine diphosphate
XRE	xenobiotic response element

CHAPTER 1

1.1 Overview of liver physiology

The liver is critical to the maintenance of physiological homeostasis. It plays an important role in the synthesis of plasma proteins, lipoproteins, amino acids and cholesterol. The liver serves as a storage depot for many essential nutrients including glycogen and vitamins. In addition, the liver is uniquely positioned to receive the majority of its blood supply from the portal vein, which drains blood from the gastrointestinal (GI) tract directly into the liver. As a result, the liver is the first organ exposed to the nutrients, bacterial products, and xenobiotics such as drugs and environmental contaminants absorbed from the GI tract into the systemic circulation. Therefore, the metabolic functions of the liver are of particular importance. The liver is responsible for the majority of xenobiotic metabolism and plays a critical role in the breakdown of proteins and carbohydrates (Grisham, 2009).

The unique anatomical structure of the liver parenchyma allows for a distinctive blood flow that facilitates the liver's many functions. Blood vessels comprise approximately 22% of the volume of the liver and under normal physiological conditions the liver contains 12% of the body's blood supply (Lautt and Greenway, 1987). Blood enters the liver from two sources; the portal vein, which supplies 75% of the liver's blood volume, provides deoxygenated, nutrient-rich blood from the GI tract, whereas the hepatic artery supplies the remaining 25%, which is oxygen-rich blood. At the most terminal level these two sources along with the biliary ducts comprise a structure known as the portal triad. At this juncture the blood from the afferent vasculature (portal vein and hepatic artery) mixes and flows into the hepatic sinusoids that run perpendicular to the larger vessels. The hepatic sinusoids represent about 60% of the liver's total

vascular volume. The sinusoids function as the capillary beds of the liver and are the location where interactions occur between hepatic parenchymal and immune cells and exchange of nutrients and metabolic byproducts with the circulating blood take place in the liver. The unique features of the sinusoidal endothelial cells (SECs) that line the sinusoids facilitate this interaction. These cells differ from normal capillary endothelial cells in that they lack a basal membrane and possess 150-170 nm openings called fenestrae. These features allow for increased diffusion of fluid and solutes from the perfusing blood into the space of Disse which separates the SECs from adjacent hepatocytes (Wisse *et al.*, 1985). Hepatocytes are the major liver parenchymal cell and are responsible for most of the synthetic and metabolic functions of the liver. Hepatocytes represent approximately 60% of the cells in the liver but comprise 80% of the liver's cellular volume (Blouin *et al.*, 1977). At the efferent portion of the hepatic sinusoid the blood flow exits via the central hepatic vein. The liver's overall structure is composed of these repeated functional units delineated by the specific hemodynamics of blood flow into the liver sinusoids from the portal triad and exiting through the central hepatic vein.

1.2 Liver as an immunological organ

The liver anatomy and position in the circulatory system make it an ideal location for immune surveillance. In addition to being the first organ to receive nutrients from the GI tract, the blood entering the liver from the portal vein contains antigens and microflora. The liver is the first organ exposed to these immune triggers and is uniquely structured for the processing of these molecules to either evoke or suppress an immune response (Parker and Picut, 2005). Antigens are filtered from the blood in the hepatic sinusoids by the reticulo-endothelial system composed of SECs, Kupffer cells, and dendritic cells (Racanelli and Rehermann, 2006). The cells of the reticuloendothelial system in the liver function to modulate local and peripheral immune responses evoked by the presence of circulating antigens. The liver possesses cells associated with both the innate and adaptive immune response. As the liver is exposed to large amounts of antigens from the GI tract, the intrahepatic immune cells play a major role in both peripheral immune tolerance of the adaptive immune system as well as control of the localized innate immune response (Knolle and Gerken, 2000; Thomson and Knolle, 2010). The intrahepatic immune cells must be capable of generating an effective immune response to eliminate pathogens and prevent chronic infection while at the same time maintaining a balance so as to not cause unnecessary tissue damage in response to innocuous foreign antigens derived from the GI tract.

1.3 Hepatic immune system

The cells of the immune system are typically differentiated into innate or adaptive immunity by the types of signals to which they respond and the time frame of that response. Cells of the innate immune system are often characterized by their ability to mount an immediate, non-specific response. In contrast, cells of the adaptive immune system recognize specific antigens and generate a delayed response that results in clonal expansion of antigen-specific effector cells and generation of immunological memory to expedite responses upon subsequent exposure. The innate immune cells in the liver can be subdivided into two general categories. The first category is composed of phagocytic cells including macrophages and neutrophils that are involved in non-specific phagocytosis of antigens. The second category includes lymphocytic cell types such as natural killer (NK) cells, NKT cells, as well as other T cell subgroups that are involved in direct cell killing of infected parenchymal cells. Cells in both categories also generate numerous inflammatory mediators when activated that can inhibit or exacerbate the immune response. The adaptive immune system in the liver is primarily composed of conventional $CD4^+$ and $CD8^+$ T cells and B cells responsible for the production of humoral factors such as pathogen specific antibodies critical to the efficiency of the adaptive immune response.

In the innate immune system a response is generally triggered by the recognition of pathogen-associated molecular patterns (PAMPs) that are detected by pattern recognition receptors (PRRs) expressed on the surface of immune cells (Meylan *et al.*, 2006). PRRs such as toll-like receptors (TLRs) differ from the lymphoid receptors present on the cell surface of cells associated with the adaptive immune system

(Janeway and Medzhitov, 2002). Unlike the T and B cell receptors of the adaptive immune system that are generated against specific antigens through a complex series of gene rearrangements, PRRs on innate immune cells detect conserved molecular patterns present on bacteria, viruses, and fungi in order to differentiate them from host cells. For example, various TLRs can detect the lipopolysaccharide present in the cell wall of gram-negative bacteria or the peptidoglycan on gram-positive bacteria (Hashimoto *et al.*, 1995). Different immune cells express different repertoires of PRRs and thus can respond differently to the presence of various pathogens (Schwabe *et al.*, 2006).

Hepatic Macrophages

The liver has the largest population of organ-specific macrophages in the body. These fixed macrophages, called Kupffer cells, represent 20% of hepatic non-parenchymal cells (Mackay, 2002). They provide immune surveillance by adhering loosely to the sinusoidal endothelials cells within the lumen of the hepatic sinusoids, particularly in the periportal regions. Kupffer cells are the major scavengers of PAMPS and immune complexes via the expression of an extensive array of PRRs (Smedsrod *et al.*, 1994). These hepatic macrophages are characterized by an actively phagocytic phenotype and remove bacteria and dead cells from the hepatic sinusoids via the expression of C3 and Fc receptors. Upon activation by PAMPs, Kupffer cells are capable of expressing a large number of cytokines that play an important role in hepatic immune modulation by stimulating other innate immune cells or instigating an acute phase response within hepatic parenchymal cells (Parker and Picut, 2005). Macrophage-derived cytokines such as tumor necrosis factor alpha (TNFα) and

interleukin (IL) -6 can drastically alter hepatocyte function and even induce cell death. Other cytokines such as IL-12 can activate and increase the cytolytic activity of natural killer cells as well as act on hepatic T cells to induce the polarization of the subsequent adaptive immune response (Takahashi *et al.*, 1996).

Table 1

Summary of relative lymphocyte populations in various tissues

Murine lymphocyte populations				
Cell Types	**Liver**	**Spleen**	**Thymus**	**Lung**
αβ T cells	44%	64%	70%	52%
γδ T cells	5.4%	3%	1.5%	6%
NKT cells	28%	1%	4%	0.8%
NK cells	12.1%	7%	0.1%	13%

Human lymphocyte populations		
Cell Types	**Peripheral Blood**	**Liver**
αβ T cells	72%	37%
γδ T cells	2%	5%
NK cells	2%	31%
NKT cells	2%	12%

Data compiled from:

(Doherty and O'Farrelly, 2000; Mackay, 2002; Dong *et al.*, 2004; Gao *et al.*, 2008)

Hepatic Natural Killer cells

The liver contains a unique population of NK cells. NK cells are a lymphoid population with potent cytolytic activity. First identified as Pit cells due to their increased granularity, these hepatic NK cells are capable of killing virus-infected hepatocytes or tumor cells (Wiltrout *et al.*, 1984; Wiltrout *et al.*, 1985; Nakatani *et al.*, 2004). The cytolytic action of NK cells is mediated by the release of perforin and granzymes in addition to the induction of cell death receptor-mediated apoptosis via the expression of TNFa related apoptosis inducing ligand (TRAIL) and FasL (Fas Ligand) (Smyth *et al.*, 2001). Activated NK cells can produce a range of cytokines such as TNFα, IL-10, and Interferon gamma (IFNγ) (Smyth *et al.*, 2002b).

Many of the immune functions attributed to NK cells are related to their ability to produce copious amounts of IFNγ. The production of IFNγ can suppress tumor metastasis and viral infection, as well as increase antigen presentation and facilitate leukocyte interactions (Street *et al.*, 2001). In addition, IFNγ release plays a central role in NK and NKT cell-mediated cytotoxicity (Smyth *et al.*, 2002a) and can inhibit hepatocyte proliferation via cell cycle arrest and induction of apoptosis (Sun *et al.*, 2006). NK cell function and activation is regulated by a complex interaction between activating and inhibitory receptors (Lanier, 2005). The increased expression of activating receptors such as NKG2D leads to increased cytolytic activity toward parenchymal cells and enhanced cytokine production (Bauer *et al.*, 1999; Smyth *et al.*, 2002b; Boyton and Altmann, 2007). In contrast, the actions of NK cells are held in check by the presence of inhibitory receptors such as NKG2A which detects the presence of MHC class 1-like molecules on host parenchymal cells and blocks NK cell

activation (Borrego *et al.*, 1998). Although NK cells are prevalent in other organs besides the liver, hepatic NK cells have been identified to be phenotypically distinct (Bouwens *et al.*, 1987; Vanderkerken *et al.*, 1993). Hepatic NK cells demonstrate both functional and morphological differences from NK cells found in either the spleen or peripheral circulation. Hepatic NK cells express increased levels of TRAIL compared to splenic NK cells (Ochi *et al.*, 2004). In addition, hepatic NK cells have increased expression levels of perforin and granzyme compared to NK cells isolated from the spleen or peripheral blood (Vermijlen *et al.*, 2002). Experimentally, hepatic NK cells are phenotypically similar to splenic NK cells that have been activated by IL-2 and demonstrate enhanced cytolytic activity towards tumor cells that are resistant to splenic NK cells (Luo *et al.*, 2001; Ishiyama *et al.*, 2006).

Hepatic T cells

In addition to the many types of innate immune cells, a large population of T lymphocyte cells can be found in the liver. However, the hepatic lymphocyte population differs greatly in both phenotype and function from that found in other organs such as the spleen or thymus and that of the peripheral circulation (Nemeth *et al.*, 2009). Hepatic T cells have a higher ratio of $CD4^+$ to $CD8^+$ cells than what is observed for T cells of the spleen or lymph nodes (Ohteki and MacDonald, 1994). In addition, there is a higher percentage of double positive ($CD4^+$,$CD8^+$) and double negative ($CD4^-$,$CD8^-$) T cells in the liver. Approximately 15% of hepatic T cells express the δγ T cell receptor (TCR) rather than the conventional αβ TCR. In other organs, such as the spleen, the population of δγ T cells is much lower and comprises only 3% of the T cell population (Ohteki *et al.*, 1991). δγ T cells have an increased contribution to innate immune

responses and play an role in development of autoimmune disease (Pang *et al.*, 2012). The hepatic lymphocyte population is enriched in T cells that perform innate immune functions. In fact, although rare in other organs, approximately 60% of T cells in the liver are NKT cells (Watanabe *et al.*, 1995; Tsukahara *et al.*, 1997).

Natural killer T cells

NKT cells are T lymphocytes that express NK cell receptors in addition to the TCR and bridge the activities of the innate and adaptive immune response (Hashimoto *et al.*, 1995; Exley and Koziel, 2004). NKT cells are typically identified by the expression of the CD3 TCR complex and NK cell receptors such as CD56 in humans and either DX5 or NK1.1 in mice. NK and NKT cells form a first line of defense against microbial pathogens and tumor metastasis (Cui *et al.*, 1997).

NKT cells become activated as part of the innate immune system and exert immunological control prior to the activation of the adaptive immune response from classical T cells and B cells (Seki *et al.*, 2000). The NKT cell population expresses intermediate levels of TCR and can be classified into classical and non-classical NKT cells based on their TCR receptor and the expression of other TCR co-receptors such as CD4 or CD8 (Watanabe *et al.*, 1995). Both classical and non-classical NKT cells are greatly enriched in the liver. NKT cells comprise nearly 30% of all intrahepatic lymphocytes; whereas in immune organs such as the thymus, spleen, and lymph nodes, NKT cells represent less than 4% of lymphocytes. (Ohteki and MacDonald, 1994; Benlagha *et al.*, 2000; Matsuda *et al.*, 2000). The classical (type-1) NKT cells express an invariant TCR composed of an alpha chain consisting of variable region α14 and joining segment α18 with a bias towards a beta chain with variable region β7, 8, or 2 in

mice (Vα14-Jα18/Vβ7, 8 or 2) and in humans the corresponding classical NKT cell population expresses a TCR composed of Vα24-Jα18 with a bias for Vβ11 (Lantz and Bendelac, 1994; Godfrey *et al.*, 2004). Due to the lack of variability in the rearrangement of the TCR on these type-1 cells they are often referred to as invariant NKT cells or iNKT cells. This type represents the majority of NKT cells in the liver and is also the most thoroughly studied subset (Eberl *et al.*, 1999). Other subsets of NKT cells are referred to as non-classical (type-2) NKT cells and NKT-like cells. In contrast to type-1 NKT cells, both of these subsets express diverse TCR repertoires and the two subgroups are differentiated primarily by their responsiveness to CD1d. Both type-1 NKT cells and type-2 NKT cells express TCRs that react to the non-classical MHC molecule CD1d whereas NKT-like cells bind a range of MHC molecules (Bendelac *et al.*, 1995; Kawano *et al.*, 1997; Benlagha *et al.*, 2000; Matsuda *et al.*, 2000).

CD1d is a class 1-like major histocompatibility complex that contains hydrophobic binding grooves that allow for the presentation of glycolipid ligands rather than peptide antigens. The most extensively studied ligand is alpha-galactoceramide, which has been used to characterize much of what is known about CD1d biology. CD1d is expressed in the thymus, as well as on antigen presenting cells, myeloid cells, some peripheral T cells and on hepatic parenchymal cells. In the spleen dendritic cells are the primary presenters of CD1d-bound antigens, however in the liver the Kupffer cells are the main CD1d APC and the primary activators of hepatic NKT cells (Schmieg *et al.*, 2005). The expression of CD1d has been found to increase under inflammatory conditions (Castano *et al.*, 1995; Porcelli and Modlin, 1999; Brigl and Brenner, 2004).

Whereas the liver contains a larger number of the type-1 NKT cells responsive to CD1d than that found in any other organ, populations of type-2 NKT cells and NKT-like cells exist in significant numbers in other immune tissues. The NKT cells in the spleen and bone marrow are primarily NKT-like cells that express CD1d-independent TCRs and respond to a varied range of glycolipid antigens. These cells also primarily co-express CD8. In contrast, most type-1 NKT cells found in the liver co-express CD4. Not only are NKT cells more abundant in the liver compared to other organs, thymic and hepatic NKT cells also display phenotypic differences from NKT cells in other organs. The type-1 NKT cells found in the liver express an activation-like phenotype with increased basal CD69 expression, decreased expression of CD62L and decreased expression of the inhibitory NK cell receptor molecule LY49A compared to the type-2 NKT cells found in bone marrow and spleen (Eberl *et al.*, 1999; Emoto and Kaufmann, 2003). For the purposes of this introduction we will focus our attention on the classical or type-1 NKT cells and subsequently will refer to them simply as NKT cells.

NKT cells play an important role in the development of an immune response. These cells are capable of expressing a diverse array of proinflammatory and anti-inflammatory cytokines that can influence the subsequent differentiation of T helper (Th) cells into Th1 and Th2 phenotypes (Doherty *et al.*, 1999; Wilson and Delovitch, 2003). NKT cells can produce large amounts of IFNγ (Th1) and IL-4 (Th2) as well as other cytokines (Godfrey *et al.*, 2000). Interestingly, NKT cells isolated from the liver produce more IFNγ and IL-4 than similar cells isolated from the thymus (Matsuda *et al.*, 2000). In addition to the production of cytokines, NKT cells share similar cytolytic activity as NK cells. NKT cells utilize both FasL and perforin to perform cytolytic killing of parenchymal

cells. NKT cells are stimulated by IL-12 and IL-2 (Hashimoto *et al.*, 1995; Satoh *et al.*, 1996). The expression of IL-12 receptor has been shown to increase upon NKT cell activation. IL-12 stimulation induces an increase in perforin and IFNγ production (Watanabe *et al.*, 1995; Kawamura *et al.*, 1998; Kitamura *et al.*, 1999). The production of IFNγ by NKT cells is critical for the subsequent activation of NK cells in models of liver injury (Carnaud *et al.*, 1999; Hayakawa *et al.*, 2001). NKT cells also interact with dendritic cells to induce the activation of cytotoxic T cells (Stober *et al.*, 2003).

As a result of the diverse functions, NKT cells participate in the direct killing of parenchymal cells and also play an important role in the induction of tolerance or activation of other immune cells to exacerbate a response to stimulus (Smyth *et al.*, 2002a). It is perhaps not surprising then that NKT cells have been proposed to play a central role in the development of autoimmune diseases. A number of reviews have covered this issue (Sharif *et al.*, 2002; Hammond, 2003; Wilson and Delovitch, 2003; Mars *et al.*, 2004; Van Kaer, 2004; Linsen *et al.*, 2005; Cardell, 2006; Yamamura *et al.*, 2007; Berzins *et al.*, 2011; Novak and Lehuen, 2011). In addition to autoimmune liver diseases, NKT cells contribute to the pathogenesis of liver injury in a number of models, including septic peritonitis (induced by cecal ligation and puncture), intracellular bacterial infection, and administration of bacterial super antigens (Seki *et al.*, 2000).

Sinusoidal Endothelial Cells

In addition to the innate immune cells, SECs are an important component of the innate immune system in the liver. SECs constitute approximately fifty percent of hepatic nonparenchymal cells (Knolle and Limmer, 2001). SECs play an important role in hepatic immune regulation (Doherty and O'Farrelly, 2000). They protect hepatocytes by

providing a physical anatomical barrier against circulating immune cells in the hepatic sinusoids. This is important due to the increased generation of neoantigens by hepatocytes during the performance of their metabolic functions. In addition, the fenestrations in SECs function to regulate the trafficking of soluble antigens through the hepatic sinusoids and space of Disse (Wack *et al.*, 2001). SECs are also capable of releasing immunomodulating cytokines following the detection of PAMPs by PRRs expressed on their luminal cell surface (Knolle and Limmer, 2001). SECs express mannose and scavenger receptors that facilitate the endocytic uptake of antigens. SECs function as efficient organ-specific antigen presenting cells (APCs) and express major histocompatibility complex (MHC) II in addition to the co-stimulatory molecules CD80 and CD86 (Lohse *et al.*, 1996). The presence of organ specific APCs in the liver is another mechanism to modulate the typical immune response. The APC activity of SECs allows for antigen presentation in the context of the hepatic microenvironment. As a result, SECs play a major role in the induction of peripheral immune tolerance to dietary antigens. SECs modulate the immune response via the production of mediators such as transforming growth factor beta (TGFβ) and prostaglandins (Knolle and Limmer, 2001).

1.4 Autoimmune liver disease

The hepatic immune system is susceptible to dysfunction leading to prolonged inflammatory injury. Primary biliary cirrhosis (PBC), autoimmune hepatitis (AIH) and primary sclerosing cholangitis (PSC) are three major types of autoimmune liver disease. Each has distinct serological, clinical and histological profiles (Czaja, 2010). PBC results in destruction of small bile ducts leading to cholestasis and eventually fibrosis of the liver. AIH is characterized by interface hepatitis of a lymphocytic nature in the periportal regions of the liver lobule that results in progressive destruction of the hepatic parenchyma. PSC is characterized by inflammation of the bile ducts eventually leading to obstruction. (Czaja and Manns, 2010). A review of epidemiological data by Feld and Heathcote (Feld and Heathcote, 2003) reported the prevalence of autoimmune liver disease to be highest in the United Kingdom and United States. In the United States, the prevalence of PBC was 40.2 per 100,000 people. The prevalence of AIH was 16.9 per 100,000 people and the prevalence of PSC was the lowest at 1-4 per 100,000 people. A major diagnostic biomarker of each autoimmune disease is the detection of self-directed antibodies in the serum of patients. A hallmark of PBC is the presence of anti-mitochondrial antibodies in the serum, whereas, AIH is associated with detection of antinuclear and smooth muscle antibodies, as well as antibodies against liver and kidney microsomal proteins. In addition, the detection of other antibodies in serum have been proposed as prognostic markers (Czaja, 2010). While the clinical presentation of antibodies is well described and indicative of disease, the presence of self-reactive antibodies does not appear to contribute to development of disease (Krawitt, 1996). The pathogenesis of the various diseases remains obscure.

1.4.1 Contributing factors

Numerous studies have been conducted to better understand the epidemiological factors contributing to the etiology of autoimmune liver diseases. All autoimmune liver diseases appear to share contributing factors: in each disease there exists self-reactive immune cells, the inappropriate recognition of liver antigens as foreign, and failure to properly regulate the activation of immune cells (Czaja and Donaldson, 2000). A number of causative factors have been associated with increased prevalence and susceptibility. These include the contribution of genetic, immune cell-mediated and xenobiotic factors (Longhi *et al.*, 2010).

Genetic factors

Increased susceptibility has been attributed to a confluence of genetic factors (Czaja and Donaldson, 2000). Human leukocyte antigens (HLAs) are the human homolog of major histocompatibility complexes that function to regulate immune responses by the expression of antigens to circulating lymphocytes. Expression of particular polymorphisms in HLAs have been causally linked to autoimmune disease (Oliveira *et al.*, 2011). In particular, polymorphisms in HLAs associated with MHC type II activity correlate well with the incidence of AIH in British and North American patients (Strettell *et al.*, 1997). In addition, polymorphisms in cytotoxic T-lymphocyte antigen 4 (CTLA-4) which is expressed on T cells has been linked to development of AIH and PBC (Agarwal *et al.*, 2000a; Agarwal *et al.*, 2000b). CTLA-4 normally functions to downregulate the T cell response and the polymorphism appears to disrupt the function of this off switch on T cells leading to detrimentally enhanced activity. Polymorphisms in

genes for cytokines that influence immune function such as TNFα, have also been correlated with enhance susceptibility to AIH (Cookson *et al.*, 1999).

Role of hepatic immune cells

Development of autoimmune diseases of the liver appears to be primarily cell-mediated rather than a result of the production of humoral factors. In addition to the activation of conventional $CD4^+$ and $CD8^+$ T cells in the adaptive immune response, numerous innate immune cell types play a role in the pathogenesis of disease. A role for NKT cells has been proposed in PBC due to the detection of an increased number of NKT cells in the livers of PBC patients compared to healthy individuals (Kita *et al.*, 2002). In particular, increased NKT cells appear localized to the portal tracts proximal to interlobular bile ducts where injury occurs (Harada *et al.*, 2003). NK cells isolated from patients with PBC had increased cytotoxic activity compared to those from healthy patients (Chuang *et al.*, 2006). NK cells are also frequently observed in close association with destroyed small bile ducts in PBC patients (Gao and Bertola, 2011; Shimoda *et al.*, 2011). A role for hepatic macrophages has been well characterized in AIH with increased numbers reported in patients compared to healthy subjects. Marked increase in pro-inflammatory cytokine expression was also reported, as well as increased expression of PAMPs (Longhi *et al.*, 2010).

Xenobiotic factors

It has been hypothesized that xenobiotic exposure can trigger the development of autoimmune liver disease in susceptible individuals (Gilbert, 2010). A study conducted at the Mayo clinic attributed the development of AIH in 9% of the study group to drug treatment (Bjornsson *et al.*, 2010). One mechanism by which drug exposure is

thought to contribute to the development of autoimmune liver disease is by the generation of neoantigens during the metabolism of drugs (Liu and Kaplowitz, 2002). Phase I and Phase II metabolism of drugs can generate reactive metabolites that bind to liver proteins and are subsequently mistaken as foreign antigens by hepatic immune cells.

It has been suggested that exposure to environmental pollutants could induce autoimmune disease by the same process, in addition to other proposed mechanisms. Metabolism of environmental pollutants can also result in the generation of neoantigens. Since exposure to environmental xenobiotics is often not documented as well as drug therapy, most of the research data are from studies conducted in animal models of autoimmune liver disease. One such study demonstrated that exposure to carbon tetrachloride, a hepatotoxic environmental pollutant that was once widely used in a number of industrial and agricultural applications, augments the development of a T cell-mediated autoimmune hepatitis (Aparicio *et al.*, 2009). Exposure of mice to the environmental pollutant trichloroethylene resulted in immune-mediated injury (Tang *et al.*, 2008) and induced autoimmune hepatitis by activating CD4+ T cells and promoting a Th1 cytokine profile characterized by increased IFNγ production (Griffin *et al.*, 2000).

Exposure to environmental contaminants that activate signaling of the aryl-hydrocarbon receptor (AhR) is a growing area of research regarding susceptibility to autoimmune diseases (Burchiel and Luster, 2001; Kobayashi *et al.*, 2008; Veldhoen *et al.*, 2008). The biology of AhR and its potential role in autoimmune disease will be discussed in more detail later in the Introduction.

1.4.2 Animal models of autoimmune liver disease

Multiple models have been developed in laboratory animals in an attempt to recapitulate the pathogenesis of autoimmune disease of the liver (Peters, 2002). The models generally fall into three basic categories. The first approach to induce autoimmune-like liver injury is to administer homogenized liver proteins simultaneously with adjuvants to induce immune response against liver self-antigens. This experimental autoimmune hepatitis is T-cell mediated and develops approximately four weeks after administration of antigen. It has a subacute duration with injury resolving by 24 weeks (Lohse and Meyer zum Buschenfelde, 1993).

Another method of producing autoimmune liver injury in mice is through the development of transgenic mice that express antigens such as ovalbumin or hepatitis B antigen (Ando *et al.*, 1993; Buxbaum *et al.*, 2008). The adaptive immune cells in these mice develop tolerance to the expressed transgene as a normal part of the negative selection process. However, syngenic T cells expanded against the antigen can be adoptively transferred into the transgenic mice and can induce liver injury.

The third type of animal model utilizes the administration of polyclonal antigens such as endotoxins and plant lectins to induce immune cell activation. Three of the most popular models in this category are the administration of large doses of lipopolysaccharide (LPS), co-treatment with LPS and D-galactosamine, and treatment of mice with the T cell mitogen concanavalin A (Tiegs *et al.*, 1992; Wang *et al.*, 2012). Concanavalin A-induced hepatitis has gained the most popularity among these models for the study of AIH pathogenesis. It has a number of advantages over the other two models. For example, compared to Con A, the model using administration of LPS only

induces moderate elevations in liver transaminase levels. In addition, Con A treatment induces the production of clinically relevant inflammatory cytokines such as IL-2, IL-4 and IFNγ that are known to contribute to AIH development (Zhang *et al.*, 2010). This is an important consideration for a model designed to help elucidate the mechanisms of AIH pathogenesis. Finally, Con A-induced liver injury appears to be primarily mediated by T cells, whereas LPS and LPS-galactosamine treatments exert their inflammatory effects mainly through the activation of hepatic macrophages (Sass *et al.*, 2002).

The induction of hepatitis via administration of Con A is a model of autoimmune liver disease that is particularly relevant to the research presented here as this model was used in the studies presented in this dissertation. This model activates multiple types of immune cells and ultimately results in cell-mediated killing of parenchymal cells. Concanavalin A-induced hepatitis and the cell types contributing to hepatic injury will be discussed in more detail in chapters 2 and 3.

1.5 Aryl hydrocarbon receptor

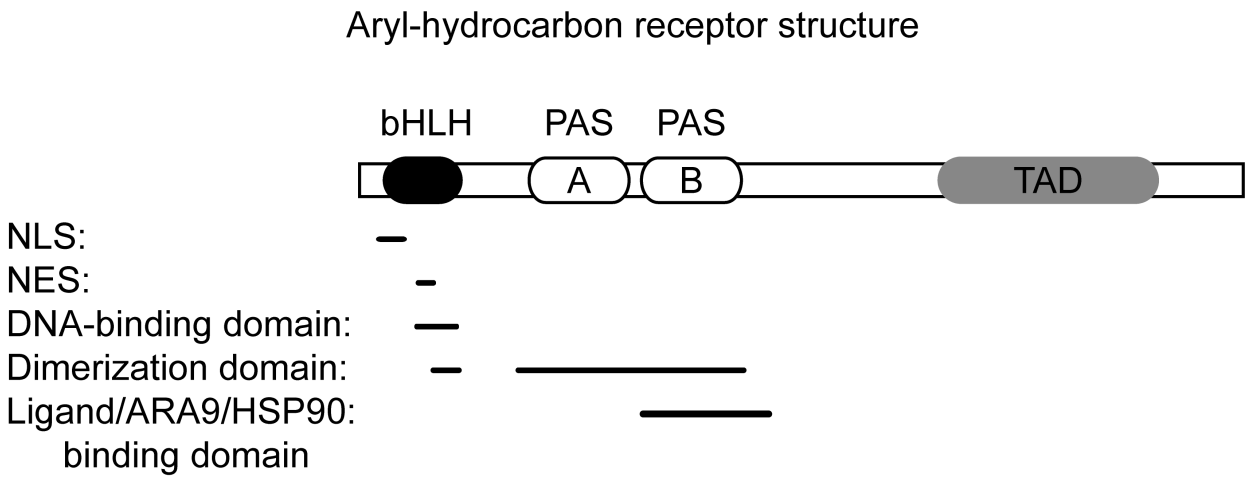
As was previously mentioned it has been hypothesized that activation of the AhR is a susceptibility factor for autoimmune liver disease. In my studies, I evaluated 2,3,7,8-tetrachlorodibenzo dioxin as a prototypical AhR ligand. Accordingly, this section contains a review of AhR biology. The AhR is highly evolutionarily conserved among invertebrates and vertebrates (Hahn *et al.*, 1997). AhR appears to be involved in numerous biological functions and recognizes a wide range of xenobiotics and endogenous ligands (Nebert *et al.*, 2000; Denison and Nagy, 2003). These AhR ligands are capable of inducing a diverse array of physiological and toxicological effects (Poellinger, 2000; Abel and Haarmann-Stemmann, 2010). As a result, the signaling and biology of AhR has been a major focus of research in the fields of toxicology, immunology and cellular biology.

1.5.1 AhR structure

The aryl hydrocarbon receptor is a ligand-dependent transcription factor that belongs to the Per-Arnt-Sim (PAS) family of proteins. These proteins function in the detection of intracellular and environmental changes such as sensing light and oxygen or responding to the redox potential of cells (Gu *et al.*, 2000). The two PAS homology domains on the AhR function in DNA recognition, ligand binding and chaperone interactions (Fukunaga *et al.*, 1995). AhR also contains a basic helix-loop-helix (bHLH) domain near the N terminus that functions as a DNA binding and dimerization motif (Dolwick *et al.*, 1993; Stevens *et al.*, 2009). In the cell, the unliganded AhR forms a multiprotein complex in the cytosol via interaction with a number of chaperone proteins that contribute to its stabilization and function (Poellinger, 2000). The AhR interacts with two heat shock protein 90 (Hsp90) chaperones that prevent nuclear translocation of the unliganded receptor and, in collaboration with the aryl hydrocarbon receptor associated 9 (ARA9) protein also known as XAP-2, maintain the AhR in a conformation to allow for ligand binding. Another 23-kDa co-chaperone protein (p23) functions to stabilize the interaction between AhR and Hsp90 and protects the receptor from proteolytic degradation (Meyer *et al.*, 1998; Kazlauskas *et al.*, 1999).

One factor that has greatly affected risk assessment studies has been differences in AhR structure that result in altered affinity for ligands and difference in AhR signaling. Structural differences in the AhR have been suggested to play a role in the interspecies variability observed in the magnitude of response following exposure to AhR ligands. Even within a species, differences in the AhR are known to exist that influence responsiveness to ligands (Poland *et al.*, 1976; Poland and Glover, 1980).

For example, inbred mouse strains have been classified into high and low responders based on the expression of either a low response AhR^d allele present in DBA/2, NOD, or 129 mouse strains and a high response AhR^b allele expressed in C57Bl/6 mice (Okey *et al.*, 1989). The differences between the high and low responder forms of the receptor can be attributed to a change in a single amino acid residue, but result in a 10-fold difference in sensitivity to AhR ligands (Poland *et al.*, 1994). The human AhR receptor has been hypothesized to have a 10-fold decrease in sensitivity compared to the mouse. However, polymorphisms in the human AhR exist that may increase or decrease the sensitivity of the receptor (Connor and Aylward, 2006). These polymorphisms might explain differences observed in epidemiological data from humans exposed to AhR ligands such as susceptibility to the development of chloracne, which is thought to be one of the most common effects of AhR ligands in humans (Bertazzi *et al.*, 1998).



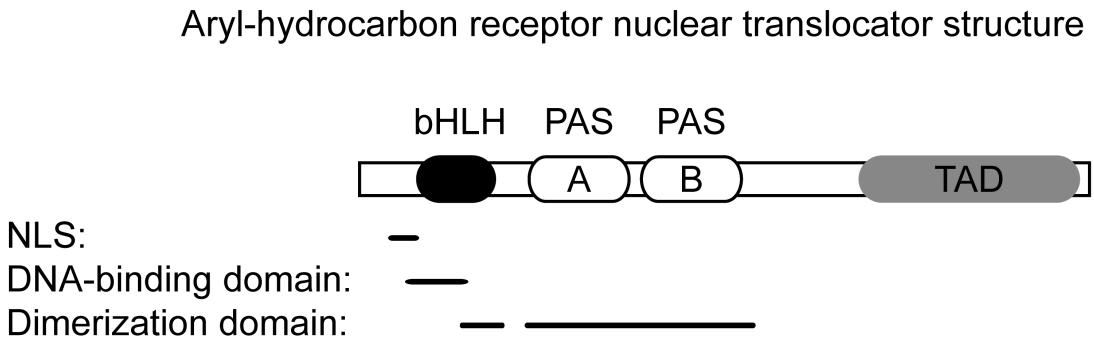


Figure 1: Structures of the aryl-hydrocarbon receptor (AhR) and the Aryl-hydrocarbon receptor nuclear translocator (ARNT). Basic helix loop helix (bHLH) region, Per-ARNT-Sim (PAS) domain, Nuclear localization sequence (NLS), Nuclear export sequence (NES).

1.5.2 AhR function

Ligand binding to the AhR induces a conformational change that causes the receptor to disassociate from the chaperone proteins and exposes a nuclear localization sequence that directs the translocation of the receptor to the nucleus. In the nucleus the AhR binds to the aryl hydrocarbon receptor nuclear translocator (Arnt) to form a heterodimer complex with high DNA binding affinity (Hankinson, 1994; Hankinson, 1995). The heterodimeric AhR-Arnt complex functions as a transcription factor to modulate expression of a vast array of genes.

Regulation

Numerous mechanisms have been elucidated that regulate the ligand-activated signaling of AhR. It has been observed that following ligand binding and translocation to the nucleus the AhR is rapidly downregulated. Studies assessing the fate of AhR following nuclear translocation indicate that the AhR is exported from the nucleus and removed via ubiquitination and degradation by the 26S proteasome. It has been demonstrated in cultured cells that inhibition of the proteasomal pathway increases both the magnitude and duration of AhR signaling and gene expression. This suggests that degradation of the receptor is a critical mechanism to attenuate AhR-driven gene transcription (Davarinos and Pollenz, 1999; Ma and Baldwin, 2000; Pollenz, 2002).

In addition to proteasomal degradation the activity of ligand-bound AhR is controlled via a negative feedback loop. Ligand-activated AhR dimerizes with Arnt and induces the expression of the aryl hydrocarbon receptor repressor (AhRR) protein. AHRR functions to inhibit AhR signaling by competing with the receptor for binding to the dimerization partner Arnt. The AhRR contains a transcriptional repressor domain

and therefore serves as another mechanism by which AhR signaling can be attenuated. (Mimura *et al.*, 1999; Baba *et al.*, 2001).

In addition to the induction of the AhRR, activation of the AhR induces the transcription of a number of metabolic enzymes that are capable of catalyzing the metabolism of some but not all AhR ligands. The induction of the metabolic enzymes can serve to eliminate the presence of AhR ligands and thus can down-regulate signaling. One hypothesis to explain the differences in potency among AhR ligands is based on the susceptibility of the ligands to metabolic breakdown that attenuates the signaling (Bohonowych and Denison, 2007).

Co-activators modulate the transcriptional activity by modifying the chromosomal structure to alter the configuration of nucleosomes. These factors include histone acetyltransferases and methyltransferases that regulate the structure of histones to increase or decrease their interaction with DNA and alter downstream transcriptional activity. The AhR-Arnt complex interacts with a number of transcriptional co-activators to influence the expression of AhR responsive genes. Interestingly, the AhRR-Arnt complex is also capable of recruiting co-repressors to further inhibit AhR gene transcription (Abel and Haarmann-Stemmann, 2010). The interaction with various different co-activators could offer one explanation why AhR-induced gene expression is different in various cell types and tissues (Hankinson, 2005).

Gene expression

The biological effects induced by activation of the AhR are due primarily to the transcriptional activity of AhR and the altered expression of a wide array of genes (Puga *et al.*, 2000). The heterodimeric AhR-Arnt complex binds to a substitution-intolerant

core nucleotide sequence of 5'-GCGTC-3' referred to as a xenobiotic response element (XRE) or dioxin response element (DRE) that serves as a regulatory element for gene expression. Other nucleotides adjacent to the core sequence have been shown to modulate the transcriptional response of AhR (Denison *et al.*, 1988; Swanson *et al.*, 1995). These DREs are widely distributed throughout the genome and serve as regulatory elements that facilitate AhR-induced gene expression. Comprehensive bioinformatics analysis has identified thousands of genes in the mouse, human and rat genome that contain putative DREs in the promoter regions, including over 1,000 orthologous genes in these three species (Dere *et al.*, 2011a; Dere *et al.*, 2011b). Expression of the AhR has been demonstrated in a number of different tissues and cell types, which may partially explain its role in numerous biological responses (Frericks *et al.*, 2007). AhR activation induces gene transcriptional changes that effect lipid metabolism, increase expression of drug metabolic enzymes, as well as alter endocrine system development and immune function (Kerkvliet, 2009; Lo *et al.*, 2011).

1.5.3 AhR ligands

Classical exogenous ligands

The most widely studied ligands for the AhR can be subdivided into two groups. The first is a large group of polycyclic aromatic hydrocarbons (PAHs). This includes chemicals such as 3-methylcholanthrene, benzyo(a)pyrene, benzoflavones and benzanthracenes. These ligands are generally considered to be of less importance to the toxicity mediated by AhR because PAHs have binding affinities in the nanomolar to micromolar range and typically result in only transient activation of the AhR because they are more susceptible to the metabolic enzymes induced upon AhR activation (Denison and Nagy, 2003). Therefore, PAHs induce a different range of AhR effects than the more potent agonists that have sustained binding to the receptor.

The other group of AhR ligands that have been extensively studied is the halogenated aromatic hydrocarbons (HAHs). This includes a number of brominated or chlorinated chemicals. The ligands that possess particular affinity for the AhR are of a similar planar structure and include chlorinated dibenzo-para-dioxins, chlorinated dibenzofurans and some polychlorinated biphenyls. In particular, while the group of HAHs includes 75 possible chlorine-substituted dibenzo-p-dioxins, 135 various chlorinated dibenzofurans and 209 different polychlorinated biphenyls, only a few meet the structural requirements to be considered potent AhR agonists (Poland and Knutson, 1982). Only a few congeners in this group demonstrate the ability to induce widespread AhR-mediated biological responses and toxicity. This level of activity appears to be limited to HAH congeners with 3 or 4 chlorine substitutions. These chlorinated ligands are particularly resistant to enzymatic metabolism and induce AhR-mediated gene

expression that can persist for days to weeks and even years in some cases. In particular, this level of AhR ligand-mediated activity is limited to 7 polychlorinated dibenzo-p-dioxins (PCDDs), 10 polychlorinated dibenzofurans (PCDFs), and 12 polychlorinated biphenyls (PCBs). These compounds possess higher binding affinities in the nanomolar to picomolar range, are insensitive to metabolic degradation and possess the ability to potently bind and activate AhR signaling to alter gene expression (Poland and Knutson, 1982; Denison and Nagy, 2003; Van den Berg *et al.*, 2006).

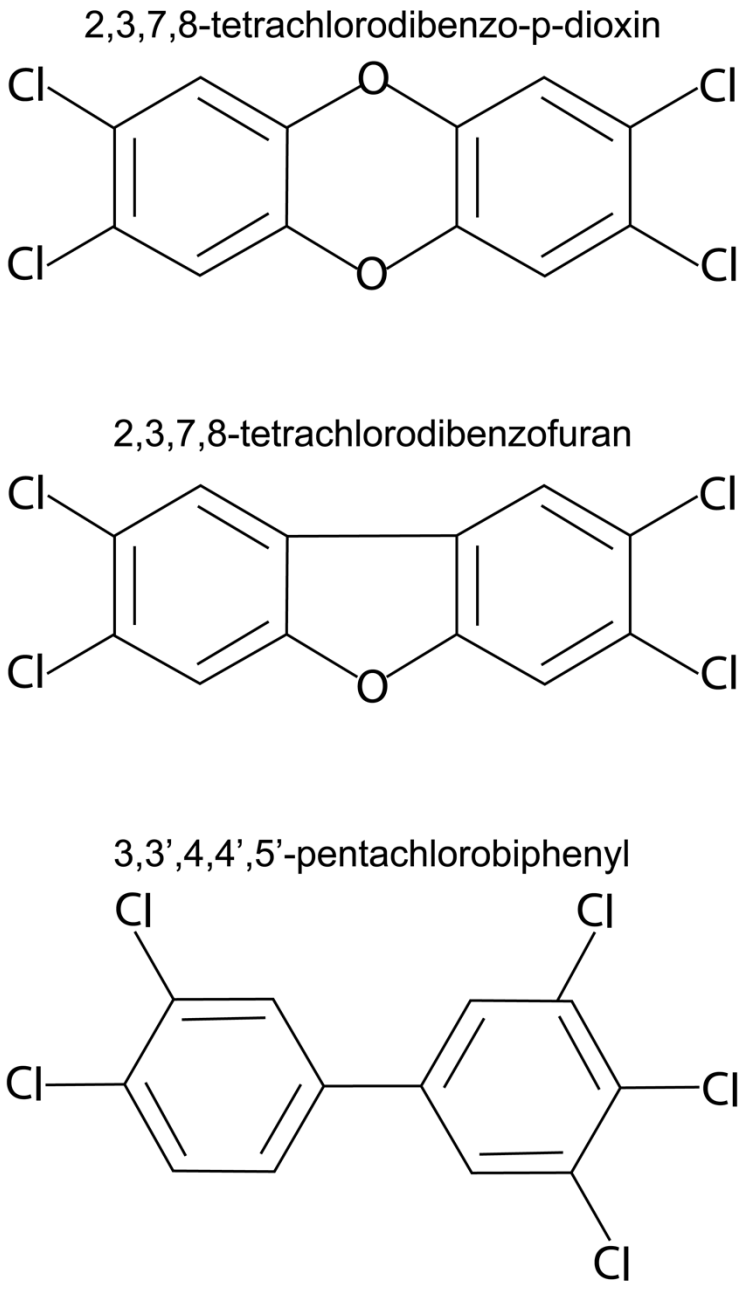


Figure 2: Examples of classical exogenous ligands for the aryl-hydrocarbon receptor.

Non-classical exogenous ligands

In addition to the classical ligands, a number of synthetic ligands exist that do not share structural characteristics with the PAHs and HAHs. These compounds possess diverse chemical structures and in general are low affinity ligands. This group contains pharmaceuticals such as the non-steroidal anti-inflammatory drugs diclofenac and sulindac as well as other man-made chemicals such as the pesticide carbaryl (Denison *et al.*, 2002; Ciolino *et al.*, 2006; Abel and Haarmann-Stemmann, 2010).

Sources of exogenous ligands

Generally speaking, all of the most potent ligands for the AhR are ubiquitous environmental contaminants. The PAHs and HAHs are almost exclusively anthropogenic in origin. The primary source of PAHs is the burning of organic matter such as fossil fuels that releases large amounts of these chemicals into the atmosphere. The majority of the high affinity HAH ligands were either produced as commercial products or unintentionally produced as contaminants in the manufacturing of other products. In particular, the production of organochlorine products is a prevalent source of PCDD and PCDF contaminants (Safe, 1986; Kerkvliet, 2002). Organochlorine products were produced in large amounts and widely used for a variety of applications. Examples of HAH contaminated products include pentachlorophenol (a wood preservative), the disinfectant hexachlorophene, as well as herbicides such as the compound Agent Orange (a 1:1 mixture of the butyl esters of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid) that were used extensively as defoliants in the Vietnam war. HAHs are also produced by the chlorine bleaching performed during pulp and paper manufacturing; as well as the incineration of municipal, hospital and

industrial wastes (Holsapple *et al.*, 1991; Sun *et al.*, 2012). In contrast to the inadvertent production of PCDDs and PCDFs, PCBs were extensively produced for a variety of purposes due to their desirable chemical properties. PCBs were included in coolants, flame retardants, adhesives, plasticizers, and hydraulic fluids. The United States produced approximately one billion pounds of PCBs prior to banning production in 1977 due to environmental concerns. As a result of this production, PCBs as well as to a lesser extent PCDDs and PCDFs are widespread environmental pollutants. The same chemical properties that made PCBs so useful in industry have made the environmental contamination of HAHs problematic. HAHs are extremely persistent in the environment as a result of their chemical stability that resists both environmental and metabolic degradation. This stability combined with their lipophilic nature leads to significant bioaccumulation of HAHs in the food chain, and background levels can be found in all humans, with significantly increased body burdens detected in those individuals living in industrialized areas (Poland and Knutson, 1982; Schecter *et al.*, 2006).

Endogenous ligands

For many years there were no identified endogenous ligands for the AhR. However, it has been believed that such ligands must exist due to the wide range of physiological and developmental abnormalities that occur in AhR-deficient mice. AhR knockout mice exhibit decreased body weight and slowed growth. In particular, numerous hepatic defects have been reported in these mice including reduced liver weight and damage to the hepatic sinusoids caused by improper development of the hepatic vascular system which is hyperproliferative and prone to causing hepatic

fibrosis. Additional developmental defects have been observed in a wide range of organs such as the heart, spleen, thymus and kidney (Schmidt *et al.*, 1996; Gonzalez and Fernandez-Salguero, 1998; Lahvis *et al.*, 2000; Lin *et al.*, 2001). These phenotypic abnormalities suggest that AhR must be responsible for more than just inducing a response to xenobiotic stimuli. In considering the wide range of physiological functions in which AhR has been found to play a role, it is not surprising that in recent years endogenous ligands have begun to be identified (Denison and Nagy, 2003). In comparison to the extensive collection of data available on the effects of HAHs on AhR activity, very little is known about the physiological effects of endogenous AhR ligands. It is now known that endogenous ligands include arachidonic acid metabolites such as lipoxin A4, and reporter assays have detected a number of prostaglandins with weak AhR agonist activity. Heme metabolites such as bilirubin also bind and activate AhR (Nguyen and Bradfield, 2008). Finally, while most of the endogenous ligands identified are weak AhR agonists, a photooxidation product of the amino acid tryptophan exhibits binding activity comparable to some of the most potent HAHs. The metabolite 6-formylindolo[3,2-b]carbazole (FICZ) appears to be a potent agonist for AhR activation and gene signaling and is a growing area of interest regarding the physiological role of endogenous ligands (Rannug *et al.*, 1987; Helferich and Denison, 1991; Stockinger *et al.*, 2011).

Toxic equivalency factors

Due to the widespread contamination of AhR ligands in the environment, regulatory agencies have struggled to gather enough data on the biological responses induced by each individual ligand to make separate risk assessment recommendations

on the safe exposure levels of these chemicals. Most of the information regarding the toxicity of AhR ligands is derived from experimental data assessing the effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). TCDD is widely accepted to be the most potent AhR ligand and as a result is the most commonly utilized in researching AhR biology and AhR-mediated toxicity. In the environment TCDD is not found in isolation. Instead HAHs are almost always found in mixtures. This is true for samples of both animal and human tissues. Furthermore, in these samples TCDD typically represents only a small percentage of the total Ahr ligands present (Birnbaum and DeVito, 1995). Since most of the data on biological responses induced by AhR activation are only available for TCDD, a system of toxic equivalency factors (TEFs) has been developed in order to draw comparisons between TCDD and other AhR ligands. The TEFs are a measure of the potency of a particular congener relative to a dose of TCDD that would result in the same biological effect. TEFs have been applied primarily to other HAHs that share enough chemical characteristics to TCDD in order to reliably assume a similar mechanism of action. These molecules all have chlorine or bromine atoms in lateral positions, a pattern of halogenation that is important in AhR ligand binding and the resistance to degradation by AhR-induced metabolic enzymes. In addition, the potent HAHs all share a planar conformation that is necessary for high affinity AhR binding. This is of particular relevance to the PCBs, which can assume either a non-coplanar or coplanar conformation depending on the location of the halogen atoms. The coplanar conformation is structurally similar to the chemical structure of the PCDDs and PCDFs and has a greatly increased binding affinity for AhR (Safe, 1986; Van den Berg *et al.*, 1998).

The TEFs are calculated on an order of magnitude basis using the available data for each congener in comparison to TCDD, which is given a value of 1. In general, the available data for each congener consists of assays to determine receptor binding affinity and induction of the canonical metabolic enzymes associated with AhR activation such as cytochrome p450 (CYP) 1A1. Although Cyp 1A1 induction does not correlate well with many TCDD-induced toxicities, it is nevertheless some of the only data available with which to draw comparisons between HAHs. Using this system, the most commonly studied HAH ligands for AhR rank as follows: TCDD (1.0), 2,3,7,8-tetrachlorodibenzofuran (0.1) and 3,3',4,4',5-pentachlorobiphenyl or PCB 126 (0.1) (Van den Berg *et al.*, 2006).

In addition to making comparisons among HAHs, the use of TEFs allows for the calculation of the total potency for mixtures of congeners. The toxic equivalency of a mixture can be determined by multiplying the TEF for that chemical by the concentration in a mixture. This technique essentially treats these mixtures as if they were composed 100 percent of the prototypical ligand TCDD. This value is referred to as the toxic equivalent (TEQ).

1.5.4 AhR biology

As previously stated the physiological role of the AhR has been characterized through the use of multiple AhR knockout mouse strains. The numerous physiological and developmental abnormalities in these mice suggest a wide array of functions for AhR-mediated gene expression (Fernandez-Salguero *et al.*, 1995; Schmidt *et al.*, 1996; Gonzalez and Fernandez-Salguero, 1998). In the absence of treatment with any exogenous ligand, gene expression analysis has demonstrated almost 400 differentially regulated genes in AhR knockout mice compared to wild-type controls, indicating substantial activity of the AhR even in the absence of treatment (Tijet *et al.*, 2006). The widespread induction of gene expression by AhR activation can be roughly categorized into two pathways (Stevens *et al.*, 2009).

Adaptive pathway

The induction of metabolic biotransformation enzymes was one of the first biological responses characterized for the AhR. PAH ligands for AhR cause a subsequent increase in expression of metabolic enzymes that is mediated by AhR activation (Sawyer and Safe, 1982). Those enzymes then catalyze the metabolism of PAHs reducing the half-life in the body and help protect against subsequent PAH exposure, whereas HAHs are significantly insensitive to these metabolic enzymes and subsequently are known to have increased persistence in bodily tissues (Abel and Haarmann-Stemmann, 2010). The expression of the CYP 1A1 by AhR ligands has served as the primary model by which the mechanism of AHR-mediated gene expression has been determined (Schmidt and Bradfield, 1996). In addition to the CYP enzymes induced by AhR activation, a number of other enzymes that perform metabolic

biotransformation of xenobiotics have DREs in their gene promoters and are induced by AhR ligands. This includes glutathione s-trasferase, aldehyde dehydrogenase, UDP-glucuronosyltransferases, and enzymes involved in antioxidant responses (Bock and Kohle, 2006). Interestingly, the metabolism of PAHs can lead to bioactivation of reactive intermediates that have a different spectrum of toxicities associated with them (Burchiel and Luster, 2001; Nebert *et al.*, 2004). Therefore, susceptibility of various ligands to the action of metabolic enzymes induced by AhR activation is one of the primary reasons for the differences in biological and toxic effects induced by PAHs versus HAHs (Bohonowych and Denison, 2007).

Toxic pathway

The toxicity of HAHs has been studied for decades and occurs in many different species (McConnell and Moore, 1979; Poland and Knutson, 1982). Activation of the AhR by HAHs induces involution of lymphoid organs such as the thymus and spleen. Many HAHs cause hepatotoxicity and alter lipid metabolism in an AhR-dependent manner (Birnbaum *et al.*, 1990; Chang *et al.*, 2005; Kopec *et al.*, 2008). HAHs alter endocrine system function and can also act as tumor promoters (Pitot *et al.*, 1980). AhR agonists have also been extensively studied for their ability to modulate the adaptive immune response (Kerkvliet, 2009). The ability of AhR activation to influence the immune response is of particular importance to the research discussed in this dissertation and will be discussed in more detail regarding the prototypical ligand TCDD later in the Introduction.

It is difficult to discuss the toxic effects of HAHs without focusing on the extensive amount of research conducted on TCDD. As the most potent inducer of AhR activity,

TCDD represents an ideal agonist for the study of AhR mediated toxicity because it can be used at smaller doses than other agonists to induce an equivalent response, which limits the potential for off target effects. For this reason the remainder of the discussion about AhR induced toxicity will focus primarily on effects observed with TCDD treatment or exposure.

1.6 TCDD-induced toxicity

Toxicokinetics

As previously mentioned, the lateral chlorine atoms and lipophilic characteristics of TCDD result in increased chemical stability. TCDD is extremely persistent in both the environment and in living tissues where it is highly resistant to metabolism. In humans the half-life of TCDD is estimated at 5-11 years depending on the dose, while in rats the half-life is approximately 30 days and in mice 8-12 days (Birnbaum, 1986; Hakk *et al.*, 2009). TCDD is rapidly absorbed through the lung and GI tract, while absorption through the skin is slower. However, the route of exposure does not significantly affect the spectrum of toxicity observed. Due to the increased lipophilic nature of TCDD it is commonly distributed to adipose tissues. In addition, TCDD appears to accumulate disproportionately in the liver (Thoma *et al.*, 1990). The ratio of liver to adipose tissue concentration increases as the dose of TCDD is increased (Abraham *et al.*, 1988). The hepatic distribution of TCDD is partially explained by increased binding to metabolic enzymes induced by AhR activation, such as CYP1A2. However, the binding of TCDD to CYP1A2 does not contribute significantly to metabolism of TCDD. Instead the sequestration of TCDD by CYP1A2 binding is thought to contribute to the increased elimination half-life due to decreased availability of TCDD to be metabolized by other enzymes (Hakk *et al.*, 2009). Detectable levels of TCDD can also be found in a number of other tissues including skin, adrenal glands, thyroid, pancreas spleen, lymph nodes, lung and bone marrow (Diliberto *et al.*, 1995).

Toxicity of TCDD

The administration of TCDD induces a number of toxicities in a range of animal models. These toxicities include development of a wasting syndrome, tumor promotion, altered endocrine function, teratogenicity and other reproductive toxicities as well as many others observed in various animal experiments. The toxicity of TCDD has been detailed in a number of excellent review articles (Poland and Knutson, 1982; Birnbaum, 1994; Grassman *et al.*, 1998; Birnbaum and Tuomisto, 2000; Mandal, 2005; Schecter *et al.*, 2006). For the purposes of this Introduction a focus will be placed on the hepatotoxic and immunotoxic effects of TCDD.

Hepatotoxicity

The liver is a major target organ for TCDD, and exposure induces hepatotoxicity in many different animal species. This toxicity manifests as hepatic necrosis and steatosis characterized by fatty degeneration of the liver and accumulation of lipids in the hepatocytes (Birnbaum and Tuomisto, 2000). This toxicity is mediated by AhR as determined by protection observed in a number of studies in which AhR signaling was impaired (Fernandez-Salguero *et al.*, 1996; Thurmond *et al.*, 1999; Shimizu *et al.*, 2000; Bunger *et al.*, 2003; Chang *et al.*, 2005). In addition, mouse strains expressing a low responsive AhR allele are less susceptible to hepatotoxicity (Birnbaum *et al.*, 1990). The gene expression pattern induced by TCDD is cell-type specific, and the hepatotoxicity of TCDD appears to be mediated by AhR transcriptional effects within hepatocytes because mice with conditional hepatocyte-specific AhR knockout are protected (Walisser *et al.*, 2005; Hayes *et al.*, 2007). To some extent a role exists for a secondary effect mediated by inflammatory immune cells in TCDD hepatotoxicity

(Pande *et al.*, 2005). The role of TCDD in immunotoxicity will be discussed in section 1.7, but it is noteworthy to state that the dose required to cause acute hepatotoxicity is typically an order of magnitude higher than a dose that alters immune cell activity (DeVito *et al.*, 1995).

1.6.1 Human exposure

Background exposure

Exposure to TCDD and other HAHs generally occurs via three routes: environmental, occupational, and accidental. TCDD contamination is widespread in the environment, however background levels appear to be higher in individuals living in industrialized countries. The body burden of TCDD is estimated using lipid-adjusted serum concentrations. This measurement of body burden is based on the assumption that TCDD distributes evenly to the adipose tissue and that TCDD concentrations in serum lipids are equivalent to those in whole body lipid. This association does not hold true for all HAHs, but for TCDD there is a high correlation between lipid-adjusted serum levels and body lipid levels (Gochfeld *et al.*, 1989). Importantly, this technique may underestimate the concentration of TCDD in the hepatic tissues. As stated previously, there is a disproportionate accumulation of TCDD in the liver compared to adipose tissue, particularly at higher doses. Studies on the average environmental exposure of the general population in the United States have estimated that the levels of HAHs range from 8-17 TEQ ng/kg lipid for dioxin-like PCBs and 28-41 TEQ ng/kg lipid for the PCDDs and PCDFs. While TCDD exposure accounts for only 15% of the total TEQ value measured in this study, the background exposure to the U.S. population is equivalent to a body burden of 36-58 ng TCDD/kg lipid (DeVito *et al.*, 1995). Although these levels are not insignificant given that very low dose effects on enzyme induction have been observed in animals, of particular interest to this research are the groups of individuals exposed to TCDD and other HAHs via the occupational or accidental routes.

Epidemiological studies

Individuals who work in chemical manufacturing (especially those working with chlorophenol) and those who work at incinerators are exposed to levels of TCDD far above background levels in the general population (Schecter *et al.*, 2006). A number of studies have been performed on cohorts exposed to large levels of TCDD either occupationally or due to industrial accidents. Table 2 includes a number of studies conducted to assess health effects and body burdens of various cohorts involved in occupational or accidental exposure to TCDD and other HAHs.

Unfortunately only a few of the studies conducted have measured immunological parameters (indicated in Table 2) and often different parameters were measured in different studies decreasing our ability to make comparisons across exposure cohorts. However, a few interesting findings are particularly relevant to the research presented here. In a study conducted by the National Institute for Occupational Safety and Health (NIOSH) a number of lymphocyte sub-populations were assessed, and a decrease in activated T cells was associated with increased TCDD exposure (Halperin *et al.*, 1998). In contrast, another study from a cohort of workers in Hamburg reported that an increased proportion of CD8+ T cells and a decrease in naïve T cell phenotype was associated with TCDD exposure (Ernst *et al.*, 1998). A study in chemical workers from the United Kingdom assessed a number of immune parameters in individuals that were last occupationally exposed to TCDD 17 years earlier. This study reported that there was no change in the populations of T or B lymphocytes in TCDD-exposed individuals, but a significant increase in the number of NK cells was detected in peripheral blood. Additionally, the presence of antinuclear antibodies was detected more frequently in

TCDD exposed individuals (Jennings *et al.*, 1988). This result is consistent with measurements from a cohort consisting of Vietnam war veterans which detected small but significant increases in serum IgA concentrations (Greene *et al.*, 2003). In general, the inconsistent measurement of immune parameters, coupled with conflicting results in different cohorts make interpreting the data a difficult task. This is further complicated by the issue of the delay between the last exposure of a particular cohort and the time the studies were conducted, such that in some cases over 40 years had passed before effects on immune parameters were measured. Add to this the fact that no definitive adverse effect on immune function has been demonstrated, even in highly exposed human cohorts, and the application of the extensive supply of data on TCDD-induced modulation of immune responses in animals becomes difficult to interpret from a human risk assessment standpoint.

Table 2

Summary of human epidemiological studies

Study Cohort	Estimated years since last exposure	Lipid-adjusted serum levels (TEQ)	Immune Parameters measured	References
US adults (Background exposure)	N/A	36-58 ng/kg	No	(DeVito *et al.*, 1995)
NIOSH	15-37	2-3389 ng/kg	Yes	(Halperin *et al.*, 1998)* (Sweeney *et al.*, 1990)
Hamburg	5-37	15-300 ng/kg	Yes	(Ernst *et al.*, 1998)* (Flesch-Janys *et al.*, 1996)
Netherlands	18-43	1.9-194 ng/kg	No	(Heederik *et al.*, 1998)
UK chemical Workers	N/A		Yes	(Jennings *et al.*, 1988)*
Superfund	N/A	2-94 ng/kg	No	(Cranmer *et al.*, 2000)
Dow Chemical TCP workers	24-63	2-176 ng/kg	No	(Collins *et al.*, 2006)
BASF Germany	36-37	1-553 ng/kg	Yes	(Ott *et al.*, 1994)*
Chemical Workers in US	20-36	ND-3400 ng/kg	No	(Piacitelli *et al.*, 1992)
Austria	N/A	98-659 ng/kg	No	(Neuberger *et al.*, 1991)
Seveso, Italy	1	ND-56,000 ng/kg	Yes	(Mocarelli *et al.*, 1991) (Greene *et al.*, 2003)* (Needham *et al.*, 1997) (Needham *et al.*, 1999)
Times Beach, Missouri	N/A	Not reported	Yes	(Webb *et al.*, 1989)*
Air Force Ranch Hands	16-25	5-313 ng/kg	Yes	(Greene *et al.*, 2003)*

N/A: data was not available for this study cohort

1.7 Effects of TCDD on the immune system

The effect of TCDD and role of AhR signaling in the modulation of immune responses have been steadily increasing as more information is discerned about AhR-mediated gene transcription and the expression of AhR in different immune cell types. For many years TCDD has been regarded as a potent immunotoxicant capable of inhibiting the activity of T and B cells in the adaptive immune system. However, as new results are reported on the effects of TCDD and other AhR ligands in various animal models it has become apparent that the ability of AhR to modulate the immune response extends far beyond a standard immunosuppressive action.

Dioxin response elements have been identified in a large number of immunologically relevant genes (Kerkvliet, 2009). Evidence indicates that activation of AhR is an important factor in the differentiation of T regulatory cells and Th17 cells (Kimura *et al.*, 2008; Quintana *et al.*, 2008). These cell types play important roles in shaping the development of an immune response by releasing mediators that can either suppress or exacerbate the activity of other immune cells (Sakaguchi *et al.*, 2008; Hammerich *et al.*, 2011). The following is a brief description of some of the reported effects that TCDD and other AhR ligands have on cells of the adaptive and innate immune system.

1.7.1 Adaptive immune cells

TCDD is a well known suppressor of the adaptive immune system including cell-mediated immunity and the humoral response (Holsapple *et al.*, 1991). Although the exact mechanism by which TCDD inhibits the adaptive immune response is still unclear many of the effects TCDD exerts on the adaptive immune cells have been well characterized (Kerkvliet, 1995; Kerkvliet *et al.*, 1996).

B cells

B cells have not been shown to play a role in the Con A-induced hepatitis model utilized in the studies presented in this dissertation and as such, I will refrain from discussing at length the effects of TCDD on B cell function and instead focus on cell types that are particularly relevant to the experimental model discussed here. However, there is an extensive amount of research that has been conducted regarding the effects of TCDD on B cells and the following summary is not meant to be an exhaustive description of those findings. In short, TCDD acts on B cells at multiple points to decrease the generation of a humoral response.

The function of B cells is directly suppressed by TCDD and involves inhibition at multiple points in their development. TCDD also inhibits the proliferation of B cells and decreases the differentiation of activated B cells into plasma cells. Furthermore, TCDD treatment can suppress the transcription of components of the IgM antibody, decreasing antibody production by plasma cells (Sulentic and Kaminski, 2011).

T cells

The suppressive effect of TCDD on cytotoxic T cells is AhR-dependent. Congenic mice expressing the responsive AhR^b allele were more sensitive than AhR^d

mice to TCDD-induced suppression of the cytotoxic T lymphocyte response to injection of allogeneic tumor cells (Kerkvliet *et al.*, 1990b). In another model, suppression of T cell response to sheep red blood cells (SRBCs) by TCDD was dependent on AhR (Kerkvliet *et al.*, 1990a)

Increasing evidence points to a role for AhR modulated T cell lineage determination via altered expression of lineage-specific transcription factors. The transcription factors critical for the differentiation of T cells into Th2, Th17 and T regulatory cells, GATA3, RORc (γt), and Foxp3 respectively, all contain multiple putative DRE sequences in their promoter regions (Sun *et al.*, 2004; Kerkvliet, 2009).

Importantly, some subsets of T cells express extremely high levels of AhR, including the Th17 and T regulatory subtypes. These T cell subsets appear particularly sensitive to AhR ligands such as TCDD. In addition, the cytokines IL-17(A and F) and IL-22 are known to have DREs in the promoter regions of their genes. IL-17 and IL-22 contribute to the pathogenesis of multiple diseases and alteration of their synthesis may be one mechanism by which AhR signaling can alter immune responses (Veldhoen *et al.*, 2008; Hammerich *et al.*, 2011). Interestingly, Th17 cells have similar effector functions as the γδ T cells enriched in the liver. While γδ T cells function in a primarily innate immune capacity, they share similar gene expression characteristics with Th17 cells and, following treatment with AhR ligands, both cell types produce increased IL-17 upon stimulation (Martin *et al.*, 2009).

In addition to Th17 cells, AhR activation of T regulatory (Treg) cells has received much attention for the ability of Tregs to suppress the adaptive immune response. In a model of graft-versus-host response TCDD-treated mice were protected through the

induction of T regulatory cells that suppress the immune response by the release of IL-10 (Marshall *et al.*, 2008). The immune response in this model was not impaired when T cells from AhR knockout mice were used (Kerkvliet *et al.*, 2002).

Dendritic cells

Compared to the research on T and B cells, less information exists on the precise effects of TCDD on dendritic cells (Bankoti *et al.*, 2010a; Bankoti *et al.*, 2010b). However, these cells play an important role in the development of the adaptive immune response and thus any modulation of their function by AhR activation could have widespread implications (Lau and Thomson, 2003). Interestingly, rather than developing a suppressed phenotype dendritic cells isolated from the spleens of TCDD-treated mice had increased expression of accessory molecules involved in T-cell activation including MHC Class II, CD54, and CD40. Compared to cells isolated from vehicle-treated mice, dendritic cells from TCDD-treated mice also expressed increased levels of IL-12, a cytokine that stimulates the immune response and increases the activity of innate immune cells (Vorderstrasse and Kerkvliet, 2001; Vorderstrasse, 2003). However, in a model of transplant tolerance, AhR activation in dendritic cells results in the promotion of T regulatory cells that promote allograft-specific tolerance (Hauben *et al.*, 2008). These different responses indicate that the effects of AhR activation in dendritic cells is likely to be dependent on the context of the immune response.

1.7.2 Innate immune cells

It has always been assumed that AhR is widely expressed in innate immune cells based on the TCDD-mediated effects observed in neutrophils and macrophages (Kerkvliet, 2009). However, unlike cells of the adaptive immune system only a few cell-type specific AhR expression studies have been performed. AhR expression in macrophages has been verified by gene array studies, but the degree to which other innate immune cells express AhR and to what extent that expression level may be biologically relevant is still uncertain (Frericks *et al.*, 2007; Esser *et al.*, 2009). It is known that a number of genes relevant to the function of innate immune cells possess putative DREs and expression is increased by TCDD treatment. These genes include chemokine receptors, cytolytic mediators such as granzyme B and cathepsins, interleukin receptors, and chemokines (Sun *et al.*, 2004; Stevens *et al.*, 2009).

Macrophages

Mice treated with TCDD exhibit increased sensitivity to endotoxin and have increased production of the macrophage-derived cytokine TNFα. Compared to vehicle treatment, TCDD-treatment resulted in increased plasma concentration of TNFα after endotoxin injection, and the response appeared to segregate with the high- and low-responsive AhR expressed in congenic mouse strains (Clark *et al.*, 1991). Compared to vehicle treatment, TCDD treatment of primary isolated peritoneal macrophages or a peritoneal macrophage cell line (IC-21) resulted in increased TNFα production and secretion when cells were stimulated with LPS (Moos *et al.*, 1997). TCDD treatment alone is sufficient to induce TNFα expression in a THP-1 macrophage cell line. The exact mechanism of TCDD-induced expression of TNFα in this cell line is unknown, but

it appears to be mediated by AhR and the activation of the epidermal growth factor receptor (Cheon *et al.*, 2007).

TCDD treatment also increased the expression of monocyte chemotactic protein 1 (MCP-1) and keratinocyte chemoattractant (KC) in the liver, thymus, kidney, adipose tissue and heart of mice as many as 7 days after treatment. The increase in chemokines was associated with increased detection of F4/80, a macrophage cell marker, in those tissues (Vogel *et al.*, 2007).

Neutrophils

In both high and low responder mouse strains acute doses of TCDD increased the number of circulating neutrophils compared to controls in a dose-dependent manner (Birnbaum *et al.*, 1990). TCDD-treated mice with allogeneic tumor cells injected into the spleen had increased neutrophils in the blood compared to vehicle control mice. Isolated neutrophils from the TCDD-treated mice also had increased oxidative bursts but decreased tumoricidal capacity compared to those isolated from vehicle-treated mice (Choi *et al.*, 2003).

TCDD treatment is also known to enhance the stimulus-induced recruitment of neutrophils to various organs. TCDD treatment increased the number of neutrophils in the lungs of influenza-infected mice in an AhR-dependent manner. The increase in pulmonary neutrophils resulted in exacerbated damage to the lung parenchymal tissue and decreased mouse survival (Teske *et al.*, 2005).

Similarly, increased numbers of infiltrating neutrophils and monocytes were observed in the peritoneum of TCDD-treated mice injected with sheep red blood cells (SRBC) (Kerkvliet and Oughton, 1993).

NK cells

The influence of TCDD on NK cells has not been extensively studied, however a few studies indicate a potential for TCDD to modulate NK cells. Assessment of influenza-infected mice showed increased NK cells in the lungs of TCDD-treated mice compared to controls. However, TCDD treatment did not elicit any changes in function of the NK cells, and IFNγ levels were not altered (Neff-LaFord *et al.*, 2003). In contrast, NK cells isolated from the blood and spleen of TCDD-treated mice had increased cytolytic activity and displayed increased cytotoxicity towards YAC-1 cells in culture. This increase was observed even when cells were isolated from mice treated with TCDD 90 days earlier, indicating that TCDD may have lasting effects on NK cell activity (Funseth and Ilback, 1992). In addition, putative DREs have been identified in various NK cell receptor genes including Klra, Klrb1, Klrc, Klrg, and Klrk (Sun *et al.*, 2004). The expression of these costimulatory or inhibitory receptors on NK and NKT cells has the potential to drastically alter cytolytic activity and cytokine production (Bauer *et al.*, 1999; Zhang *et al.*, 2008).

1.7.3 TCDD effects on autoimmune disease

As previously mentioned, environmental xenobiotics have been demonstrated to contribute to the development of various autoimmune diseases. AhR ligands such as TCDD represent a likely group of candidates capable of modulating immune function and increasing susceptibility or development of autoimmune diseases and this possibility has been addressed in a number of review articles (Veldhoen *et al.*, 2008; Esser *et al.*, 2009; Stockinger *et al.*, 2011).

Experimentally, AhR ligands alter the development and severity of autoimmune diseases in animal models. AhR activation exacerbates pathophysiology associated with rheumatoid arthritis (Kobayashi *et al.*, 2008). In a model of experimental autoimmune encephalomyelitis, AhR-knockout mice developed disease with delayed kinetics, and AhR ligands such as TCDD and FICZ to alter the onset and severity of disease. Interestingly, in these studies the two AhR ligands had different effects. TCDD treatment decreased the severity of disease in mice while administration of FICZ increased the severity and shortened onset (Quintana *et al.*, 2008; Veldhoen *et al.*, 2008). The differences observed appear to be related to the ability of the ligands to induce either Th17 or T regulatory cells, which exacerbate and protect against injury respectively.

In a SNF_1 mouse model that develops spontaneous autoimmune nephritis prenatal treatment with TCDD increased the development of autoreactive T cells in adult mice and exacerbated disease. An increase in autoantibodies was observed, and phenotypic changes were also seen in B cells isolated from the bone marrow and spleen (Mustafa *et al.*, 2009a; Mustafa *et al.*, 2009b; Holladay *et al.*, 2011). These

results indicate that AhR ligands such as TCDD can alter the development and severity of a range of autoimmune diseases. However, the effects of TCDD on the development of autoimmune liver disease are currently unknown.

1.8 Rationale for studies

The following studies were proposed to address a gap in knowledge regarding the role of environmental contaminants such as TCDD and their potential to enhance the development of inflammatory liver disease. The effects of AhR activation by TCDD has not been explored in a model of autoimmune liver injury in which emphasis was placed on the effector role of intrahepatic innate immune cells that are known to contribute to the development of these diseases. Given that the liver represents a major target organ for TCDD distribution and toxicity it is likely this would also be a major site of immune modulation via the AhR. Due to the fact that the liver is an important site for immune surveillance and must maintain a delicate balance between response and tolerance any modulation of the immune response could have far reaching consequences. These studies will utilize the concanavalin A-induced immune-mediated model of liver injury to determine if exposure to TCDD modulates intrahepatic immune cell function; resulting in altered pathogenesis and severity of hepatotoxicity.

1.9 Hypothesis and specific aims

TCDD pretreatment enhances the response of intrahepatic immune cells to the inflammatory stimulus concanavalin A resulting in increased hepatotoxicity in a mouse model of autoimmune liver disease.

Specific Aim 1: To evaluate the effect of TCDD pretreatment on the sensitivity of mice to Concanavalin A administration in a model of autoimmune liver injury.

i. Determine the dose-response relationship for Concanavalin A-induced inflammatory liver injury in mice in the presence or absence of TCDD pretreatment (Chapter 2)

ii. Develop a detailed time course of plasma inflammatory cytokine levels (Chapter 2)

Specific Aim 2: To determine whether TCDD treatment alters the activation/function of innate immune cells following stimulation with the inflammatory stimulus Concanavalin A.

i. Determine the roles of natural killer cells and natural killer T cells in TCDD/Con A-induced liver injury (Chapters 2 and 3)

ii. Assess the role of macrophages in TCDD/Con A-induced liver injury (Chapter 3)

iii. Determine the role of neutrophils in the development of liver injury following TCDD/Con A co-treatment (Chapter 3)

iv. Determine the effects of TCDD pretreatment on expression of cell surface adhesion molecules, release of chemokines and accumulation of inflammatory innate immune cells in the liver after Con A administration (Chapter 2 and 3)

CHAPTER 2

2.1 Abstract

Inflammation plays a major role in immune-mediated liver injury, and exposure to environmental pollutants such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been reported to alter the inflammatory response as well as affect immune cell activity. In this study, we tested the hypothesis that TCDD pretreatment exacerbates hepatotoxicity in a murine model of immune-mediated liver injury induced by concanavalin A (Con A) administration. Mice were pretreated with 30 μg/kg TCDD or vehicle control on day zero and then given either Con A or saline intravenously on day four. Mice treated with TCDD did not develop liver injury; however, TCDD-pretreatment increased liver injury resulting from moderate doses of Con A (4-10 mg/kg). TCDD-pretreated mice had altered plasma concentrations of inflammatory cytokines, including interferon gamma (IFNγ), and TCDD/Con A-induced hepatotoxicity was attenuated in IFNγ knockout mice. At various times after treatment, intrahepatic immune cells were isolated, and expression of cell activation markers as well as cytolytic proteins was determined. TCDD pretreatment increased the proportion of activated natural killer T (NKT) cells and the percent of cells expressing Fas ligand (FasL) after Con A administration. In addition FasL knockout mice and mice treated with CD18 antiserum were both protected from TCDD/Con A-induced hepatotoxicity, suggesting a requirement for direct cell-cell interaction between effector immune cells and parenchymal cell targets in the development of liver injury from TCDD/Con A treatment. In summary, exposure to TCDD increased NKT cell activation and exacerbated immune-mediated liver injury induced by Con A through a mechanism involving IFNγ and FasL expression.

2.2 Introduction

The liver is a major site of immune cell activity and surveillance. In many inflammatory liver diseases such as viral and autoimmune hepatitis, cells of both the innate and adaptive immune system contribute significantly to the development of liver injury (Rehermann and Nascimbeni, 2005; Dong *et al.*, 2007). The inability of the hepatic immune cells to manage inflammatory stress is a key component in the progression of these diseases (Santodomingo-Garzon and Swain, 2011). Exogenous factors that affect the ability of immune cells to regulate the inflammatory response can be important contributors to sensitivity to inflammatory liver injury and disease. One factor that can affect immune cells is exposure to persistent environmental pollutants such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or other aryl hydrocarbon receptor (AhR) ligands (Esser *et al.*, 2009; Veldhoen and Duarte, 2010). TCDD exposure and AhR activation have been linked to a number of adverse effects in both animals (Poland and Glover, 1980) and humans (Sweeney and Mocarelli, 2000).

In addition to developmental and reproductive toxicity, TCDD has been widely studied in animal models for its ability to alter the function of numerous immune cell types (Birnbaum and Tuomisto, 2000). Pretreatment of mice with AhR ligands enhanced the accumulation of neutrophils and increased the severity of lung injury from influenza virus infection (Teske *et al.*, 2005). AhR activation also exacerbated pathology in experimental autoimmune encephalomyelitis (Veldhoen *et al.*, 2008). Furthermore, TCDD increased inflammatory stress in mice after administration of bacterial lipopolysaccharide or sheep red blood cells (Clark *et al.*, 1991; Moos *et al.*, 1994; Olivero-Verbel *et al.*, 2011). However, whereas the liver is known to be both a major site

of TCDD accumulation and an important target organ for the toxicity of AhR ligands (Gasiewicz *et al.*, 1983), the effects of TCDD exposure on the activation and function of hepatic lymphocytes during inflammatory liver injury have not been extensively evaluated.

In this study, we tested the hypothesis that TCDD pretreatment exacerbates hepatotoxicity in a murine model of immune-mediated liver injury induced by concanavalin A (Con A) administration. Con A administration is a widely used animal model of liver injury with mechanisms and pathology resembling immune-mediated hepatitis (Tiegs *et al.*, 1992). Con A-induced hepatitis largely depends on the activation of innate immune cells, which have received far less attention in studies of TCDD exposure than cells of the adaptive immune system. This model of inflammatory injury is characterized by strong immune-mediated killing of hepatocytes through direct cell cytolytic processes, as well as the production of inflammatory cytokines (Tiegs *et al.*, 1992; Mizuhara *et al.*, 1994; Kusters *et al.*, 1996; Seino *et al.*, 1997). The various mechanisms involved in the development of Con A-induced hepatitis allow for an in-depth analysis of the modes of action through which TCDD alters the immune response to influence the development of liver injury. The ability of TCDD to perturb any of the key mechanisms involved in the inflammatory response to Con A, such as the production of inflammatory and protective cytokines, activation of immune effector cells, and regulation of cell cytolytic activity towards hepatic parenchymal cells, was also evaluated.

2.3 Materials and methods

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. TCDD (Accustandard, New Haven, CT) was dissolved in DMSO and diluted in olive oil to a 0.2 µg/mL working solution. Rabbit anti-murine CD18 antiserum was purchased from New England Peptide (Gardner, MA).

Mice

Unless otherwise stated, all experiments were performed using male C57BL/6J mice. All mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at 10-12 weeks of age. Upon arrival, wild-type, *Ifng*^{tm1Ts}, C57BL/6-PRF1^{tm1Sdz}/J, B6Smn.C3-Fasl^{gld}/J mice were acclimated for at least one week in a 12 h light/dark cycle with access to Global Rodent diet 2018 (Harlan Teklad, Madison, WI) and spring water ad libitum. All procedures were carried out with the approval of the Michigan State University Institutional Animal Care and Use Committee. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the United States National Institutes of Health.

Experimental protocols

A single administration of 3 or 30 µg/kg TCDD or olive oil (vehicle) was given via oral gavage 4 days before Con A or saline administration. During that 4-day period, TCDD- treated mice were housed in separate cages from vehicle-treated mice. Con A (Lot 096K7011) was dissolved in saline at a concentration of 1.0 mg/mL and administered intravenously at a dose of 6.0 mg/kg body weight (unless otherwise stated). Activity of alanine aminotransferase (ALT) in plasma was measured

spectrophotometrically using Infinity-ALT reagent (Thermo Fisher Scientific, Waltham, MA). In CD18 neutralization experiments, 15 h before and 2 h after Con A administration, mice were treated intraperitoneally with 200 µL of a rabbit antiserum designed against amino acids 89-100 of murine CD18. Control mice were treated with equivalent volume of normal rabbit serum. The dosing protocol for CD18 neutralization was adopted from previous studies in our laboratory in which the antiserum effectively prevented leukocyte accumulation in the liver as determined by histopathological examination and immunohistochemistry (Shaw *et al.*, 2009).

Histopathology

Left lateral liver lobes were fixed in neutral buffered formalin for 24 h and paraffin-embedded. Lobes were sectioned and stained with hematoxylin and eosin. The sections were examined by light microscopy. Histopathology sections presented are from mice with plasma ALT activity corresponding closely with the average of each respective treatment group.

Cytokine analysis

OptEIA ELISA kits purchased from BD (Franklin Lakes, NJ) were used to measure plasma concentrations of interferon gamma (IFNγ). Plasma concentrations of tumor necrosis factor alpha (TNFα), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-10 (IL-10) and interleukin-6 (IL-6) were measured using a bead-based Milliplex MAP immunodetection array purchased from Millipore (Billerica, MA) and run on a Bio-Plex instrument (Bio-Rad, Hercules, CA).

RNA isolation and RT-PCR analysis

Liver samples were placed in TRI reagent (Molecular Research Center, Cincinnati, OH) and homogenized. Total RNA was isolated according to the manufacturer's instructions. Isolated RNA was analyzed using a nanodrop spectrophotometer (Thermo Scientific, Waltham, MA) to determine quantity and quality. iScript reverse transcription supermix for RT-qPCR (Bio-Rad Laboratories, Hercules, CA) was used to prepare cDNA from 1 µg of RNA. The expression levels of target genes were determined using specific DNA oligos and SYBR green PCR master mix (Applied Biosystems, Foster City CA) on a Step-One real-time PCR system (Applied Biosystems). Copy number was determined by comparison to standard curves of the respective genes generated from pooled cDNA. Target gene expression levels were standardized to the geometric mean of glyceraldehyde-3-phosphate dehydrogenase (Gapdh), beta-actin (Actb) and hypoxanthine guanine phosphoribosyl transferase (Hprt) gene expression. To evaluate the expression of target genes the following PCR primers were used: Gapdh (115 bp), 5'-TCAACAGCAACTCCCACTCTTCCA-3' (forward), 5'-AC CCTGTTGCTGTAGCCGTATTCA-3' (reverse); Actb (140 bp), 5'-TGTGATGGTGGGAA TGGGTCAGAA-3' (forward), 5'-TGTGGTGCCAGATCTTCTCCATGT-3' (reverse); Hprt (133 bp), 5'-GGAGTCCTGTTGATGTTGCCAGTA-3' (forward), 5'-GGGACGCAGCAAC-TGACATTTCTA-3' (reverse); IL-17A, Il17a (101 bp), 5'-TCCAGAAGGCCCTCAGAC-TA-3' (forward), 5'-TGAGCTTCCCAGATCACAGA-3' (reverse); IL-22, Il22 (107 bp) 5'-GCTCAGCTCCTGTC-ACATCA-3' (forward), 5'-TCGCCTTGATCTCTCCACTC-3' (reverse); intercellular adhesion molecule 1, Icam1 (160 bp) 5'-AGATCACATTCACGG-TGCTGGCTA-3' (forward), 5'-AGCTTTGGGATGGTAGCTGGAAGA-3' (reverse);

perforin, Prf1 (155 bp), 5'-AGCCAGCGTCTCCAGTGAATACAA-3' (forward), 5'-TAGCT-TGGTTCCCGAAGAGCAGAT-3' (reverse); granzyme B, Gzmb (127 bp), 5'-AGAATGTT TGCATTGGAGCTGGGC-3' (forward), 5'-ACATCAGCAACTTGGGTGC AACTG-3' (reverse); Fas receptor, Fas (100 bp), 5'-AGTTTAAAGCTGAGGAGGCGGGTT-3' (forward), 5'-TTTCAGGTTG-GCATGGTTGACAGC-3' (reverse). Data are reported as fold-change of standardized treatment over standardized Vehicle/Saline or Vehicle/Con A at 0 h for time course studies.

Flow cytometry

Flow cytometry was performed on hepatic leukocytes isolated and prepared as follows. Mouse livers were collected into RPMI medium supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The livers were passed through a nylon mesh, and the cell suspension was centrifuged at 50xg for 5 min to remove hepatocytes. The remaining leukocytes were centrifuged at 450xg and incubated with red cell lysis buffer (BioLegend, San Diego, CA) for 3 min followed by 2 washes with phosphate buffered saline supplemented with 5% FBS. The leukocyte cell suspension was separated using lympholyte-M (Cedar Lane, Burlington, Ontario, Canada) according to the manufacturer's instructions. The prepared hepatic leukocytes were first stained with TruStain FcX (anti-mouse CD16/CD32) to avoid nonspecific staining of Fcγ receptors. Leukocytes were then stained with fluorescein isothiocyanate-conjugated anti-NK1.1 (PK136) and allophycocyanin-cyanine dye 7-conjugated anti-CD3epsilon (145-2c11). In some experiments cells were also stained with phycoerythrin-conjugated anti-Fas Ligand (MFL3), pacific blue-conjugated anti-CD69 (H1.2F3) or pacific blue-conjugated anti-CD25 (PC61). Intracellular perforin staining

was performed using phycoerythrin-conjugated anti-perforin (eBioOMAK-D) (ebiosciences, San Diego, CA) and BD Cytofix/Cytoperm kit (Becton Dickinson, Franklin Lakes, NJ). Intracellular staining requires incubation of the leukocytes in culture with protein transport inhibitor cocktail (ebiosciences, San Diego, CA) after isolation; accordingly leukocytes were isolated at an earlier time point than cells collected for other flow cytometry measurements. Appropriate fluorescent-conjugated isotype controls were used to establish positive and negative gating parameters. All reagents and antibodies for flow cytometry staining were obtained from BioLegend (San Diego, CA) unless otherwise indicated. Staining was performed according to manufacturer's directions, and samples were analyzed on a BD FACSCanto II with data analysis performed using Kaluza software (Beckman Coulter, Brea, CA).

Statistical analyses

Results are expressed as mean $\pm$ S.E.M. Percentile data were subjected to arcsine transformation. When necessary, data were normalized by Box-Cox transformation using R-stats server (R Foundation for Statistical Computing, Vienna, Austria). Analysis of data was performed using either student's *t*-test or two-way analysis of variance (ANOVA) followed by pairwise multiple comparisons using the Student-Newman-Keuls or Tukey's method where appropriate. Non-parametric data were analyzed using Kruskal-Wallis test followed by pairwise multiple comparisons using Tukey's or Dunn's method where appropriate. The criterion for statistical significance was $p<0.05$.

2.4 Results

The effect of TCDD pretreatment on sensitivity of mice to Con A-induced hepatotoxicity.

To determine the effect of TCDD pretreatment on Con A-induced liver injury, mice were treated with vehicle (olive oil) or 30 μg/kg TCDD 96 h (4 days) before the administration of increasing doses of Con A (0-10 mg/kg) and euthanized 24 h later. This dosing protocol was chosen based on the observation that, after administration of 30 μg/kg of TCDD, the concentration of TCDD in the liver increases through 72 h (Boverhof *et al.*, 2005). Accordingly, 96 h represents a time close to the maximum accumulation of TCDD in the liver. As shown in Fig. 3A, TCDD treatment alone was not hepatotoxic at the dose employed. Con A at doses of 6 or 10 mg/kg in vehicle-pretreated mice resulted in a moderate elevation of ALT activity in plasma. In mice pretreated with TCDD, sensitivity to Con A was increased such that 4 mg/kg Con A caused a significant increase in ALT activity in plasma. Furthermore, TCDD-pretreated mice had greater ALT activity compared to vehicle-pretreated mice at all Con A doses from 4 to 10 mg/kg. A dose of 6 mg/kg Con A was chosen for all subsequent studies because larger doses sometimes led to death before 24 h in the TCDD-pretreated mice. In preliminary studies, we demonstrated that TCDD pretreatment at 3 μg/kg also sensitized mice to Con A-induced hepatitis: ALT activity in plasma of mice treated with Vehicle/Con A was 171 ± 100 U/L, and in mice treated with 3 μg/kg TCDD/Con A it was 2479 ± 1617 U/L). However, at the smaller dose of TCDD the response was more variable than at the 30 μg/kg dose used in the rest of this study.

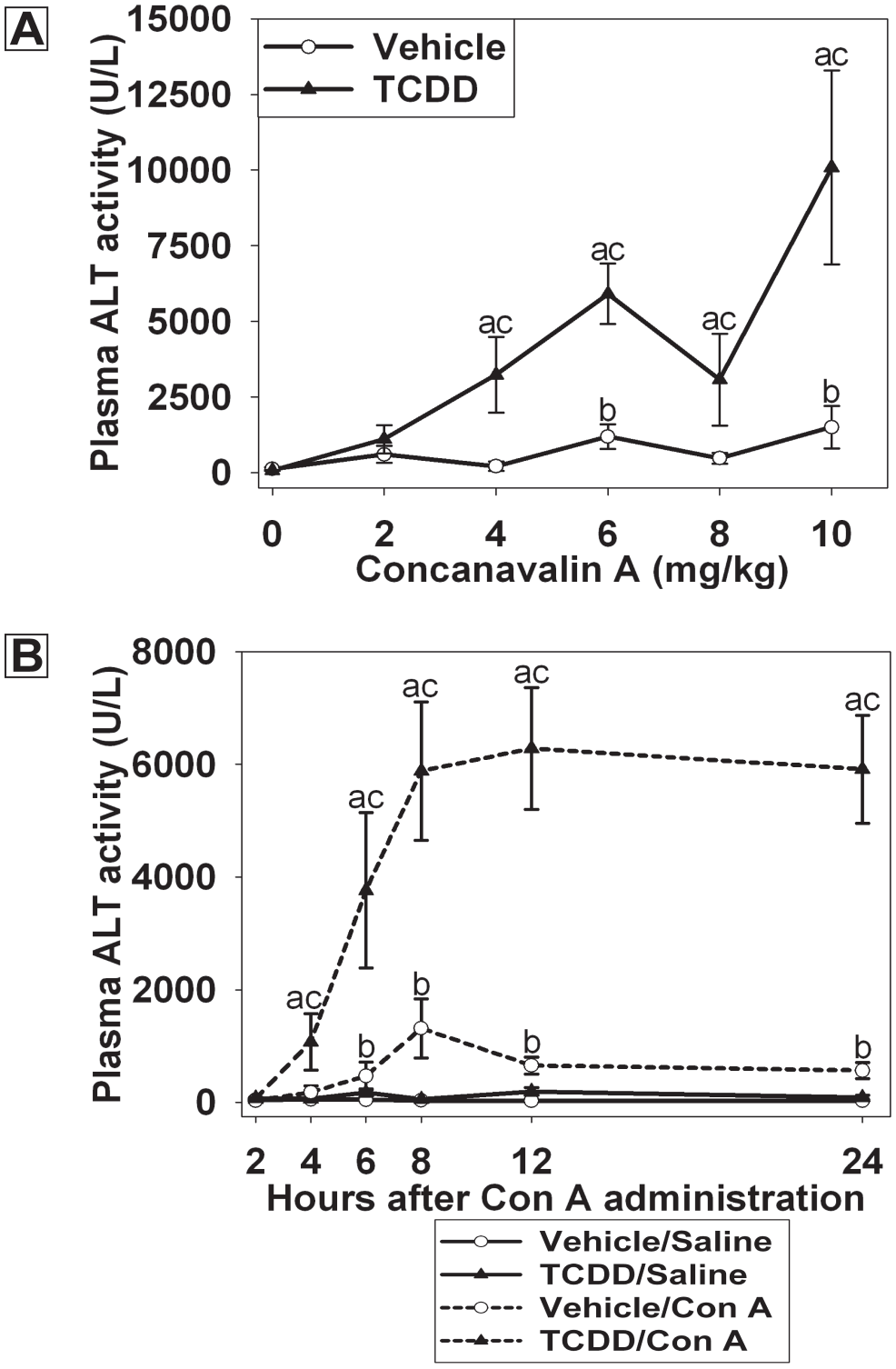


Figure 3: Dose-response and time course of Con A-induced liver injury in the presence and absence of TCDD. (A) Mice were treated on Day 0 with either 30 μg/kg

Figure 3 (cont'd)

TCDD or vehicle. Four days later mice received Con A at doses from 0 to 10 mg/kg. ALT activity in plasma was measured 24 h after Con A administration. a $p < 0.05$ TCDD/Con A versus TCDD/Saline. b $p < 0.05$ Vehicle/Con A versus Vehicle. c $p < 0.05$ TCDD/Con A versus Vehicle/Con A at the same dose. Data represent the mean ± SE of independent replicates from 2 separate experiments. At each dose: Vehicle/Con A n=3-5 and TCDD/Con A n=3-5. (B) Mice were treated with vehicle (open circle) or 30 μg/kg TCDD (black triangle) on day 0. On day 4, pretreated mice were administered saline (solid line) or 6 mg/kg Con A (dashed line). Plasma was collected at the times indicated, and ALT activity was determined. a $p < 0.05$ TCDD/Con A versus TCDD/Saline at the same time point. b $p < 0.05$ Vehicle/Con A versus Vehicle/Saline at the same time point. c $p < 0.05$ TCDD/Con A versus Vehicle/Con A at the same time point. Data represent the mean ± SE of independent replicates from 2 separate experiments. At each time point: Vehicle/Saline n=4-5, TCDD/Saline n=4-5, Vehicle/Con A n=5-9, and TCDD/Con A n=5-9.

The development of injury in TCDD/Con A-treated mice

TCDD pretreatment alone caused no change in plasma ALT activity compared to vehicle pretreatment throughout the time course (Fig. 3B). Con A caused a moderate increase in ALT activity in plasma by 6 h after administration, and this was sustained through 24 h. In TCDD/Con A-treated mice, plasma ALT activity was increased earlier, at 4 h after Con A administration, and continued to increase through 8h then maintained at a plateau through 24 h. ALT activity in TCDD/Con A-treated mice was increased compared to all other groups at all times from 4 to 24 h. Histopathological examination of the liver sections (Fig. 4) revealed that TCDD pretreatment caused no hepatocellular necrosis but increased the appearance of intermittent foci of leukocytes compared to the livers of vehicle-pretreated mice. In Vehicle/Con A-treated and TCDD/Con A-treated mice, histopathology results reflected the plasma ALT activity. Vehicle/Con A treatment resulted in focal areas of necrosis and leukocyte accumulation that were more common near the periphery of the liver lobules. In TCDD/Con A-cotreated mice, there were extensive areas of midzonal hepatocellular necrosis with infiltration of leukocytes in the necrotic regions. In addition, although not easily seen in the images shown in Fig. 2, foci of infiltrating leukocytes were observed in both Vehicle/Con A and TCDD/Con A that were not localized to areas of hepatocellular necrosis.

The effect of TCDD pretreatment on the inflammatory cytokine response to Con A administration

Inflammatory cytokines were assessed at times previously reported to be the peak plasma concentrations following Con A administration (Sass *et al.*, 2002). TCDD pretreatment alone did not cause an increase in plasma IL-6 concentration (Fig. 5A).

The concentration of IL-6 in plasma was increased at 1.5 h after Con A administration and remained elevated at 4.5 h. The plasma concentrations of TNFα and IL-10 were not detectable in either Vehicle/Saline or TCDD/Saline control groups (Fig. 5B and C). Concentrations of TNFα and IL-10 were increased at 1.5 h after Con A administration and had returned toward baseline by 4.5 h. The increase in these three cytokines by Con A was not altered by TCDD pretreatment. The plasma concentrations of Th1- and Th2-related cytokines, IL-2 and IL-4, respectively, were increased by Con A at 1.5 h; this effect was reduced by TCDD pretreatment (Fig. 5D and E). Con A administration resulted in increased concentration of IFNγ in plasma at 1.5, 4.5, and 8 h after treatment, and at 8 h pretreatment with TCDD enhanced this effect (Fig. 5F). Because of the well-characterized role of Th17 cells in immune modulation following AhR activation (Veldhoen *et al.*, 2008), the hepatic expression of IL-17A and IL-22 mRNA (Fig. 6A and B) was assessed. Hepatic IL-17A expression was increased after Con A administration, and TCDD pretreatment did not alter that induction. TCDD pretreatment alone increased the hepatic expression of IL-22 mRNA in the absence of Con A. Con A treatment led to a significant increase in IL-22 mRNA by 6 h, and this was not affected by TCDD pretreatment.

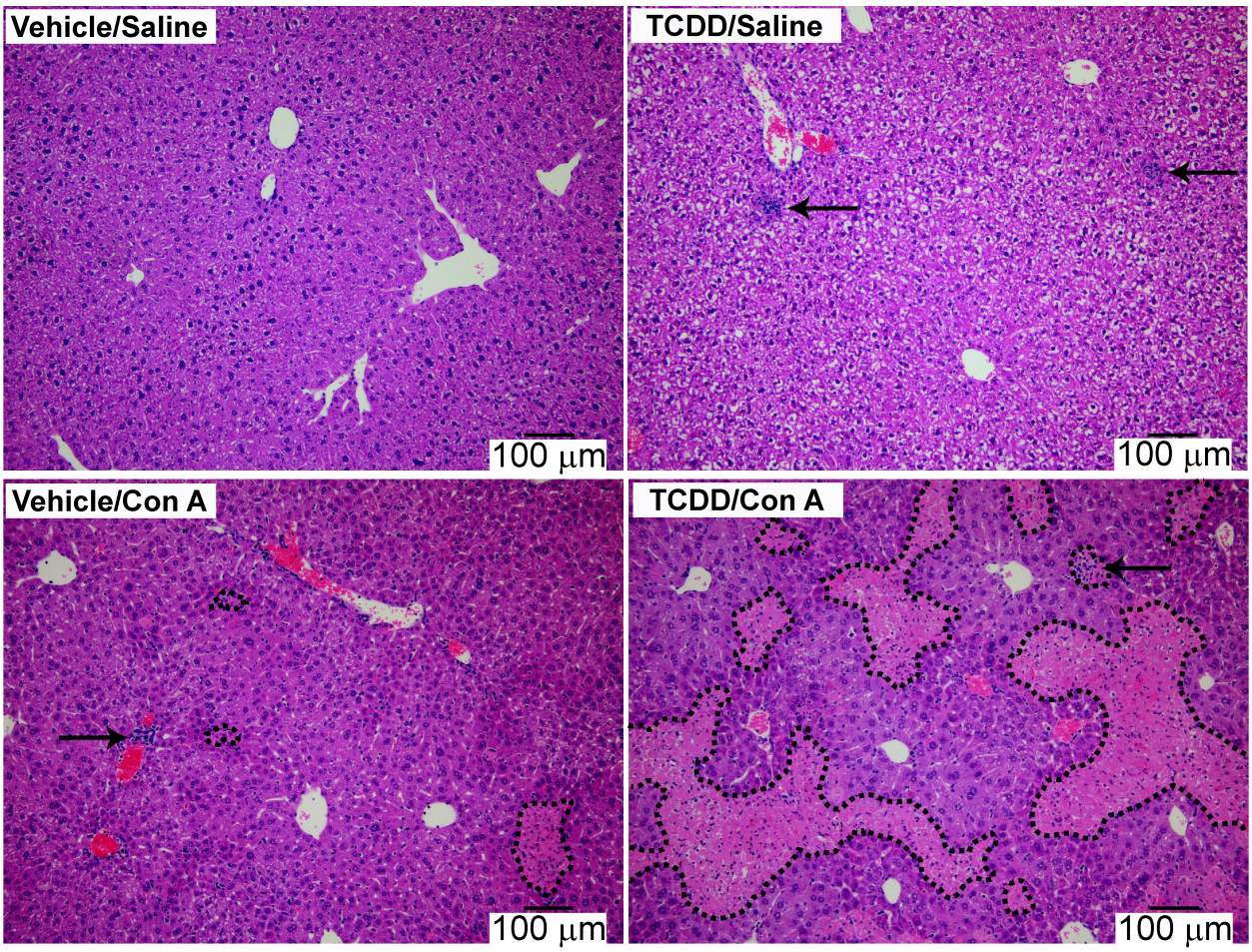


Figure 4: Histopathology of TCDD/Con A-induced liver injury. Mice were treated on day 0 with vehicle or 30 μg/kg TCDD and on day 4 with Saline or 6 mg/kg Con A. Liver sections were collected 24 h after Saline or Con A administration. H&E stained sections were photographed at 10x magnification. Dotted lines mark necrotic areas. Arrows indicate foci of leukocyte infiltration. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

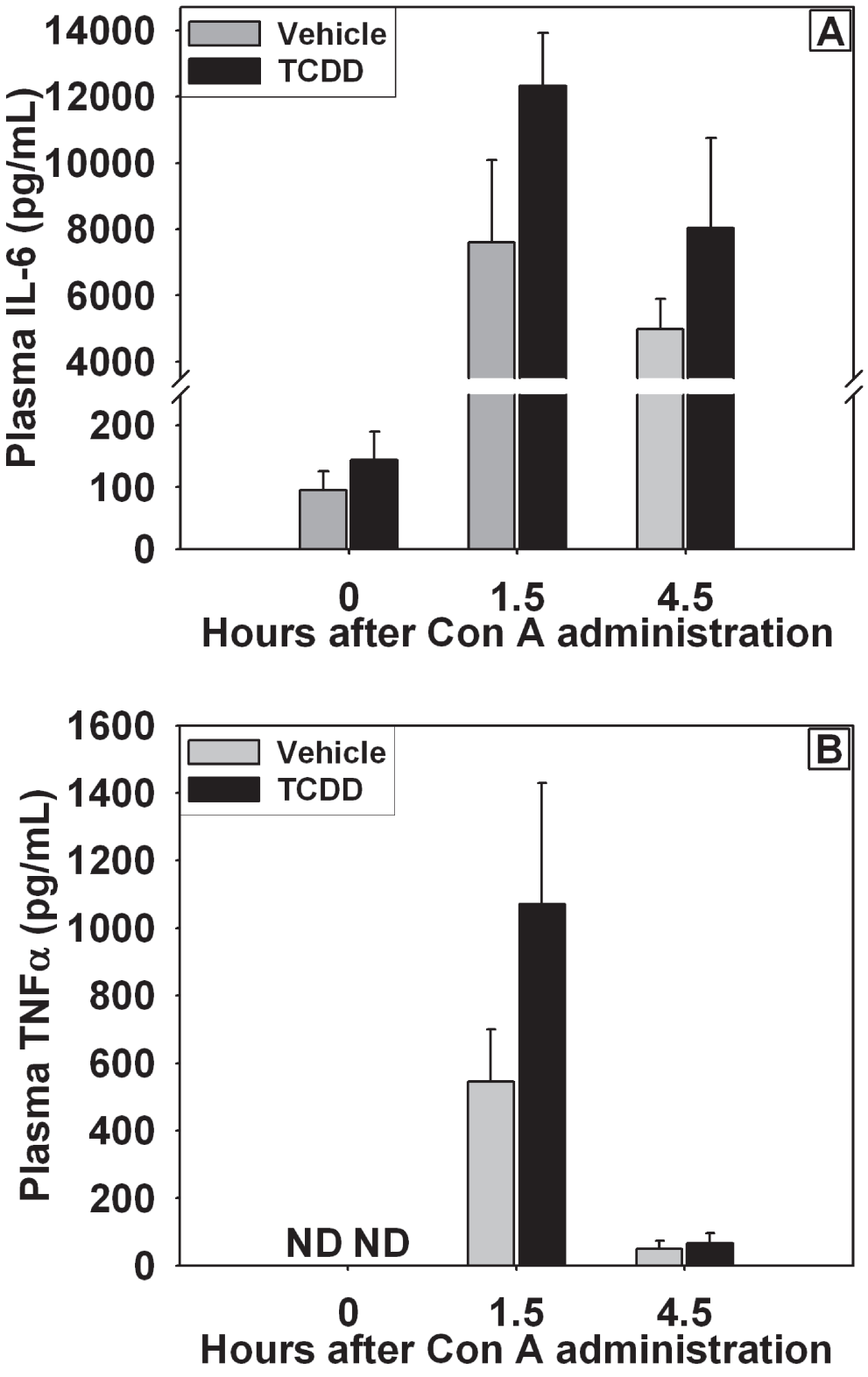
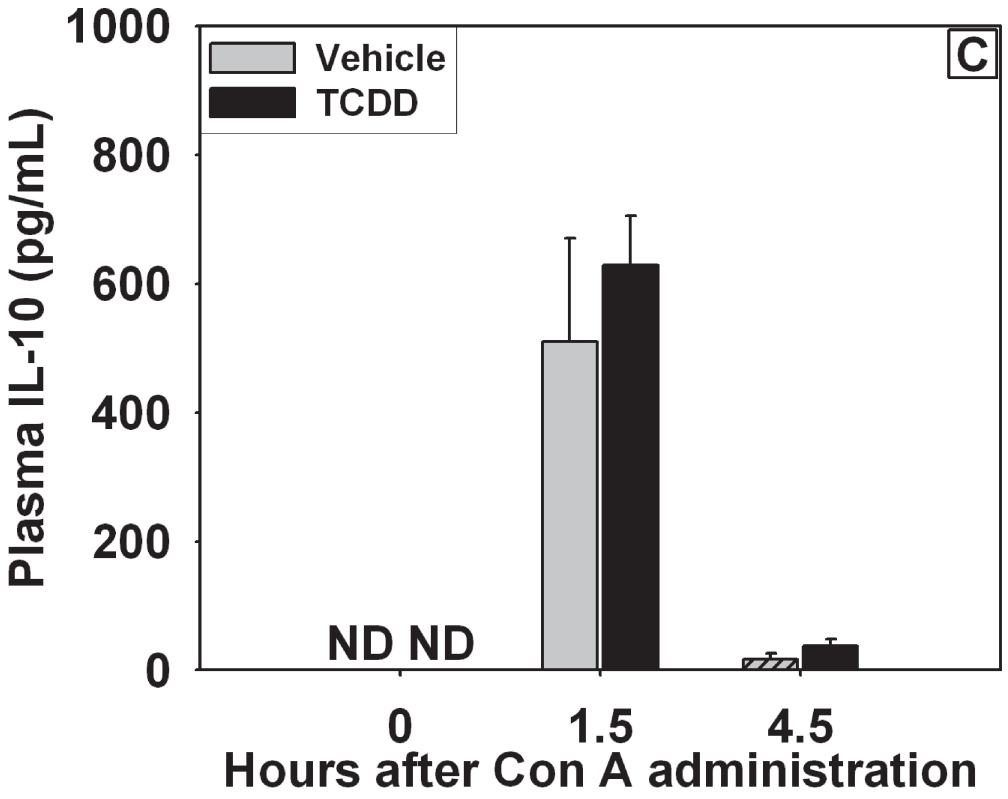


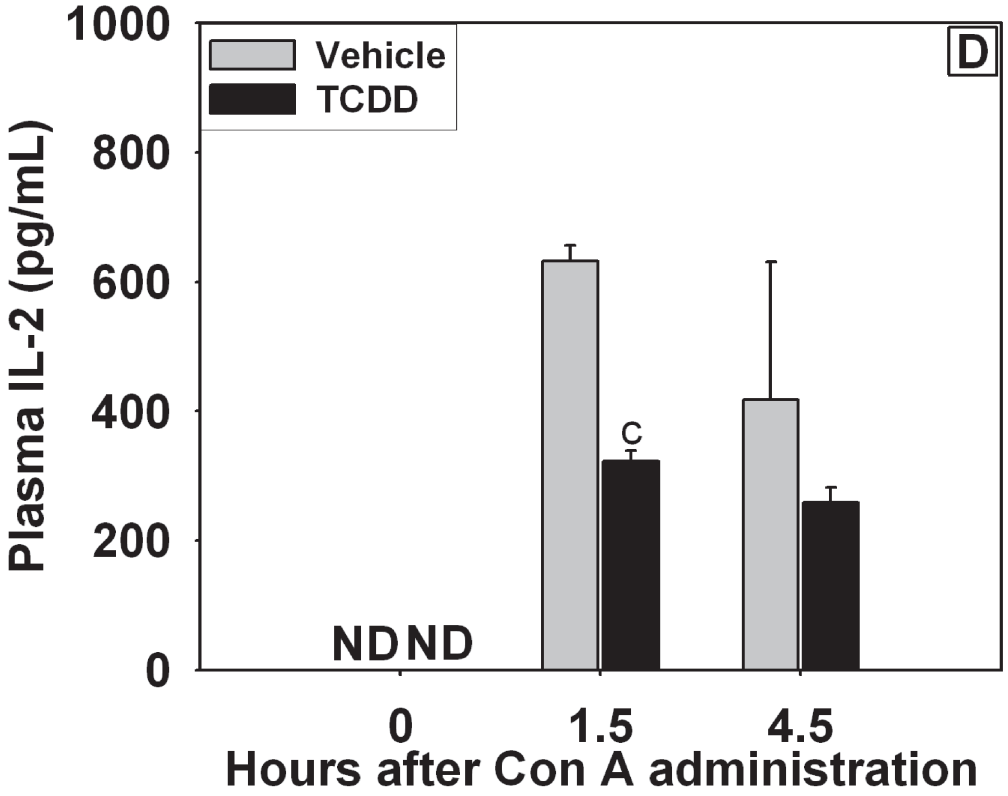
Figure 5: Concentrations of IL-6 (A), TNFα (B), IL-10 (C), IL-2 (D), IL-4 (E), and IFNγ (F) in plasma after Con A administration. Mice were treated as described in the

Figure 5 (cont'd)

legend to Figure 4 with Vehicle/Con A (gray bars) or TCDD/Con A (black bars). **c** $p < 0.05$ Vehicle/Con A versus TCDD/Con A at the same time point. ND= Not Detected (value below the limit of detection). Data represent the mean ± SE of independent replicates from 2 separate experiments. At each time point: Vehicle/Con A n=3-5 and TCDD/Con A n=3-5.

Figure 5 (cont'd)





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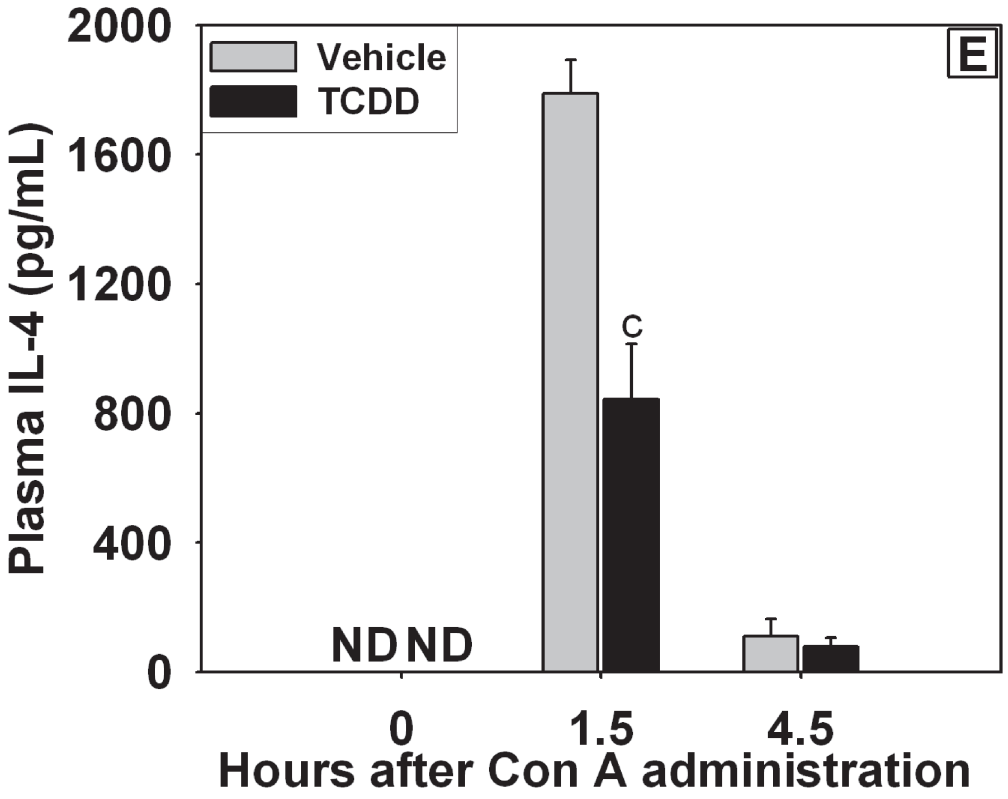
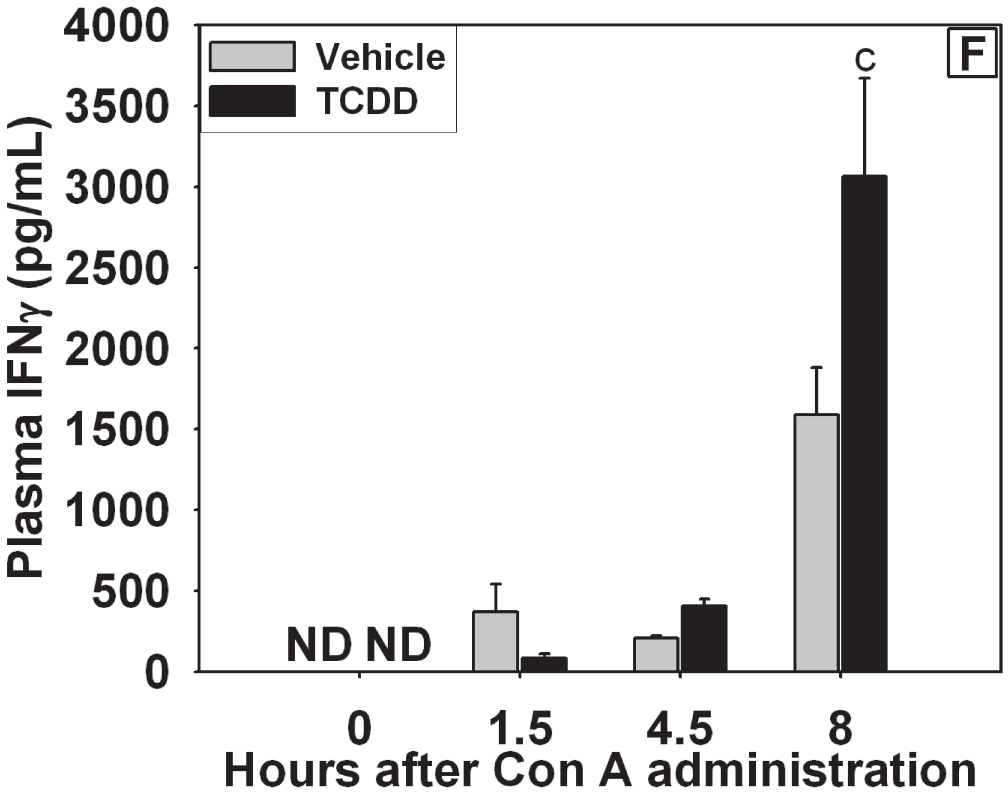




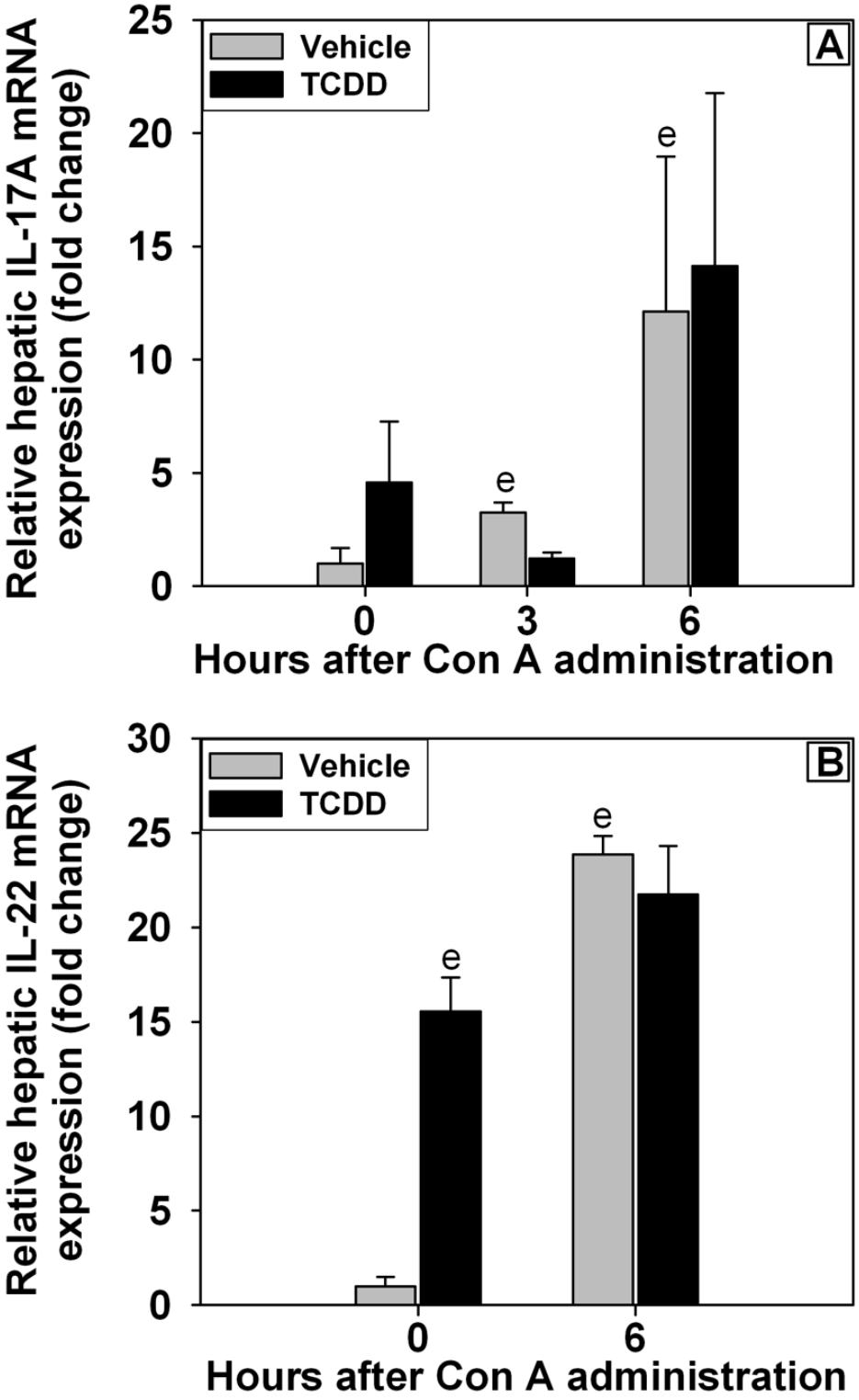


Figure 6: Expression of IL-17A (A) and IL-22 (B) mRNA in liver after Con A administration. Mice were treated as described in the legend to Figure 4 with

Figure 6 (cont'd)

Vehicle/Con A (gray bars) or TCDD/Con A (black bars). e $p < 0.05$ versus Vehicle treatment at 0 h. Data represent the mean ± SE of independent replicates from 2 separate experiments. At each time point: Vehicle/Con A n=4-6 and TCDD/Con A n=4-6.

The role of IFNγ in the development of TCDD/Con A-induced liver injury

Since TCDD enhanced the Con A-mediated increase in IFNγ in plasma and because previous studies demonstrated the importance of IFNγ in inflammatory liver injury after Con A administration (Kusters *et al.*, 1996), the role of IFNγ in the development of TCDD/Con A-induced injury was investigated using *Ifng*^{tm1Ts} (IFNγ knockout) mice. As shown in Fig. 7A, 24 h after TCDD/Con A-treatment, IFNγ knockout mice had decreased plasma ALT activity compared to wild-type mice. These findings were consistent with observations from histological examination of liver sections in which TCDD/Con A-treated IFNγ knockout mice had less necrosis compared to TCDD/Con A-treated wild-type mice (Fig. 7B).

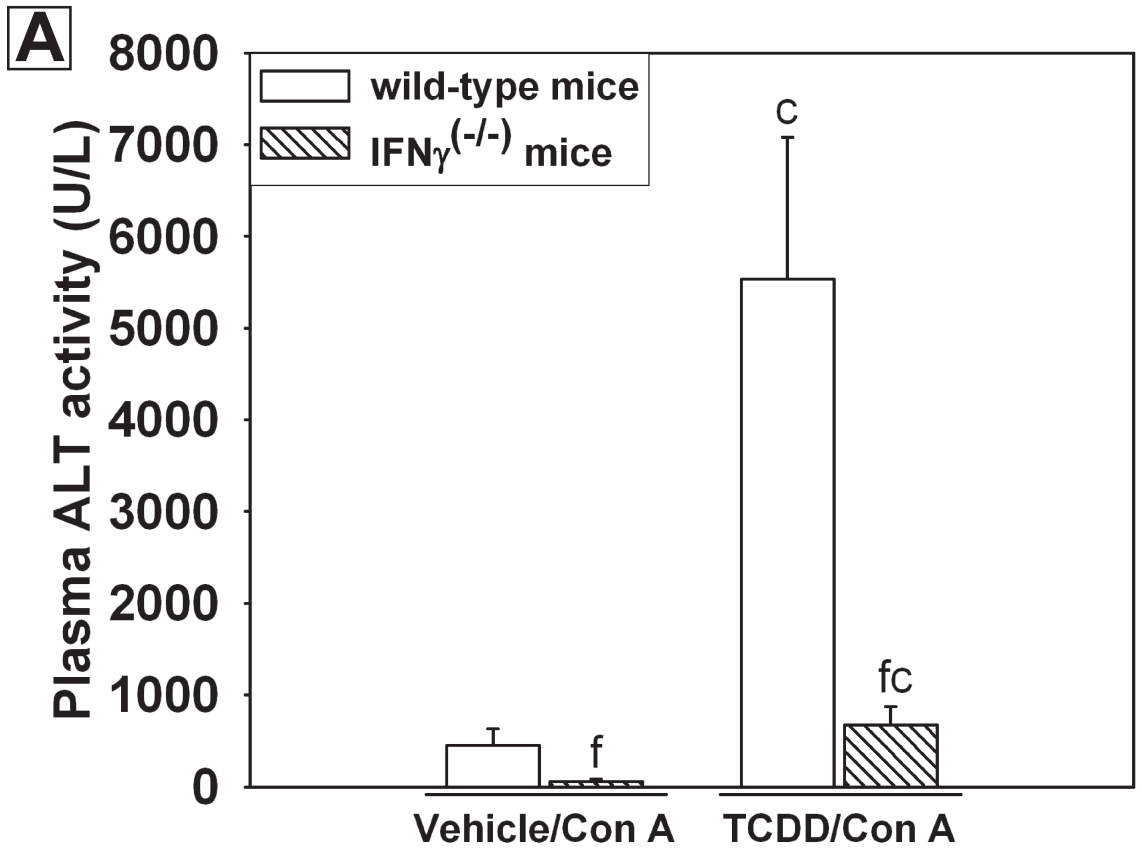


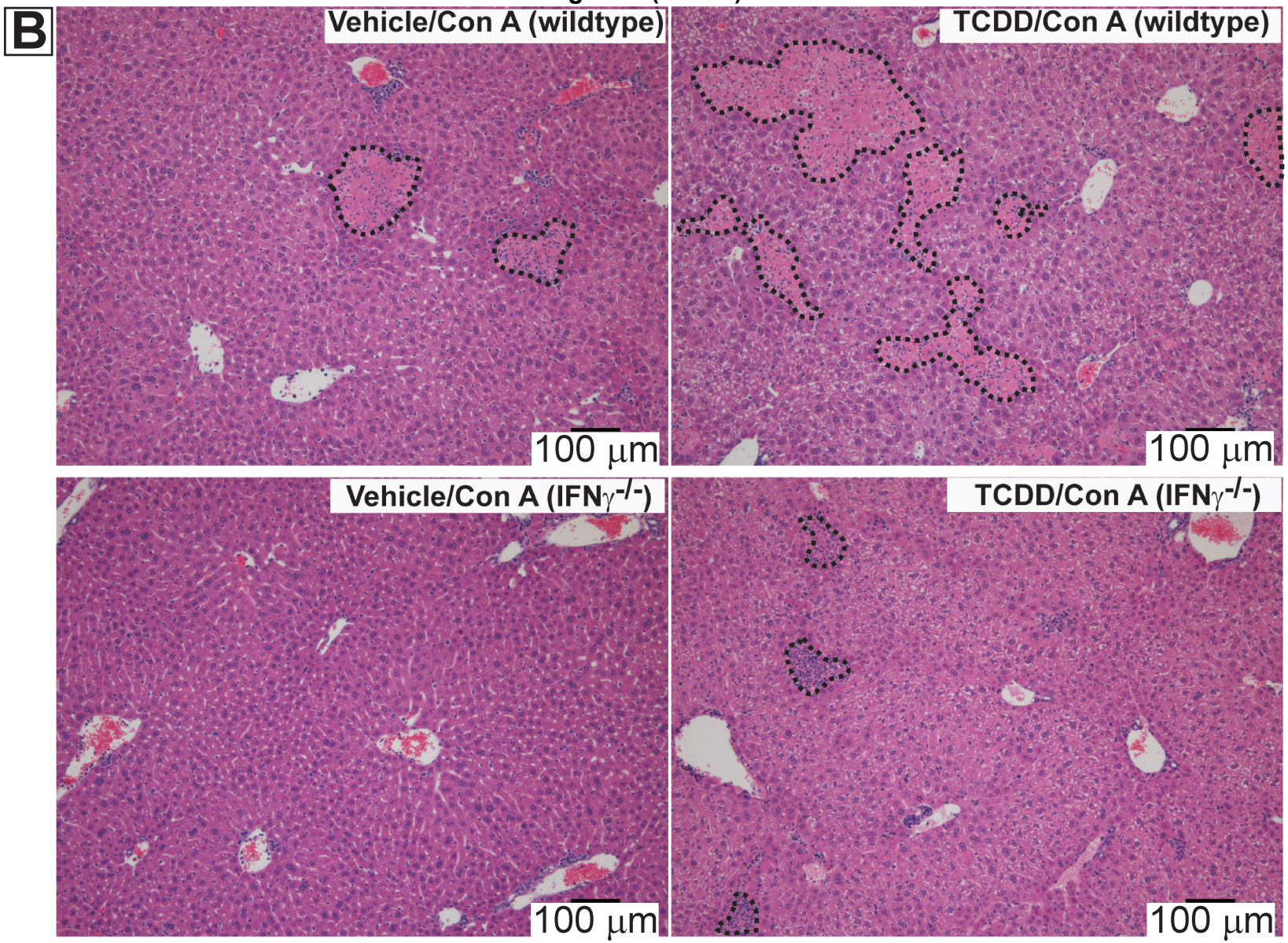
Figure 7: Liver injury after TCDD/Con A treatment in IFNγ$^{(-/-)}$ mice. (A) Wild-type (open bars) or IFNγ knockout (IFNγ$^{(-/-)}$; hatched bars) mice were treated as described in the legend to Figure 4 with Vehicle/Con A or TCDD/Con A, and plasma ALT activity was measured 24 h after Con A administration. c $p < 0.05$ TCDD/Con A versus Vehicle/Con A in the same mouse genotype. f $p < 0.05$ versus the same treatment in the wild-type control. Data represent the mean ± SE of independent replicates. For each genotype: Vehicle/Con A n=7-9, TCDD/Con A n=5-7

(B) Histopathology of TCDD/Con A treatment in *Ifng*^{tm1Ts} (IFNγ knockout) or wild-type mice. Mice were treated on day 0 with vehicle or TCDD and on day 4 with 6 mg/kg Con

Figure 7 (cont'd)

A. Liver sections were collected 24 h after Con A administration. H&E stained sections were photographed at 10x magnification. Dotted lines demonstrate necrotic areas.

Figure 7 (cont'd)



The role of hepatic lymphocytes in TCDD/Con A-induced hepatotoxicity

To determine if the response of hepatic lymphocytes to Con A administration was altered by TCDD pretreatment, lymphocytes were isolated from liver after Con A or saline administration in vehicle or TCDD-pretreated mice. Flow cytometry was used to investigate the composition of hepatic immune cells by staining for expression of NK1.1 and CD3ε to identify natural killer (NK) cells (NK1.1+,CD3ε-), NKT cells (NK1.1+,CD3ε+) and T cells (NK1.1-,CD3ε+). Con A administration caused a decrease in the percentage of NKT cells and increased the percentage of T cells (Fig. 8A and B). TCDD/Saline-treated mice had a smaller percentage of NKT cells in their livers compared to Vehicle/Saline-treated mice. TCDD-pretreatment significantly increased the percentage of T cells observed in the livers of mice 4 h after Con A administration (Fig. 8A and B).

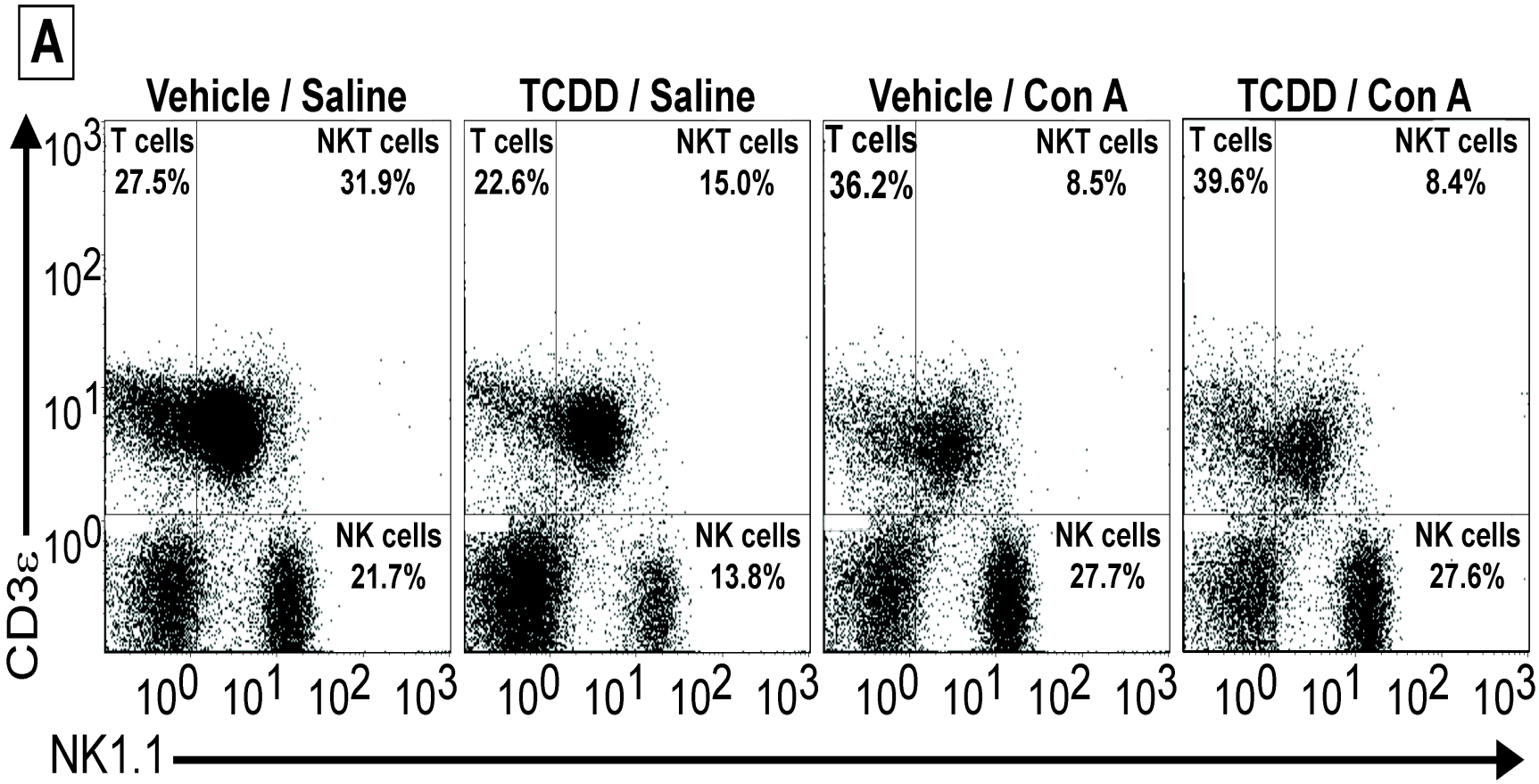
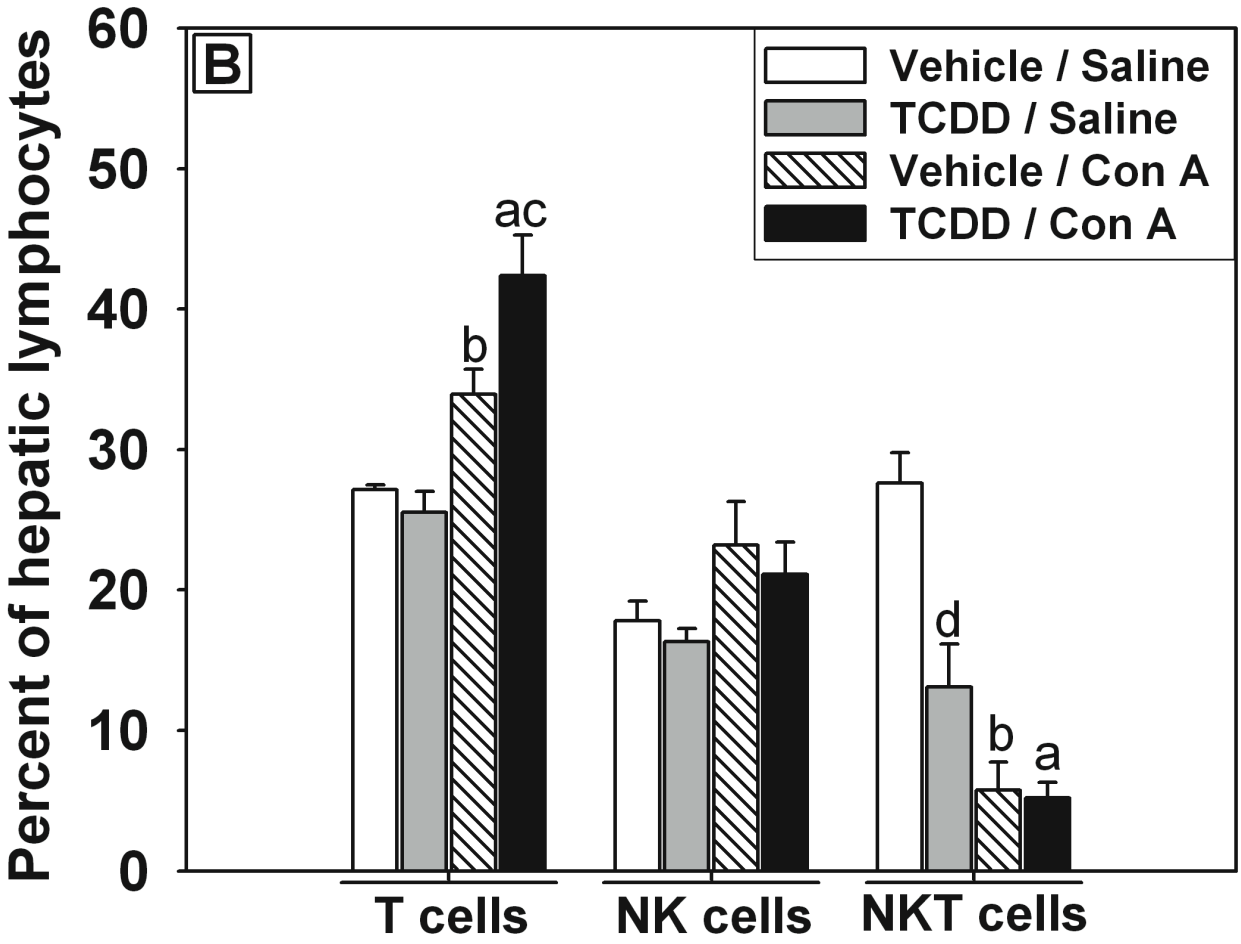


Figure 8: Hepatic lymphocyte populations after treatment with Con A in the presence and absence of TCDD.

Mice were treated as described in the legend to Figure 4 with Vehicle/Saline, TCDD/Saline, Vehicle/Con A or TCDD/Con A. Hepatic lymphocytes were isolated from mice 4 h following Con A or saline administration. Lymphocytes were stained with fluorescently labeled anti-NK1.1 and anti-CD3ε antibodies to determine the relative numbers of NKT cells

Figure 8 (cont'd)

($NK1.1^+$,$CD3\varepsilon^+$), T cells ($NK1.1^-$,$CD3\varepsilon^+$) and NK cells ($NK1.1^+$,$CD3\varepsilon^-$). (A) Representative quadrant plots show gated lymphocyte populations for each treatment group. (B) Percent of T cell, NK cell, and NKT cell populations comprising total isolated hepatic lymphocytes. a $p < 0.05$ TCDD/Con A versus TCDD/Saline. b $p < 0.05$ Vehicle/Con A versus Vehicle/Saline. c $p < 0.05$ TCDD/Con A versus Vehicle/Con A. d $p < 0.05$ TCDD/Saline versus Vehicle/Saline. Data represent the mean ± SE of independent replicates from 3 separate experiments. For each cell type: Vehicle/Saline n=4, TCDD/Saline n=6, Vehicle/Con A n=4, TCDD/Con A n=6.



Activation status of these cell types was also assessed. Hepatic lymphocytes were first gated as described previously to identify NK, NKT and T cells. Then NKT cells were further gated based on staining for expression of the early lymphocyte activation marker CD69. Positive and negative gating parameters were set using samples stained with appropriately labeled fluorochrome-conjugated isotype control antibodies. The percentage of activated hepatic NKT cells, as detected by expression of CD69, was increased by TCDD/Saline treatment compared to Vehicle/Saline treatment at all times examined (Fig. 9A and B). A similar increase in NKT cells expressing CD69 was detected in the Vehicle/Con A-treatment group (Fig. 9B). The percentage of CD69-positive NKT cells in the TCDD/Con A-treatment group was significantly increased compared to all other groups at 2, 3 and 4 h after Con A administration. TCDD/Saline treatment did not increase the percentage of T cells expressing CD69 compared to Vehicle/Saline, and administration of Con A resulted in similar increases in the percentage of T cells positively stained for CD69 in both Vehicle- and TCDD- pretreated groups (data not shown). In addition, the proportion of NKT cells expressing the activation marker CD25 was increased at 3 h by TCDD/Saline treatment compared to Vehicle/Saline treatment. Vehicle/Con A treatment resulted in increased CD25-positive NKT cells compared to either Vehicle/Saline- or TCDD/Saline-treatment groups, and the proportion of NKT cells expressing CD25 in the TCDD/Con A-treatment group was increased additively relative to treatment with either agent alone (Fig. 9C). In contrast, no significant increase was observed in CD25-positive T cells due to TCDD treatment alone. Con A administration increased the percentage of CD25-positive T cells in the Vehicle- and TCDD-pretreated groups.

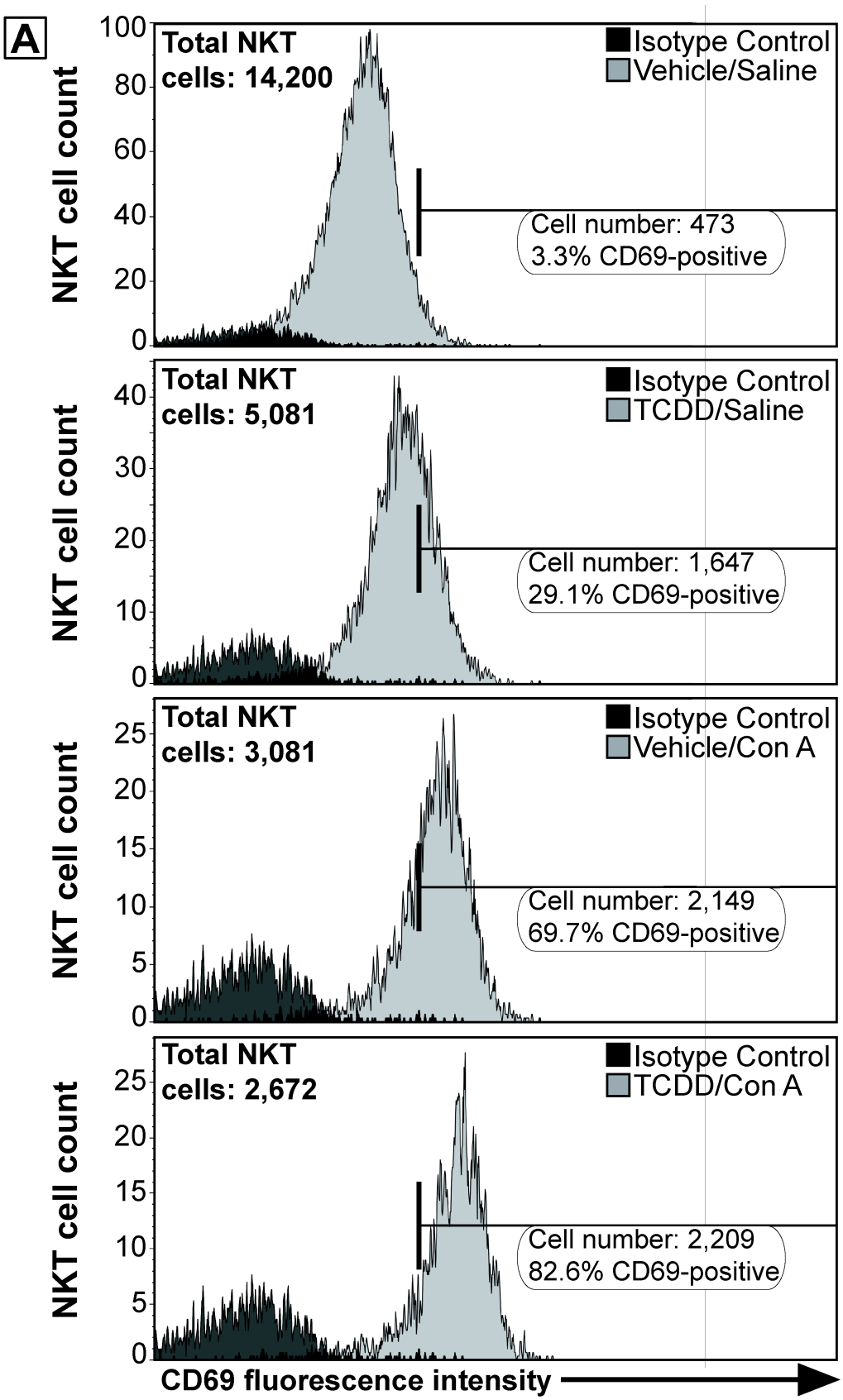
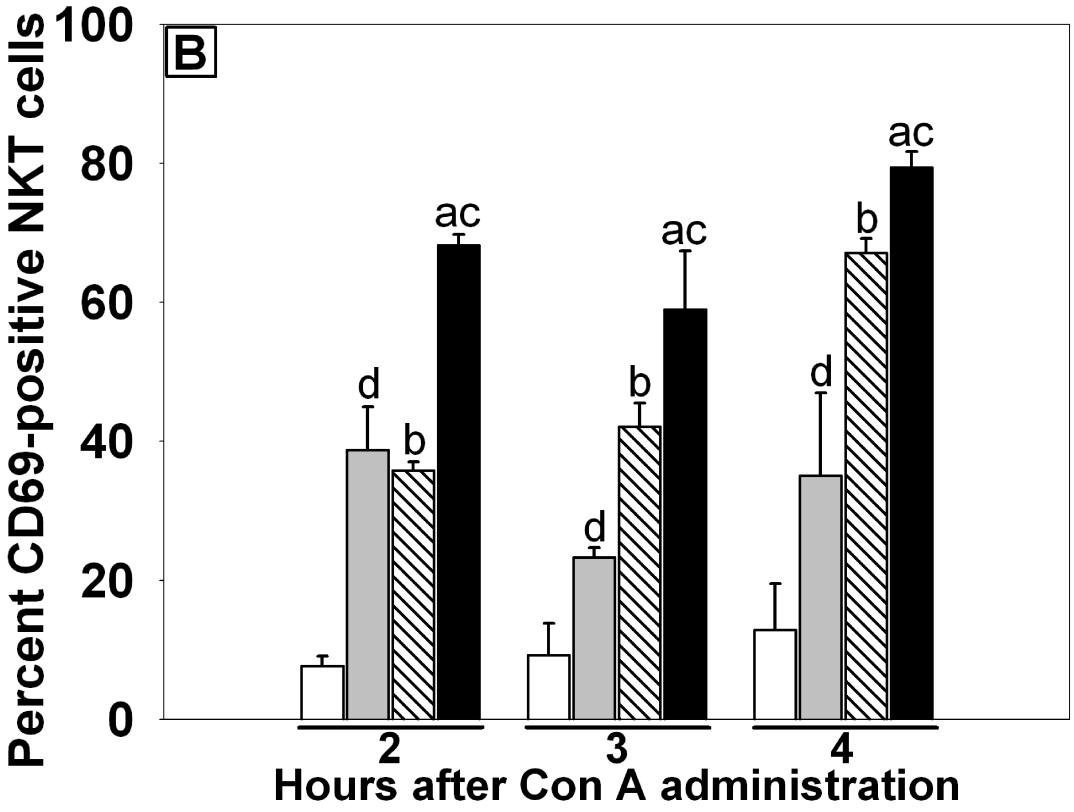


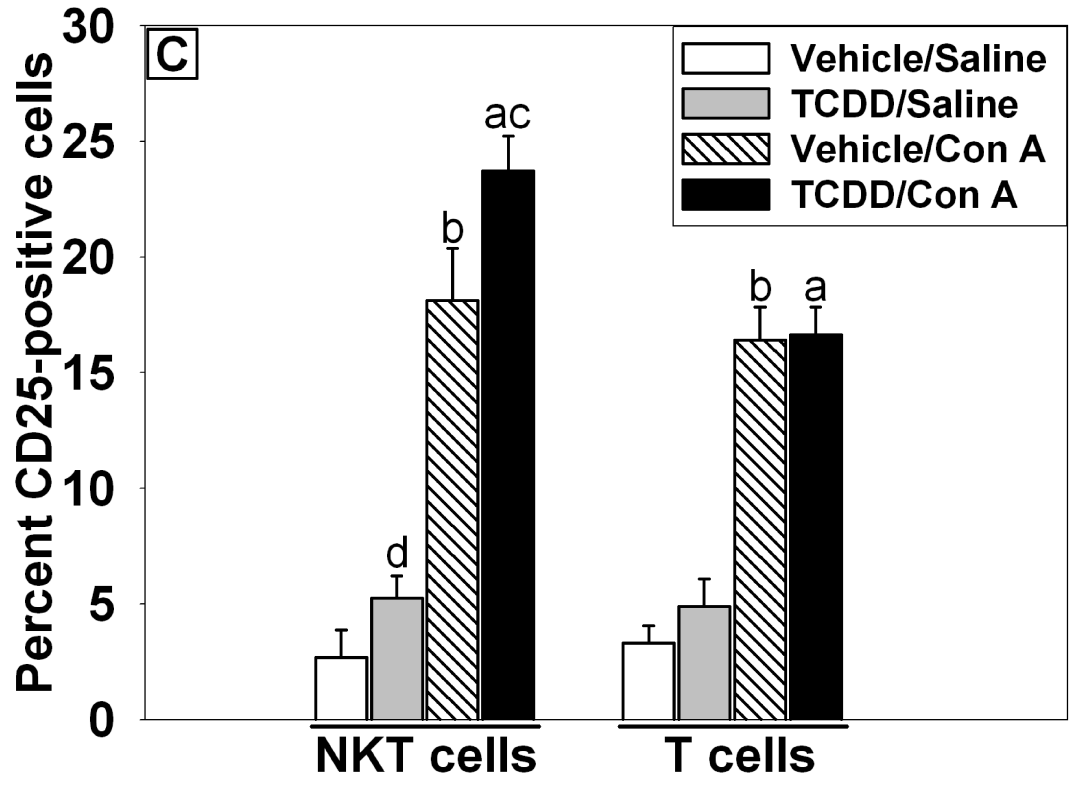
Figure 9: Activation of hepatic lymphocytes after TCDD/Con A treatment.

Figure 9 (cont'd)

Mice were treated as described in the legend to Figure 4 with Vehicle/Saline (white bars), TCDD/Saline (gray bars), Vehicle/Con A (striped bars) or TCDD/Con A (black bars). Hepatic NKT cells ($NK1.1^{+}$,$CD3\varepsilon^{+}$) and T cells ($NK1.1^{-}$,$CD3\varepsilon^{+}$), identified as described in Figure 6, were stained for the lymphocyte activation markers CD69 and CD25. (A) Representative histograms showing fluorescence intensity of CD69 staining on NKT cells 4 h after Con A or Saline administration. Treatments are indicated in the panels. The horizontal bar represents the area of positive staining based on the isotype control. (B) The percent of NKT cells staining positive for CD69 at 2, 3 and 4 h after Con A or Saline administration. a $p < 0.05$ TCDD/Con A versus TCDD/Saline at the same time point. b $p < 0.05$ Vehicle/Con A versus Vehicle/Saline at the same time point. c $p < 0.05$ TCDD/Con A versus Vehicle/Con A at the same time point. d $p < 0.05$ TCDD/Saline versus Vehicle/Saline at the same time point. (C) The percent of NKT and T cells staining positive for CD25 3 h after Con A or Saline administration. a $p < 0.05$ TCDD/Con A versus TCDD/Saline within the same cell type. b $p < 0.05$ Vehicle/Con A versus Vehicle/Saline within the same cell type. c $p < 0.05$ TCDD/Con A versus Vehicle/Con A within the same cell type. d $p < 0.05$ TCDD/Saline versus Vehicle/Saline within the same cell type. Data represent the mean ± SE of independent replicates from 2 separate experiments. For each time point or cell type: Vehicle/Saline n=5, TCDD/Saline n=5, Vehicle/Con A n=3-5, TCDD/Con A n=3-5.

Figure 9 (Cont'd)





Inhibition of immune cell extravasation with CD18 antiserum alters the development of liver injury

CD18 is a component of many cell adhesion integrins including lymphocyte function-associated antigen 1 (LFA-1) and macrophage-1 antigen (Mac-1) that are expressed on leukocytes. Neutralizing the interaction between CD18 and other intracellular adhesion molecules reduces the extravasation of leukocytes from liver sinusoids into parenchymal tissue and prevents direct cell-cell interaction between leukocytes and hepatocytes that is important for cytolytic processes involved in the killing of hepatic parenchymal cells (Klintman *et al.*, 2002). Intracellular adhesion molecule 1 (ICAM-1) is a major target for integrin binding. Hepatic mRNA expression of ICAM-1 was not altered by TCDD/Saline treatment but was increased with Con A treatment at 6 h, and TCDD pretreatment further increased expression in Con A-treated mice (Fig. 10A). The results suggest increased cellular adhesion and cell-cell interaction as a result of these treatments although in this experiment a distinction cannot be made between ICAM-1 expression on parenchymal cells and leukocytes. Treatment with neutralizing antiserum to CD18 attenuated the increase in plasma ALT activity at 24 h in both Vehicle/Con A- and TCDD/Con A-treated mice (Fig. 10B). Administration of CD18 antiserum to Vehicle/Saline-treated mice caused no adverse effects on the liver at the 24 h time point (ALT activity in plasma= 22.3 ± 0.3 U/L). Despite preventing the development of liver injury, CD18 antiserum treatment did not decrease the plasma concentration of IFNγ in TCDD/Con A treated mice (data not shown).

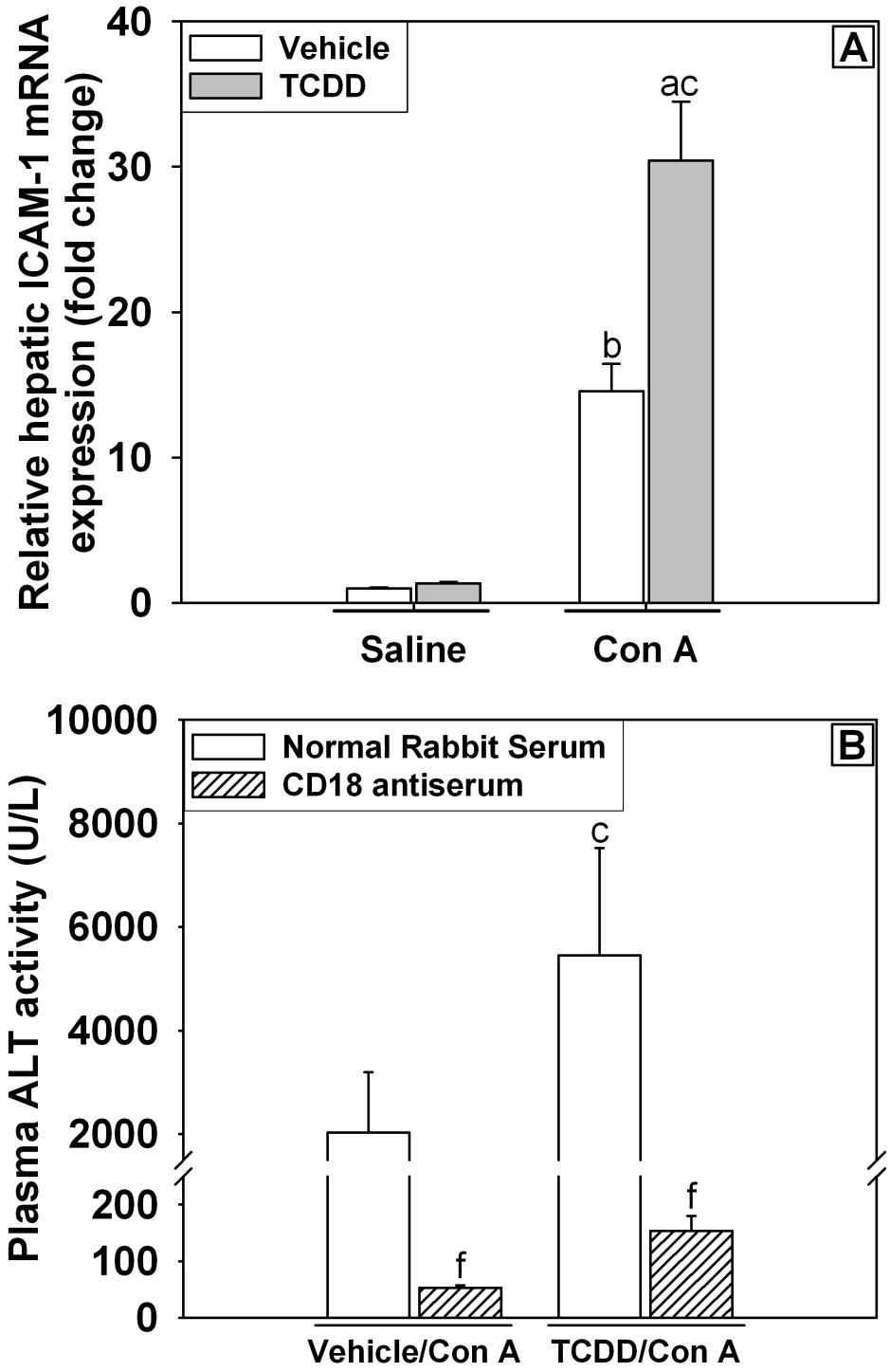


Figure 10: Increased adhesion molecule expression and the effect of neutralization of CD18 on TCDD/Con A-mediated liver injury. (A) Mice were treated as described in the legend to Figure 4 with Vehicle/Saline, TCDD/Saline, Vehicle/Con A

Figure 10 (cont'd)

or TCDD/Con A. Liver samples were collected 6 h after Saline or Con A treatment, and hepatic ICAM-1 mRNA expression was determined by RT-PCR. a $p < 0.05$ TCDD/Con A versus TCDD/Saline. b $p < 0.05$ Vehicle/Con A versus Vehicle/Saline. c $p < 0.05$ TCDD/Con A versus Vehicle/Con A. Data represent the mean ± SE of 6 independent replicates. (B) Mice were treated with vehicle or TCDD on day 0 then administered either CD18 antiserum (striped bars) or normal rabbit serum (NRS)(open bars) 15 h prior to treatment with 6 mg/kg Con A. A second administration of CD18 antiserum or NRS was given 2 h after Con A. ALT activity in plasma was measured 24 h after Con A administration. c $p < 0.05$ TCDD/Con A versus Vehicle/Con A within the same pre-treatment. f $p < 0.05$ versus the same treatment group with normal rabbit serum pre-treatment. Data represent the mean ± SE of independent replicates from 2 separate experiments. For each treatment group: Vehicle/Saline n=6, TCDD/Saline n=6, Vehicle/Con A n=4-6, TCDD/Con A n=4-6.

Cytolytic potential of NKT cells after TCDD/Con A treatment

Perforin and Fas ligand (FasL) are important factors commonly used by NKT cells to kill target cells. The percentage of NKT cells expressing perforin (Fig. 11A) was evaluated 3 h after treatment with Con A. Treatment with either TCDD or Con A increased the percentage of NKT cells expressing perforin, but no additional increase occurred in cotreated mice. The mRNA expression in liver was investigated at 6 h, a time when injury is still developing in both the Vehicle/Con A and TCDD/Con A treatment groups. Hepatic expression of perforin mRNA was increased by Con A treatment at 6 h. TCDD/Saline treatment did not significantly increase perforin expression, and TCDD pretreatment did not alter its expression in Con A-treated mice (Fig. 11B). Hepatic granzyme B expression at 6 h was increased slightly by TCDD/Saline treatment. Compared to Vehicle/Saline and TCDD/Saline, granzyme B expression was significantly increased by both Vehicle/Con A and TCDD/Con A treatments (Fig. 11C). The percentage of NKT cells expressing FasL (Fig. 11D) was evaluated at 4 h after treatment. TCDD/Saline treatment increased the proportion of FasL-positive NKT cells, and a similar increase was observed after Con A treatment. The percentage of NKT cells expressing FasL was greater in the TCDD/Con A-treatment group than in any other treatment group. The hepatic expression of mRNA for Fas receptor (CD95) was increased by Con A at 6 h after treatment, but TCDD pretreatment did not affect the expression in either Saline- or Con A- treated mice (Fig. 11E).

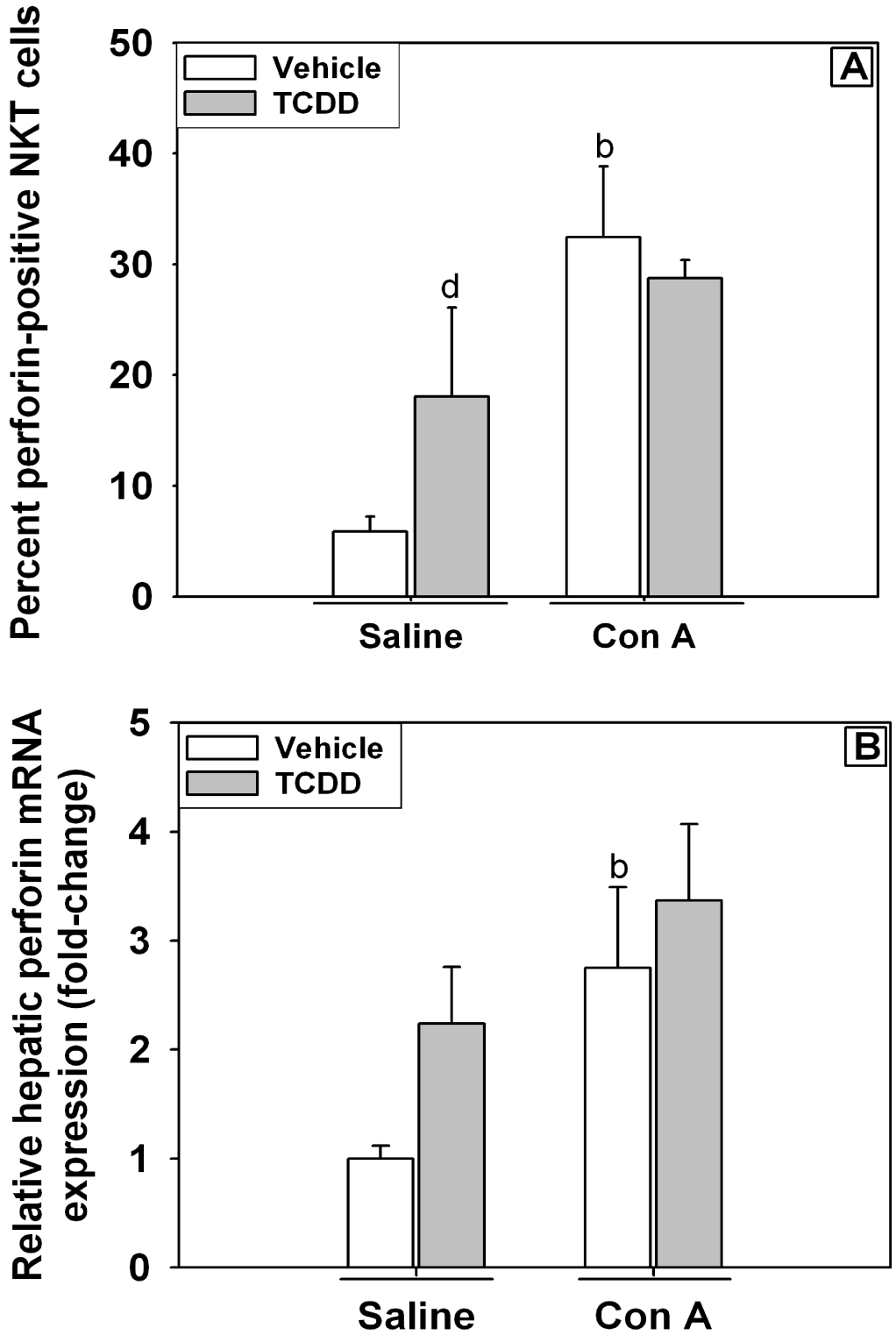
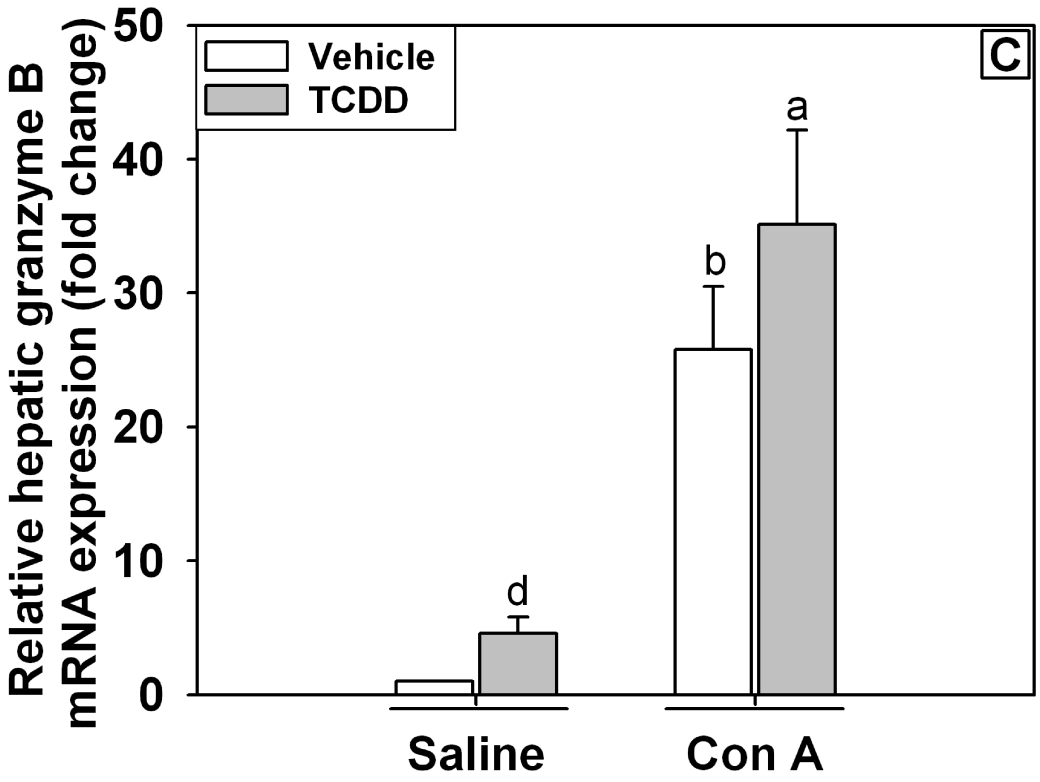


Figure 11: Expression of cytolytic markers on hepatic NKT cells and hepatic expression of perforin, granzyme B and fas in mice treated with TCDD/Con A.

Figure 11 (cont'd)

Mice were treated as described in the legend to Figure 4, and hepatic NKT cells ($NK1.1^+$,$CD3\varepsilon^+$) were isolated from mice and stained for markers of cytolytic activity. (A) NKT cells isolated 3 h after Con A or Saline administrations were stained for intracellular perforin. (D) NKT cells isolated 4 h following Con A or Saline administration were stained for expression of Fas Ligand (CD95). Liver samples were collected 6 h after Saline or Con A treatment, and hepatic perforin (B), granzyme B (C) and Fas (E) mRNA expression was determined by RT-PCR. a $p < 0.05$ TCDD/Con A versus TCDD/Saline. b $p < 0.05$ Vehicle/Con A versus Vehicle/Saline. c $p < 0.05$ TCDD/Con A versus Vehicle/Con A. d $p < 0.05$ TCDD/Saline versus Vehicle/Saline. Data represent the mean ± SE of independent replicates 2 separate experiments. For each treatment group: Vehicle/Saline n=3-6, TCDD/Saline n=3-6, Vehicle/Con A n=3-6, TCDD/Con A n=3-6.

Figure 11 (cont'd)



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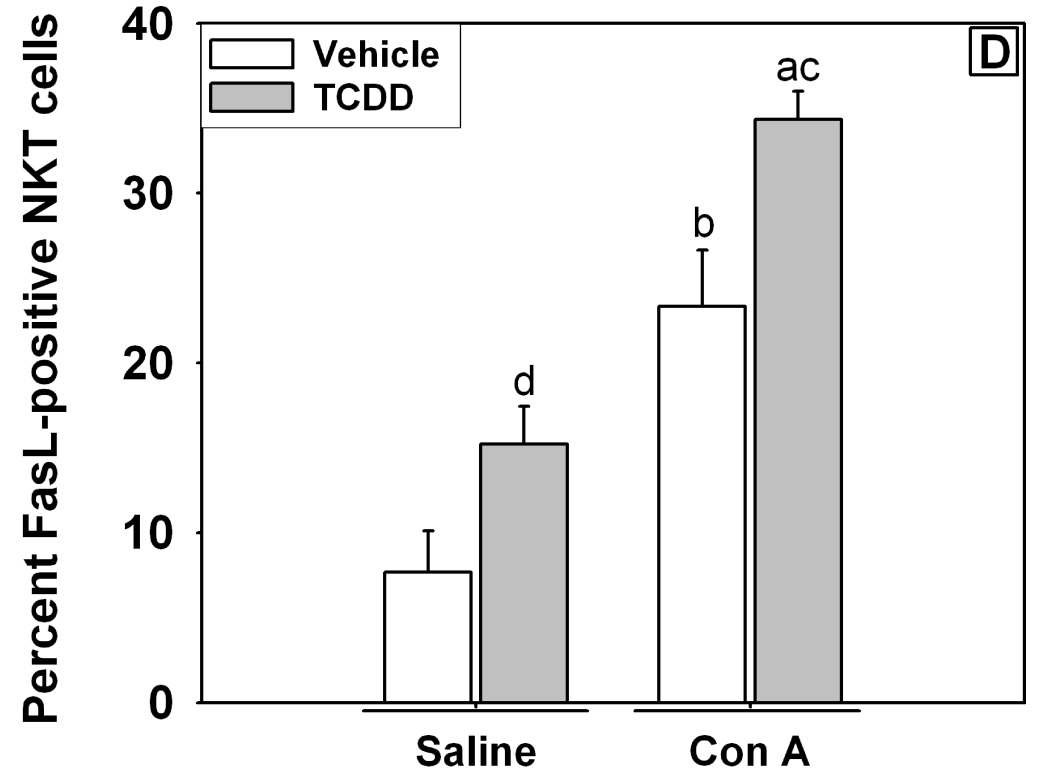
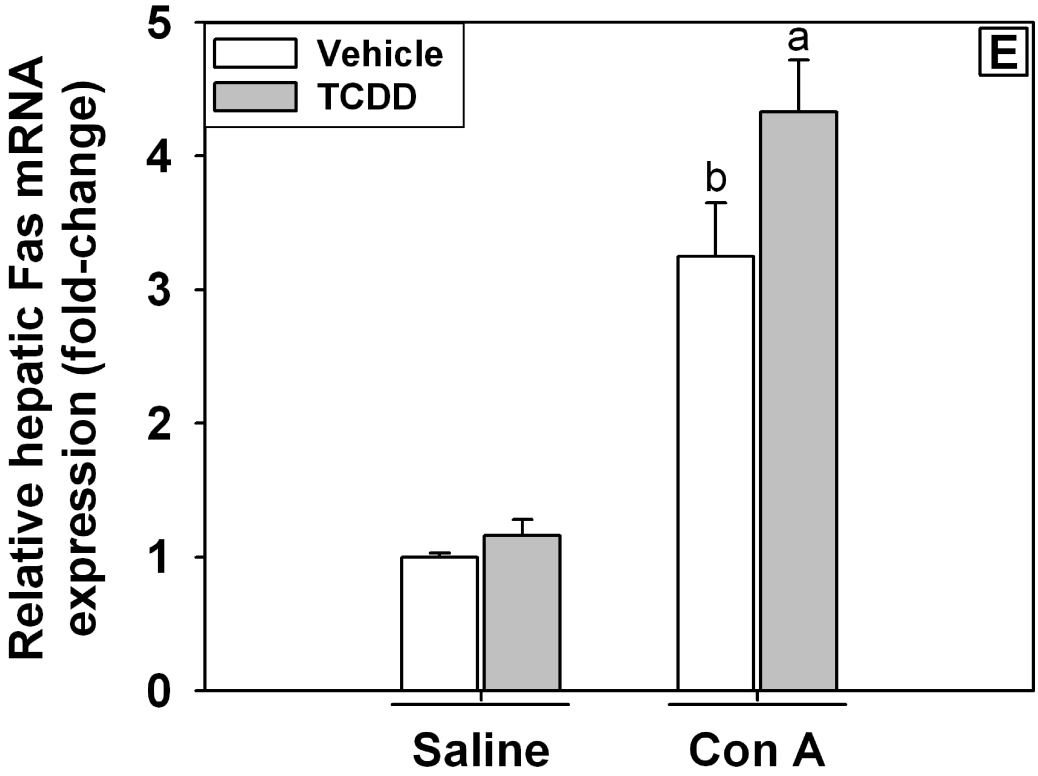




The role of FasL in the development of TCDD/Con A-induced liver injury

B6Smn.C3-Fasl^{gld}/J (*gld/gld*) mice, which possess a mutated form of FasL resulting in an inability to activate the Fas receptor on target cells, and perforin deficient C57BL/6-*Prf1*^{tm1Sdz}/J (*prf1/prf1*) mice were treated with TCDD and Con A as described above. Age-matched C57Bl/6J wild-type mice were used as controls. ALT activity in plasma was measured 24 h after Con A administration. In Vehicle/Con A- and TCDD/Con A-treated groups, *prf1/prf1* mice were not protected from liver injury. However, Vehicle/Con A- and TCDD/Con A-treated *gld/gld* mice had attenuated activity of ALT in plasma compared to wild-type controls (Fig. 12A). The plasma concentrations of IFNγ were also decreased in *gld/gld* mice (640 ± 179 pg/mL) compared to wild-type mice (1,716 ± 108 pg/mL) 8 h after TCDD-Con A-treatment. ALT activity in plasma was consistent with observed histopathological changes (Fig. 12B).

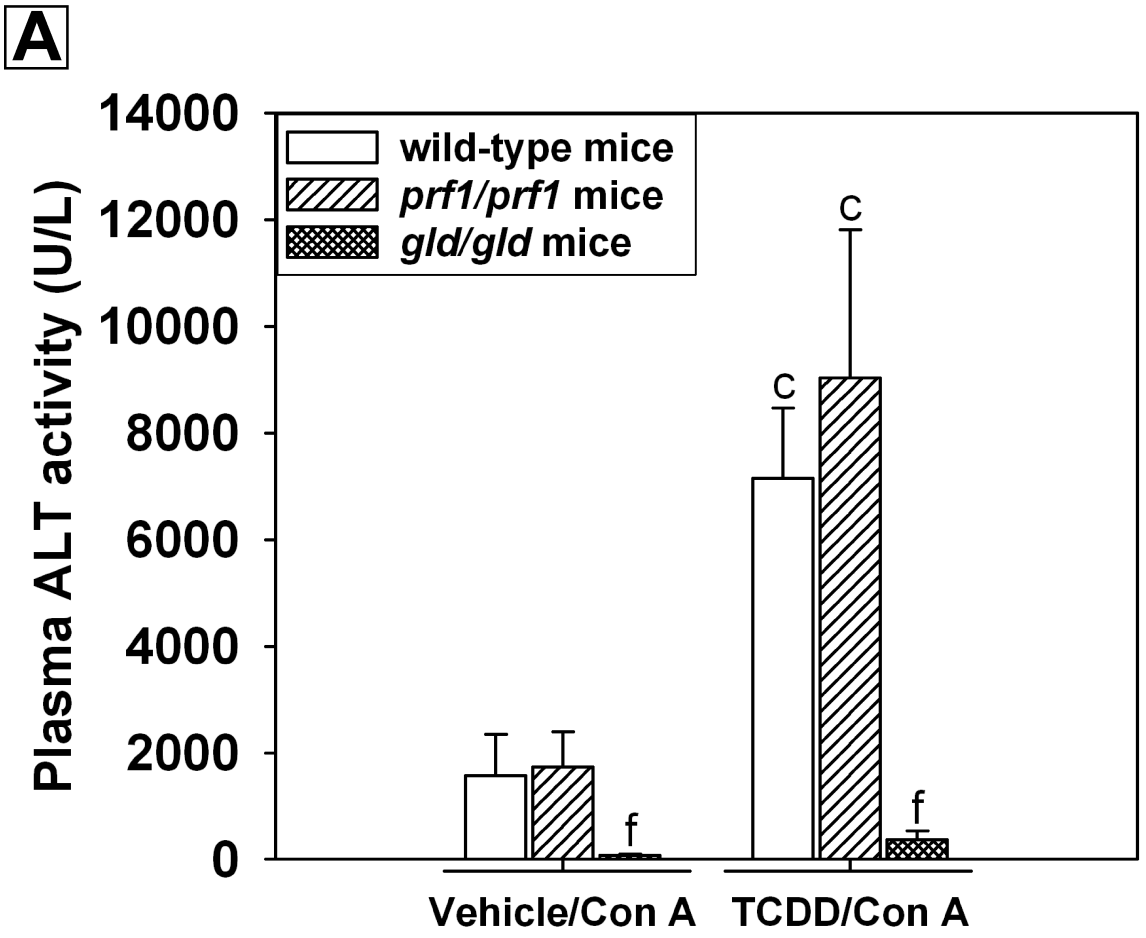


Figure 12: TCDD/Con A-induced liver injury in *prf/prf* and *gld/gld* mice.

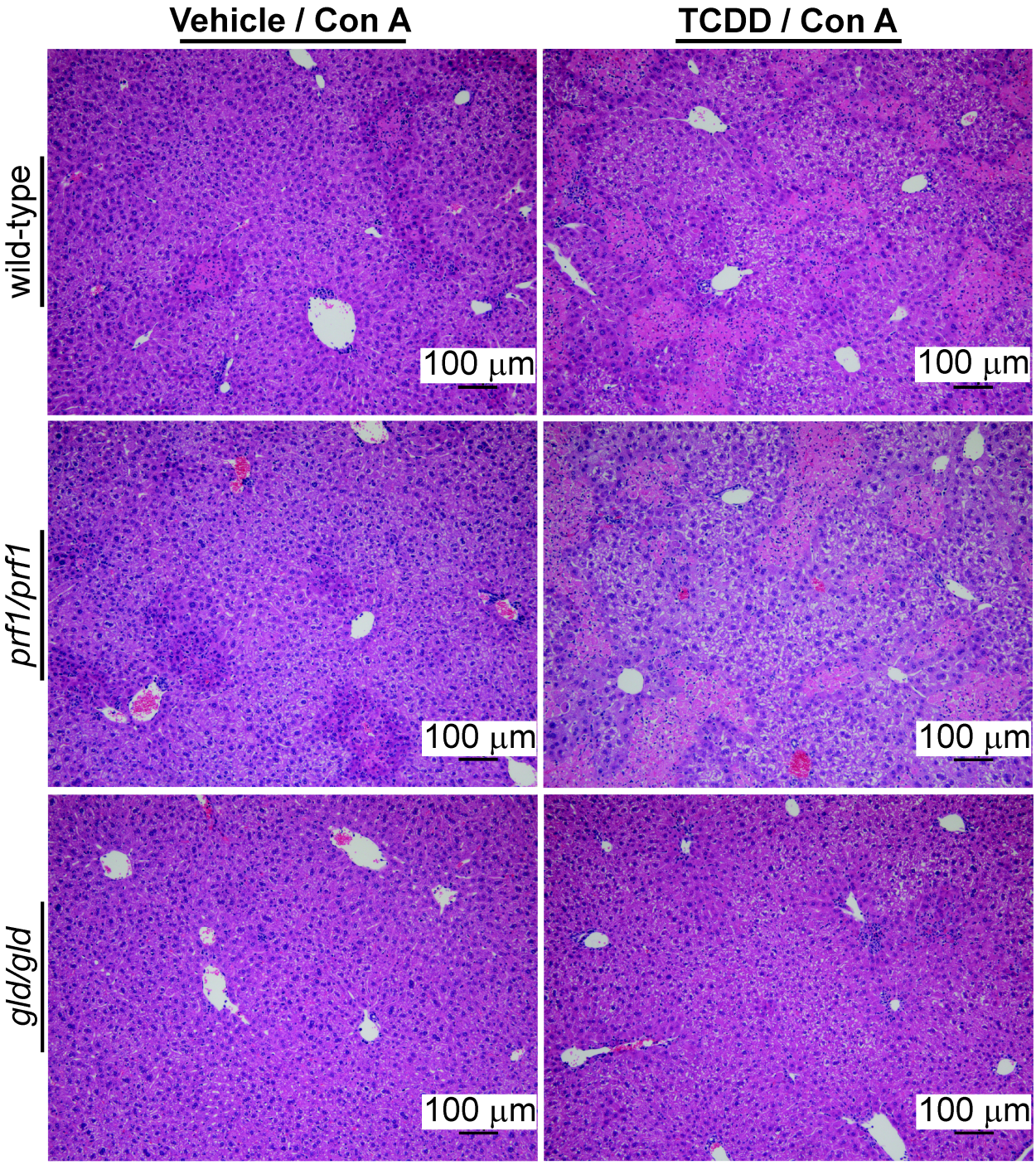
(A) Wild-type (open bars), C57BL/6-Prf1^{tm1Sdz}/J (*prf1/prf1*) (striped bars) and B6Smn.C3-Fasl^{gld}/J (*gld/gld)* (cross-hatched bars) mice were treated as described in the legend to Figure 4 with Vehicle/Con A or TCDD/Con A. Plasma ALT activity was determined at 24 h after Con A administration. c $p < 0.05$ TCDD/Con A versus Vehicle/Con A in the same mouse genotype. f $p < 0.05$ versus the same treatment in the wild-type control. Data represent the mean ± SE of 3-5 independent replicates per treatment group. (B) Histopathology of TCDD/Con A treatment in *C57Bl/6-Prf1*^{tm1Sdz}*/j* (Perforin knockout), B6Smn.C3-Fasl^{gld}/J (Fas ligand knockout) or wild-type mice. Mice

Figure 12 (cont'd)

were treated on day 0 with vehicle or TCDD and on day 4 with 6 mg/kg Con A. Liver sections were collected 24 h after Con A administration. H&E stained sections were photographed at 10x magnification.

Figure 12 (cont'd)

B



2.5 Discussion

The studies presented here were designed to investigate the effects of TCDD pretreatment on the outcome of immune-mediated liver injury and to determine what role the altered responses of immune cells play in the pathogenesis. The 30 μg/kg dose of TCDD was chosen because it has been commonly used for single oral dose exposure in this strain of mice. Using this dose enabled direct comparisons of our results to previously published work on acute toxicity (Patterson *et al.*, 2003; Boverhof *et al.*, 2005; Kopec *et al.*, 2008; N'Jai *et al.*, 2008) and humoral immunity (Smialowicz *et al.*, 1994). In addition, doses in this range were used to examine cytokine expression following innate immune cell stimulation with endotoxin (Clark *et al.*, 1991), sensitization to sheep red blood cells (Moos *et al.*, 1994) and the response to ovalbumin (Nohara *et al.*, 2002).

A major source of uncertainty regarding the toxic effects of dioxins in humans is associated with the limited data available on body burdens present in populations exposed to large doses. In particular it has been difficult to determine the human dosage at the time of exposure. Human body burdens are most commonly estimated by measuring lipid-adjusted serum concentrations (DeVito *et al.*, 1995). This measurement is used as a proxy for the concentration of dioxins in adipose tissue, a major site of distribution in the body. In the general population the body burden of dioxin-like compounds is estimated to be equivalent to 36-58 ng TCDD/kg lipid according to lipid-adjusted serum measurements (DeVito *et al.*, 1995). These measurements are based on established toxic equivalency factors (Birnbaum and DeVito, 1995). The TCDD dose used in these experiments more closely resembles

those that have been proposed to occur in cases of accidental or occupational exposure in humans, in which significantly greater body burdens of TCDD have been reported. Lipid-adjusted serum concentrations as high as 56 μg/kg have been found in workers one year after an exposure resulting from a chemical plant explosion in Seveso, Italy (Needham *et al.*, 1997). In another study of chemical plant workers, values as high as 3 μg/kg were observed despite all workers having received their last occupational exposure between 15 to 37 years earlier (Fingerhut *et al.*, 1991). These values are not unlike those measured in adipose tissue of mice given a single dose of 10 μg TCDD/kg: 37 μg/kg and 54 μg/kg at 4 and 7 days respectively (Diliberto *et al.*, 1995; Hakk *et al.*, 2009). Accordingly, the 30 μg/kg dose used in these experiments is in the range of those likely to have occurred during accidental exposure to TCDD in humans.

Con A-induced liver injury is a well-established model of hepatitis. A single, intravenous injection of this mitogenic lectin (typically 15-25 mg/kg) causes inflammatory liver injury in mice within 8-24 h after administration. For studies presented here, smaller Con A doses (0-10 mg/kg) that result in only minimal to moderate liver injury (Mizuhara *et al.*, 1998) were chosen so that exacerbation of hepatotoxicity by TCDD pretreatment could be detected. Pretreatment with TCDD resulted in an increased hepatotoxic response to a range of Con A doses, as indicated by increased plasma ALT activity and histopathology. In addition, the onset of injury after Con A administration occurred earlier in TCDD-pretreated mice. The ALT activity in the plasma of TCDD/Con A-treated mice was significantly elevated as early as 4 h after Con A administration, whereas a significant increase in Vehicle/Con A-treated mice was not observed until 6 h.

One mechanism by which TCDD might increase the sensitivity to Con A-induced liver injury is by altering the cytokine response. TNFα, IL-6, and IL-10 are known to play important roles in the modulation of injury at larger doses of Con A. For example, TNFα, a cytokine released primarily by activated macrophages and T-cells (Gantner *et al.*, 1996), is a major effector of liver injury induced by large, hepatotoxic doses of Con A; neutralization of TNFα completely protected against hepatotoxicity (Mizuhara *et al.*, 1994; Gantner *et al.*, 1995). IL-6 plays contrasting roles in the development of injury after Con A. IL-6 was protective if administered or induced prior to Con A treatment (Nishikage *et al.*, 1999), whereas recombinant IL-6 exacerbated injury when administered after Con A (Mizuhara *et al.*, 1994). The anti-inflammatory cytokine IL-10 was induced early after Con A administration. Reducing IL-10 with neutralizing antibodies increased the severity of Con A hepatotoxicity, and treatment with recombinant IL-10 before Con A administration reduced the production of TNFα, IL-12, and IFNγ and attenuated liver injury (Louis *et al.*, 1997). In studies presented here, a smaller dose of Con A had a similar effect to increase IL-6, TNFα, and IL-10 concentrations in plasma; however, TCDD-pretreatment did not alter the induction of any of these cytokines. These results suggest that the modulation of these inflammatory and protective cytokines does not play a direct role in the increased sensitivity of TCDD-pretreated mice to Con A.

The plasma concentrations of IL-2, IL-4 and IFNγ, cytokines produced by the T cell populations directly activated by large doses of Con A, are known to be increased by Con A administration. IL-2 is a marker for T cell activation, and its role in the development of Con A-induced liver injury is not fully discerned. IL-4 production by NKT

cells is partially responsible for the increase in TNFα after Con A, and neutralization of IL-4 with monoclonal antibodies completely protected mice from Con A-induced injury (Toyabe *et al.*, 1997; Kaneko *et al.*, 2000). IFNγ is essential for the development of liver injury after Con A administration (Kusters *et al.*, 1996; Tagawa *et al.*, 1997). In our studies, administration of Con A induced IL-2, IL-4, and IFNγ, confirming earlier work. Interestingly, whereas TCDD pretreatment decreased the production of IL-2 and IL-4 after Con A, the plasma concentration of IFNγ was increased. In addition, IFNγ knockout mice did not develop liver injury from treatment with TCDD/Con A. As in studies using larger doses of Con A alone, these results indicate that IFNγ plays a central role in liver injury resulting from TCDD sensitization to a smaller dose of Con A. The effects of TCDD treatment alone in IFNγ knockout mice were not determined in this study. However, given that in the absence of Con A, 30 μg/kg TCDD pretreatment failed to cause hepatocellular necrosis in wild-type mice, as determined by plasma ALT activity (Fig. 3B) and analysis of histopathology (Fig. 4), it is unlikely that this dose of TCDD alone would result in liver injury in the IFNγ knockout mice. The expression of IL-17 and IL-22 mRNA induced by Con A was unaltered by TCDD pretreatment, which indicates that TCDD does not exacerbate Con A-induced liver injury by modulating expression of Th17-type cytokines.

Liver injury from large doses of Con A is the result of a number of immune cell-mediated and cytokine-driven responses induced by activation of leukocytes in the liver. The cellular immune response is focused around the initial activation of CD4+ T-cells, in particular NKT cells. These cells are sufficient to cause injury in the absence of any other T cell population (Kaneko *et al.*, 2000; Takeda *et al.*, 2000). In addition, the

secondary activation of innate immune cell types, such as resident hepatic macrophages (Schumann *et al.*, 2000; Morita *et al.*, 2003) and infiltrating neutrophils (Bonder *et al.*, 2004), contributes to hepatotoxicity through accessory roles in the development and progression of injury. TCDD pretreatment did not alter the proportions of NK, NKT or T cells in the liver after Con A treatment. However, whereas Con A administration did result in a reduction of the percentage of NKT cells in the liver, the activation status of these critical effector cells was increased by TCDD-pretreatment as evidenced by increased expression of CD69 and CD25. This occurred at early times before the onset of liver injury. In addition, hepatic expression of ICAM-1 was significantly increased by TCDD/Con A treatment, and neutralization of CD18 greatly reduced hepatotoxicity. These findings demonstrate the importance of leukocyte extravasation and suggest a requirement for direct interaction of leukocytes with target parenchymal cells for hepatocellular killing to occur in this model. Whereas CD18 antiserum treatment did protect against the development of liver injury, it did not decrease the plasma concentration of IFNγ in TCDD/Con A-treated mice (data not shown), a result consistent with results reported previously in LFA-1 knockout mice. LFA-1 is an adhesion molecule composed of the integrin chains CD18 and CD11a. Con A administration to LFA-1 knockout mice produced plasma IFNγ concentrations that were not significantly different from wild-type mice, however LFA-1 knockout mice were protected from liver injury (Matsumoto *et al.*, 2002). This result also raised the possibility that cell cytolytic activity might play an important role in the increased liver injury caused by TCDD cotreatment.

Fas-FasL signaling represents a major mechanism by which lymphocytes can induce cell death in parenchymal cells. The role of FasL (CD95) in lymphocyte-mediated parenchymal cell death after Con A administration is still controversial. FasL knockout (*gld/gld*) mice, Fas knockout (*lpr/lpr*) mice, and mice treated with neutralizing FasL antibody were protected against liver injury induced by a hepatotoxic dose (15mg/kg) of Con A (Seino *et al.*, 1997; Takeda *et al.*, 2000). However, at even larger doses (20-30 mg/kg), *lpr/lpr* knockout mice were not protected (Leist *et al.*, 1996; Watanabe *et al.*, 1996; Tagawa *et al.*, 1998). Additionally, when mice were given a neutralizing Fas fusion protein prior to treatment with 10 mg/kg Con A, no protection was observed (Ksontini *et al.*, 1998). In studies presented here, the percentage of NKT cells expressing FasL was increased in TCDD/Con A-treated mice. In addition, *gld/gld* mice were protected from TCDD/Con A-induced liver injury. These results confirm an important role for the increased cytolytic activity of NKT cells in Con A treated mice that were pretreated with TCDD and suggest that increased FasL-mediated killing of hepatocytes is a major mechanism by which TCDD-pretreatment exacerbates liver injury from Con A.

In summary, these results demonstrate that TCDD pretreatment increases the sensitivity of mice to liver injury in a model of immune hepatitis induced by moderate doses of Con A. This increased sensitivity is characterized by an exacerbated inflammatory response and severe hepatocellular necrosis. The mechanisms underlying the exacerbated Con A response induced by TCDD pretreatment involve an increase in the production of the critical cytokine IFNγ, enhanced activation of NKT cells, and increased cytolytic activity by FasL towards hepatic parenchymal cells.

CHAPTER 3

3.1 Abstract

For many liver diseases, including viral and autoimmune hepatitis, immune cells play an important role in the development and progression of liver injury. Concanavalin A (Con A) administration is used as a model of immune-mediated liver injury resembling autoimmune hepatitis. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been hypothesized to affect the development and severity of some autoimmune diseases. Mice pretreated with TCDD develop exacerbated liver injury in response to administration of a mild dose (6 mg/kg) of Con A. Enhanced liver injury in TCDD-pretreated mice was associated with increased activation and expression of FasL in natural killer T (NKT) cells and increased plasma concentration of IFNγ, which was essential for hepatotoxicity in Con A-induced liver injury. In the present study, we tested the hypothesis that TCDD pretreatment exacerbates Con A-induced liver injury by enhancing the activation and recruitment of the accessory cell types including neutrophils, macrophages, and natural killer (NK) cells. Mice were treated on day 0 with vehicle (olive oil), 0.3, 3, 30 μg/kg TCDD, and 4, 7 or 10 days later 6 mg/kg Con A or saline was administered. Plasma samples were collected at various times after treatment, and liver injury was assessed from the activity of alanine aminotransferase in the plasma. TCDD pretreatment with doses of 3 and 30 μg/kg significantly increased liver injury resulting from Con A administered 4, 7 or 10 days later. The concentrations of neutrophil chemokines keratinocyte chemoattractant (KC) and macrophage inflammatory protein-2 (MIP-2) in plasma were significantly increased by pretreatment with TCDD at 2 and 6 h after Con A administration, respectively. NKT cell-deficient CD1d knockout mice were protected from liver injury induced by vehicle/Con A

treatment, but injury was only partially attenuated in TCDD/Con A-treated mice. RAG1 knockout mice, which lack mature T cells, were protected from liver injury induced by Con A in either vehicle- or TCDD-pretreated mice. After treatment, intrahepatic lymphocytes were isolated, and activation was determined by flow cytometry. TCDD/Con A treatment increased the percentage of NK cells expressing the activation marker CD69. Depletion of NK cells prior to treatment resulted in significant reductions in plasma IFNγ and liver injury from TCDD/Con A treatment. In summary, exposure to TCDD exacerbated the immune-mediated liver injury induced by Con A, and our findings suggest that NK cells play a critical role in this response.

3.2 Introduction

Despite increasing prevalence in the population, the etiology of autoimmune liver diseases is still not thoroughly understood (Feld and Heathcote, 2003). A number of factors appear to confer susceptibility to these diseases. In addition to known genetic risk factors, exposure to environmental xenobiotics is associated with increased incidence of autoimmune disease (Czaja and Manns, 2010; Gilbert, 2010; Longhi *et al.*, 2010). Exposure to aryl-hydrocarbon receptor (AhR) ligands such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) alters the onset and severity of injury in experimental animal models of autoimmune diseases such as joint inflammation in rheumatoid arthritis, deterioration of spinal cord neurons in experimental autoimmune encephalomyelitis and kidney damage in autoimmune lupus (Kobayashi *et al.*, 2008; Quintana *et al.*, 2008; Veldhoen *et al.*, 2008; Mustafa *et al.*, 2009a; Holladay *et al.*, 2011).

TCDD exposure alters the activity of many types of immune cells and can increase the production of inflammatory mediators particularly from innate immune cells (Kerkvliet, 1995; Esser *et al.*, 2009; Kerkvliet, 2009). In a mouse model of influenza infection, TCDD treatment increased neutrophil recruitment to the lung, resulting in exacerbated tissue injury (Teske *et al.*, 2005). An increased accumulation of neutrophils in the peritoneal cavity and increased inflammatory cytokine production was observed after administration of sheep red blood cells to TCDD-treated mice (Moos *et al.*, 1994). TCDD treatment also enhanced the response of macrophages to inflammatory stimuli such as lipopolysaccharide (Moos *et al.*, 1997). Mice treated with TCDD had increased mRNA expression of monocyte chemoattractic protein-1 (MCP-1)

and keratinocyte chemoattractant (KC) in spleen, kidney and liver tissues, and increased expression of these chemokines was associated with enhanced accumulation of macrophages in those tissues (Vogel *et al.*, 2007).

TCDD is disproportionately distributed to hepatic tissue after exposure, resulting in relatively large hepatic concentrations, and the liver is a major target organ for TCDD toxicity in many species (Poland and Knutson, 1982; Abraham *et al.*, 1988; Thoma *et al.*, 1990; Diliberto *et al.*, 1995; Birnbaum and Tuomisto, 2000). In the liver, prolonged activation of the AhR causes extensive changes in gene expression, and alterations in many AhR-regulated genes associated with immune cell activity have been identified (Kerkvliet, 2009; Stevens *et al.*, 2009; Dere *et al.*, 2011b). However, despite the importance of the liver in AhR-mediated gene expression after TCDD treatment and the increasing experimental evidence that TCDD treatment alters the development of various autoimmune diseases, the effect of TCDD exposure on the development of autoimmune liver disease has not been addressed.

The polyclonal T cell mitogen concanavalin A (Con A) has been used to produce a model of immune-mediated liver injury that resembles the pathophysiology of autoimmune hepatitis (Tiegs *et al.*, 1992). This model has been used extensively to study potential mechanisms of errant immune cell activation and destruction of hepatic parenchymal tissue similar to that which occurs in patients with autoimmune hepatitis (Peters, 2002; Wang *et al.*, 2012). In Con A-induced hepatitis, activation of a number of hepatic immune cell types contributes to the development of injury. The primary effector cells appear to be natural killer T (NKT) cells, which are required for the development of injury (Kaneko *et al.*, 2000; Takeda *et al.*, 2000). NKT cells have been

implicated in several autoimmune diseases in humans (Santodomingo-Garzon and Swain, 2011). Conventional $CD4^+$ T cells also contribute to the development of Con A-induced liver injury (Tiegs and Gantner, 1996). In addition to the activation of NKT and $CD4^+$ T cells, development of Con A-induced liver injury depends on the production of the inflammatory cytokines, tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ) (Mizuhara *et al.*, 1994; Gantner *et al.*, 1995; Kusters *et al.*, 1996; Ksontini *et al.*, 1998). Although NKT and CD4+ T cells are widely regarded as the main effectors of Con A-mediated liver injury, other hepatic immune cells appear to play accessory roles and to contribute to the development of liver injury in this model of autoimmune hepatitis. Con A administration caused a significant increase in the number of neutrophils in the liver, and neutrophil depletion reduced Con A hepatotoxicity (Bonder *et al.*, 2004; Hatada *et al.*, 2005). In addition, hepatic macrophages were reported to contribute to the development of injury through the release of inflammatory mediators including TNFα, interleukin (IL)-18, and IL-12, which can act directly on parenchymal cells or on lymphocytes to increase production of other cytokines and augment direct cytolytic activity (Faggioni *et al.*, 2000; Nicoletti *et al.*, 2000; Schumann *et al.*, 2000). It is apparent that Con A-induced hepatitis is a result of the activity of a variety of immune cell types and that the altered response of any one type has the potential to affect the development and severity of injury.

We have previously reported that pretreatment with TCDD sensitized mice to Con A-induced hepatitis. The exacerbated response to Con A was associated with increased production of IFNγ and enhanced activation of NKT cells (Fullerton *et al.*, 2013), but the effect of pretreatment with TCDD on other cell types in this model is

unknown. Given the effects of TCDD on neutrophils and macrophages in other models of inflammatory injury (Teske *et al.*, 2005; Wu *et al.*, 2011), it is pertinent to investigate whether pretreatment with TCDD confers sensitivity to Con A exclusively through increased activation of NKT cells or if an altered response by other intrahepatic immune cells is also involved. In this study we investigated the effects of TCDD on hepatic neutrophils, macrophages and NK cells to determine the role of these accessory cells in the increased sensitivity of TCDD-pretreated mice to Con A-induced liver injury.

3.3 Materials and Methods

Materials

All materials were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. TCDD was purchased from Accustandard (New Haven, CT), dissolved in DMSO and diluted in olive oil to a working concentration of 0.2 µg/mL.

Mice

C57Bl/6J, B6.129S6-Cd1d1/Cd1d2^{tm1Spb}/J and B6.129S7-*Rag1*^{tm1Mom}/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and acclimated for at least one week in a 12 h light/dark cycle with access to Global Rodent diet 2018 (Harlan Teklad, Madison, WI) and bottled spring water ad libitum. Mice were used at 10-12 weeks of age, and all procedures were carried out with the approval of the Michigan State University Institutional Animal Care and Use Committee. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the United States National Institutes of Health.

Experimental protocols

A single administration of 0.3, 3 or 30 µg/kg TCDD or olive oil (vehicle) was given by oral gavage on day 0, followed on day 4, 7 or 10 by intravenous administration of saline or 6 mg/kg Con A (Lot 096 K7011). During the course of each experiment, TCDD-treated mice were housed in separate cages from vehicle-treated mice. The activity of alanine aminotransferase (ALT) in plasma was measured spectrophotometrically using Infinity ALT reagent (Thermo Fischer Scientific, Waltham, MA). Depletion of NK cells was performed using rabbit anti mouse/rat asialoGM1 polyclonal antibody (Cedar Lane, Burlington, Ontario, Canada) according to the

manufacturer's instructions. Briefly, 50 uL of reconstituted asialoGM1 antibody (Lot DBJ5790) was administered intravenously to mice in a volume of 150 µL normal rabbit serum. AsialoGM1 antibody or control rabbit serum was given 18 h before Con A administration. We have previously demonstrated that this treatment is sufficient to deplete hepatic NK cells (Dugan *et al.*, 2011).

Histopathology

For neutrophil identification, paraffin-embedded liver sections were stained with rabbit anti-mouse neutrophil antibodies by the Michigan State University Investigative Histopathology Laboratory as described previously (Yee *et al.*, 2003). For each mouse liver section, stained neutrophils were counted in 10 randomly chosen, 40x fields, and the mean count for all fields was used as the value for one independent replicate.

Cytokine analysis

Plasma concentration of IL-12 was measured using an OptEIA ELISA kit purchased from BD (Franklin Lakes, NJ). A bead-based Milliplex MAP immunodetection array (Millipore, Billerica, MA) was used to measure plasma concentrations of KC, MCP-1 and macrophage inflammatory protein 2 (MIP-2) on a Bio-plex instrument (Bio-Rad Laboratories, Hercules, CA).

RNA isolation and RT-PCR analysis

Liver samples were homogenized in TRI reagent (Molecular Research Center, Cincinnati, OH), and isolation of total RNA was performed according to the manufacturer's instructions. The quantity and quality of isolated RNA was determined using a nanodrop spectrophotometer (Thermo Scientific, Waltham, MA). cDNA was prepared from 1 µg of RNA using iscript reverse transcription supermix for RT-qPCR

(Bio-Rad Laboratories). Expression levels of target genes were determined on a Step-one real-time PCR system (Applied Biosystems, Foster City CA) utilizing specific DNA oligos and SYBR green PCR master mix (Applied Biosystems). Copy number was determined by comparison to standard curves for each respective gene generated from pooled cDNA. The target gene expression levels were standardized to the geometric mean of expression levels of glyceraldehyde-3-phosphate dehydrogenase (Gapdh), beta-actin (Actb) and hypoxanthine guanine phosphoribosyl transferase (Hprt). To evaluate the expression of target genes, the following PCR primers were used: Gapdh (115 bp), 5'-TCAACAGCAACTCCCACTCTTCCA-3' (forward), 5'-ACCCTGTTGCTGTA GCCGTATTCA-3' (reverse); Actb (140 bp), 5'-TGTGATGGTGGGAATGGGTCAGAA-3' (forward), 5'-TGTGGTGCCAGATCTTCTCCATGT-3' (reverse); Hprt (133 bp), 5'-GGAG TCCTG-TTGATGTTGCCAGTA-3' (forward), 5'-GGGACGCAGCAACTGACATTTCTA-3' (reverse); IL-12p40, Il12b (101 bp), 5'-AAAGCTGTCTTCTGCTTGGTTGGC-3' (forward), 5'-CTGGCTCTGCGGGCATTTAACATT-3' (reverse); IL-27, Il27 (107 bp), 5'-GTGACAGGAGACCTTGGCTG-3' (forward), 5'-AGCTCTTGAAGGCTCAGGG-3' (reverse). mRNA expression data are reported as fold-change of standardized treatment over standardized vehicle/saline treatment at time zero.

Flow cytometry

Hepatic leukocytes were isolated from mice and prepared for flow cytometry analysis as follows. Mouse livers were washed with phosphate buffered saline (PBS) without calcium and magnesium. Livers were placed in RPMI medium supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin and then passed through a nylon mesh, and the resulting cell suspension was centrifuged at a speed of

50 ×g for 5 min at 4 degrees Celsius. The supernatant was removed, and the pelleted hepatocytes were discarded. The supernatant was centrifuged at 450 ×g for 5 min to pellet leukocytes. The pellet was then incubated for 4 min with red blood cell lysis buffer (BioLegend, San Diego, CA) followed by two washes with PBS containing 5% FBS. Lympholyte-M (Cedar Lane) was used according to manufacturer's instructions to further purify leukocytes. For ex-vivo stimulation experiments, hepatic leukocytes were cultured in RPMI medium supplemented with 5% FBS and 1% penicillin/streptomycin with or without 7.5 μg/mL Con A. In all other instances, hepatic leukocytes were stained and prepared immediately for flow cytometric analysis.

Hepatic leukocytes were first incubated with TruStain FcX (anti-mouse CD16/CD32) to minimize nonspecific binding of staining antibodies to Fcγ receptors. Antibodies used for staining of NK, NKT, and T cells included fluorescein isothiocyanate or phycoerythrin-conjugated anti-NK1.1 (PK136) and allophycocyanin-cyanine dye 7-conjugated anti-CD3epsilon (145- 2c11) as well as pacific blue-conjugated anti-CD69 (H1.2F3) and allophycocyanin-conjugated anti-NKG2d (CX5). Staining of hepatic macrophages and neutrophils was performed using fluorescein isothiocyanate or allophycocyanin-conjugated anti-F4/80 (BM8), phycoerythrin-conjugated anti-CD11b (M1/70) and phycoerythrin-cyanine dye 7-conjugated anti-Gr-1 (RB6-8C5). Appropriate fluorescent-conjugated isotype controls were utilized to establish positive and negative gating parameters for each antibody. Unless otherwise stated, all reagents and antibodies for flow cytometric staining were purchased from BioLegend (San Diego, CA). Hepatic leukocyte staining was performed according to manufacturer's directions, and stained samples were analyzed on a BD FACSCanto II with subsequent data

analysis performed using Kaluza software (Beckman Coulter, Brea, CA).

Statistical analysis

Experimental results are expressed as mean±SEM. Arcsine transformation was performed on percentile data. In some instances, a Box-Cox transformation was utilized to normalize data for further analysis. Statistical analysis of data was performed using either student's *t*-test or two-way analysis of variance (ANOVA) followed by pairwise multiple comparisons using Student–Newman–Keuls or Tukey's method where appropriate. Analysis of nonparametric data was performed using Kruskal–Wallis one-way analysis of variance on ranks followed by pairwise multiple comparisons using Tukey's or Dunn's method where appropriate. The criterion for statistical significance was $p<0.05$.

3.4 Results

TCDD dose-response and the persistence of sensitization to Con A after pretreatment with TCDD

We have previously demonstrated that pretreatment with a nonhepatotoxic dose of 30 μg/kg TCDD exacerbates the inflammatory liver injury induced by a dose of 6 mg/kg Con A administered 4 days later (Fullerton *et al.*, 2013). This study was undertaken to determine if smaller doses of TCDD sensitize mice to Con A-induced liver injury. When saline was administered on day 4, no liver injury developed in vehicle-pretreated mice or in TCDD-pretreated mice regardless of dose, as determined by measurements of ALT activity in plasma (Fig. 13A). When Con A was administered on day 4 an increase in ALT activity in the plasma was detected in vehicle-pretreated mice, indicating moderate hepatotoxicity. Compared to pretreatment with vehicle, mice pretreated with 3 or 30 μg/kg TCDD had significantly increased ALT activity in plasma 8 h after Con A administration.

To evaluate the persistence of TCDD-induced sensitization to Con A-induced liver injury, mice were administered Con A 4, 7 or 10 days after pretreatment with vehicle or 30 μg/kg TCDD. They were euthanized 24 h later. No significant differences in ALT activity in plasma were observed among mice given Con A 4, 7 or 10 days after vehicle pretreatment (Fig. 13B). At each time, TCDD/Con A-treated mice had increased ALT activity in plasma compared to vehicle/Con A-treated mice. In addition, TCDD-pretreated mice administered Con A after 7 days had significantly increased ALT activity in plasma compared to mice administered Con A after 4 days.

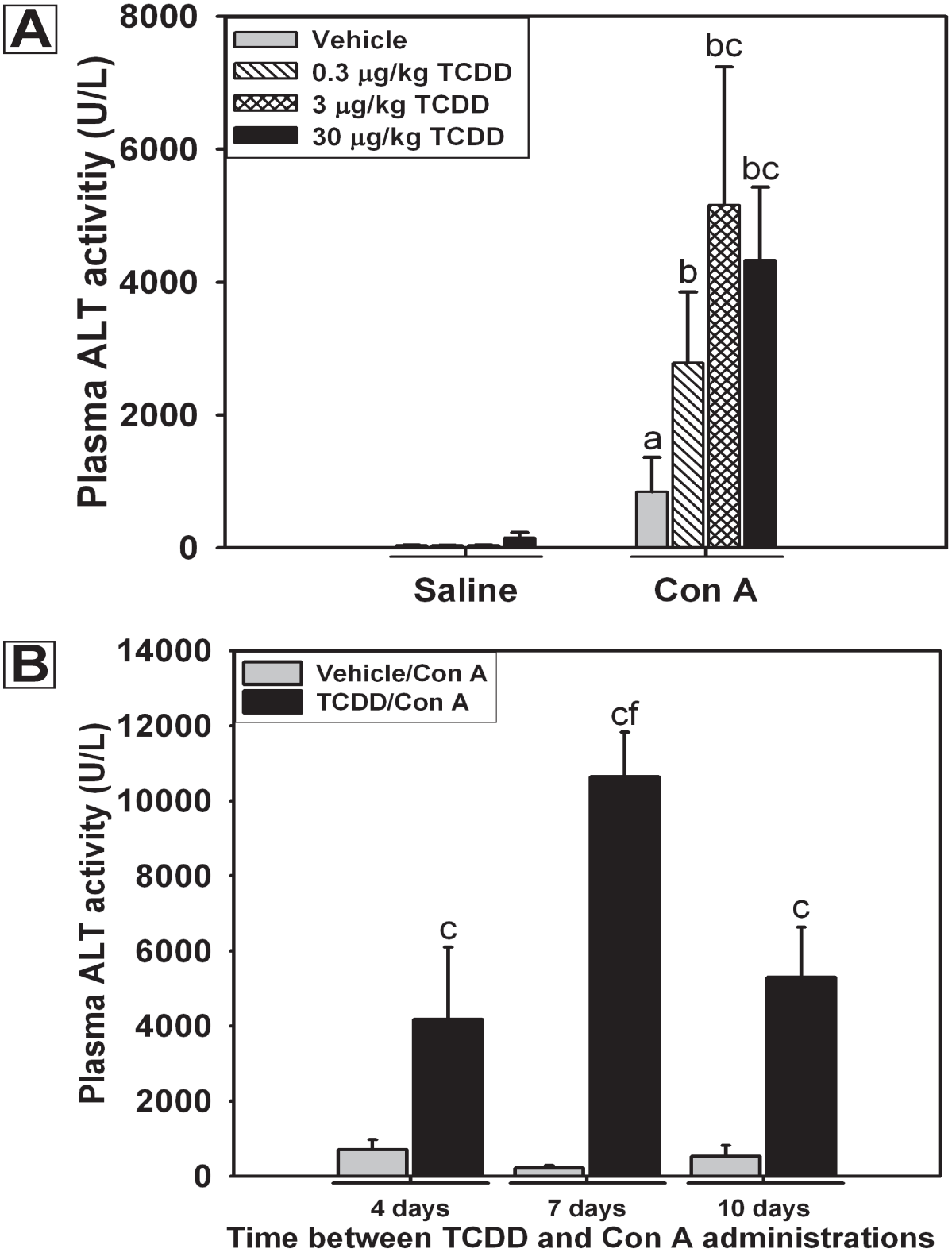


Figure 13: TCDD dose-response and temporal relationship in the exacerbation of Con A-induced liver injury. (A) Mice were treated on day 0 with either 0, 0.3, 3 or 30

Figure 13 (cont'd)

μg/kg of TCDD. Four days later they were given 6 mg/kg Con A or saline. ALT activity in plasma was measured 8 h after Con A or saline administration. a, $p<0.05$ vehicle/Con A versus vehicle/saline. b, $p<0.05$ TCDD/Con A versus the same TCDD dose with saline treatment. c, $p<0.05$ TCDD/Con A versus vehicle/Con A. Data represent the mean ± SE of 3-5 independent replicates per treatment group. (B) Mice were treated on day 0 with either 30 μg/kg TCDD or vehicle control. 4, 7 or 10 days later, 6 mg/kg Con A was administered. ALT activity in plasma was measured 24 h after Con A administration. c $p<0.05$ TCDD/Con A versus vehicle/Con A at the same treatment day. f $p<0.05$ TCDD/Con A versus the same treatment group at 4 days. Data represent the mean ± SE of 4-9 independent replicates per treatment group. Data were combined from 2 separate experiments.

Inflammatory chemokines in TCDD/Con A-induced liver injury

Inflammatory liver injury often involves the recruitment of numerous cell types to the liver. Mice treated with TCDD/Con A developed liver injury that was first evident 4 h after the administration of Con A and peaked at 8 h (Fullerton *et al.*, 2013). In this study, the production of chemokines was measured in the plasma at times prior to the peak of TCDD/Con A-induced liver injury. TCDD-pretreatment alone decreased plasma concentration of KC compared to vehicle pretreatment in the absence of Con A (Fig. 14A). Concentrations of KC, MCP-1 and MIP-2 were significantly increased 2 h after Con A administration and returned to baseline by 6 h (Fig. 14A-C). TCDD-pretreatment did not alter the induction of KC, or MCP-1 by Con A at any time but led to a significant increase in the plasma concentration of MIP-2 two h after Con A administration. In TCDD/Con A-treated mice, the concentration of KC in plasma remained elevated at 6 h compared to 0 h.

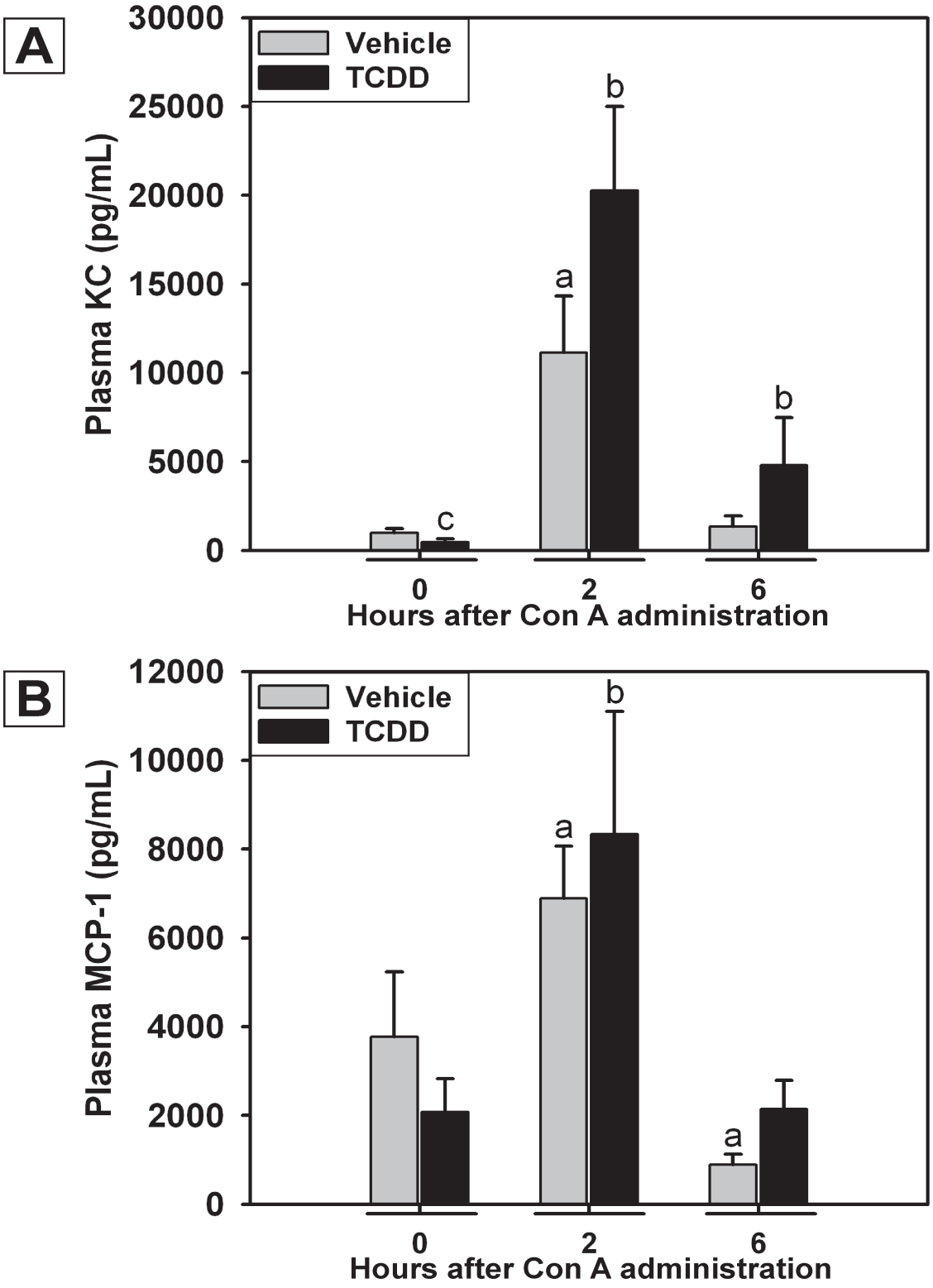
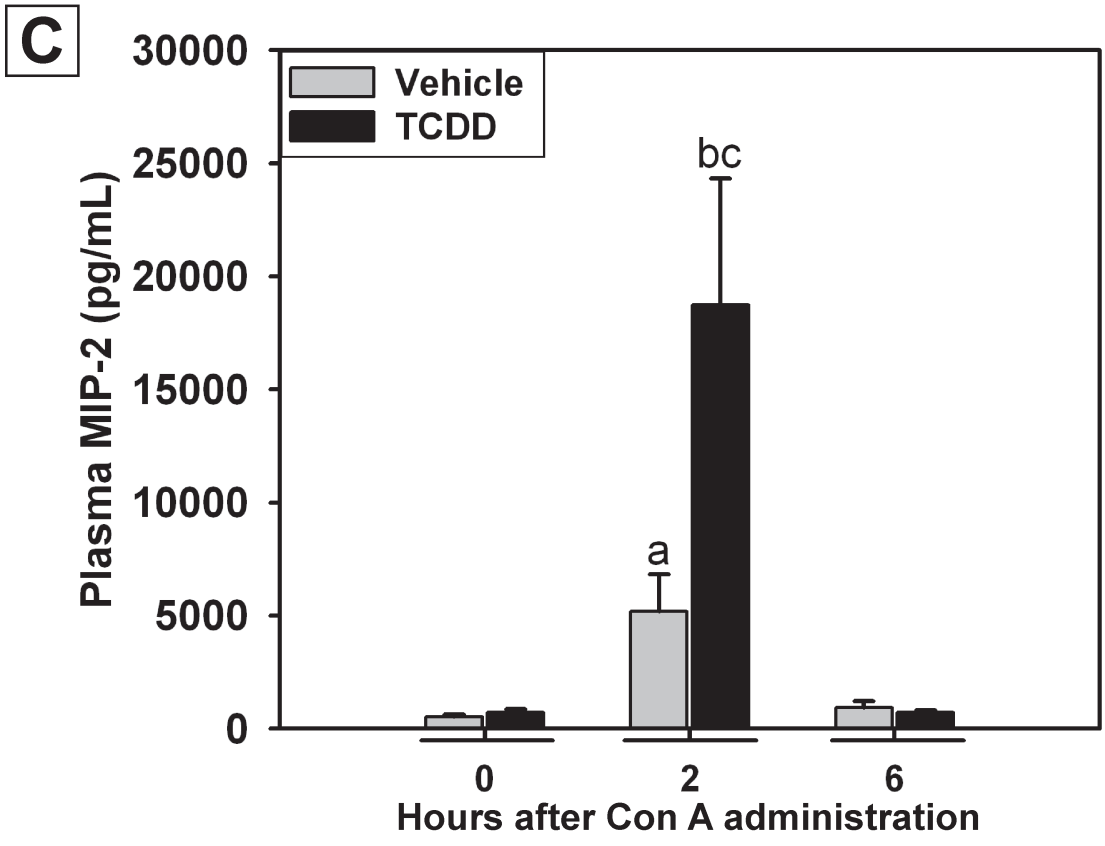


Figure 14: Concentrations of KC (A), MCP-1 (B) and MIP-2 (C) in plasma after Con A administration. Mice were treated on day 0 with vehicle (gray bars) or 30 μg/kg

Figure 14 (cont'd)

TCDD (black bars) and on day 4 with 6 mg/kg Con A. Plasma samples were collected at various times after the administration of Con A. a, $p<0.05$ versus vehicle pretreatment at 0 h. b, $p<0.05$ versus TCDD pretreatment at 0 h. c, $p<0.05$ TCDD pretreatment versus vehicle pretreatment at the same time point. Data represent the mean ± SE of 5-10 independent replicates per treatment group. Data were combined from 2 separate experiments.



Hepatic neutrophil accumulation in TCDD/Con A-induced liver injury

Accumulation of neutrophils in the liver was determined by immunohistochemical staining of liver samples collected 4 h after the administration of saline or Con A. TCDD pretreatment alone did not result in increased numbers of neutrophils in the liver (Fig. 15A and B). Con A administration significantly increased the number of neutrophils compared to vehicle/saline treatment, and TCDD pretreatment did not alter the accumulation or distribution of neutrophils in the liver. Flow cytometry was used to evaluate the percentage of neutrophils (Gr-1$^+$, CD11b$^+$ cells) in the isolated hepatic leukocyte population at 0, 2, 4 and 24 h after Con A administration. TCDD-pretreatment did not alter the percentage of neutrophils in the liver at any time after Con A administration (Fig. 15C).

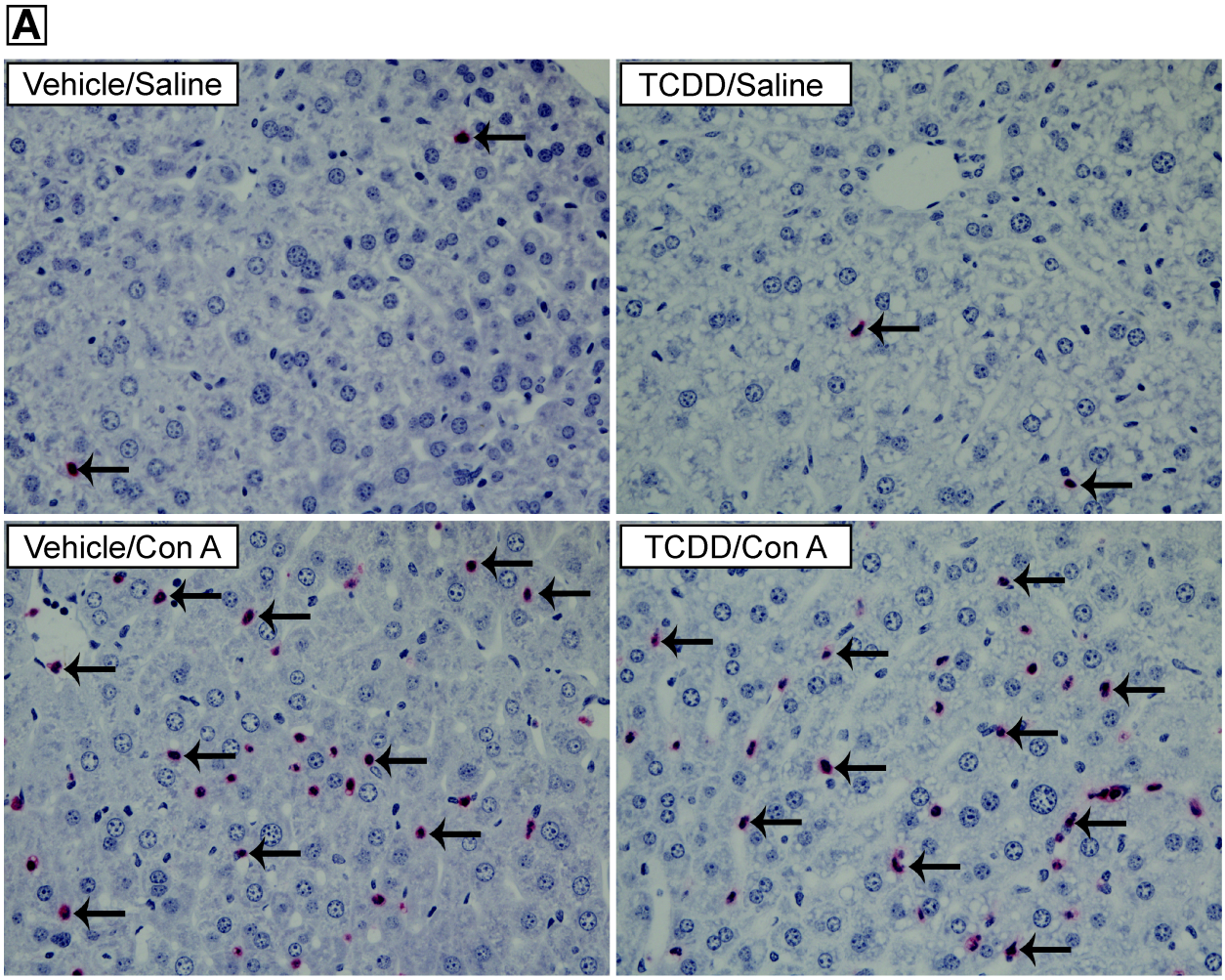
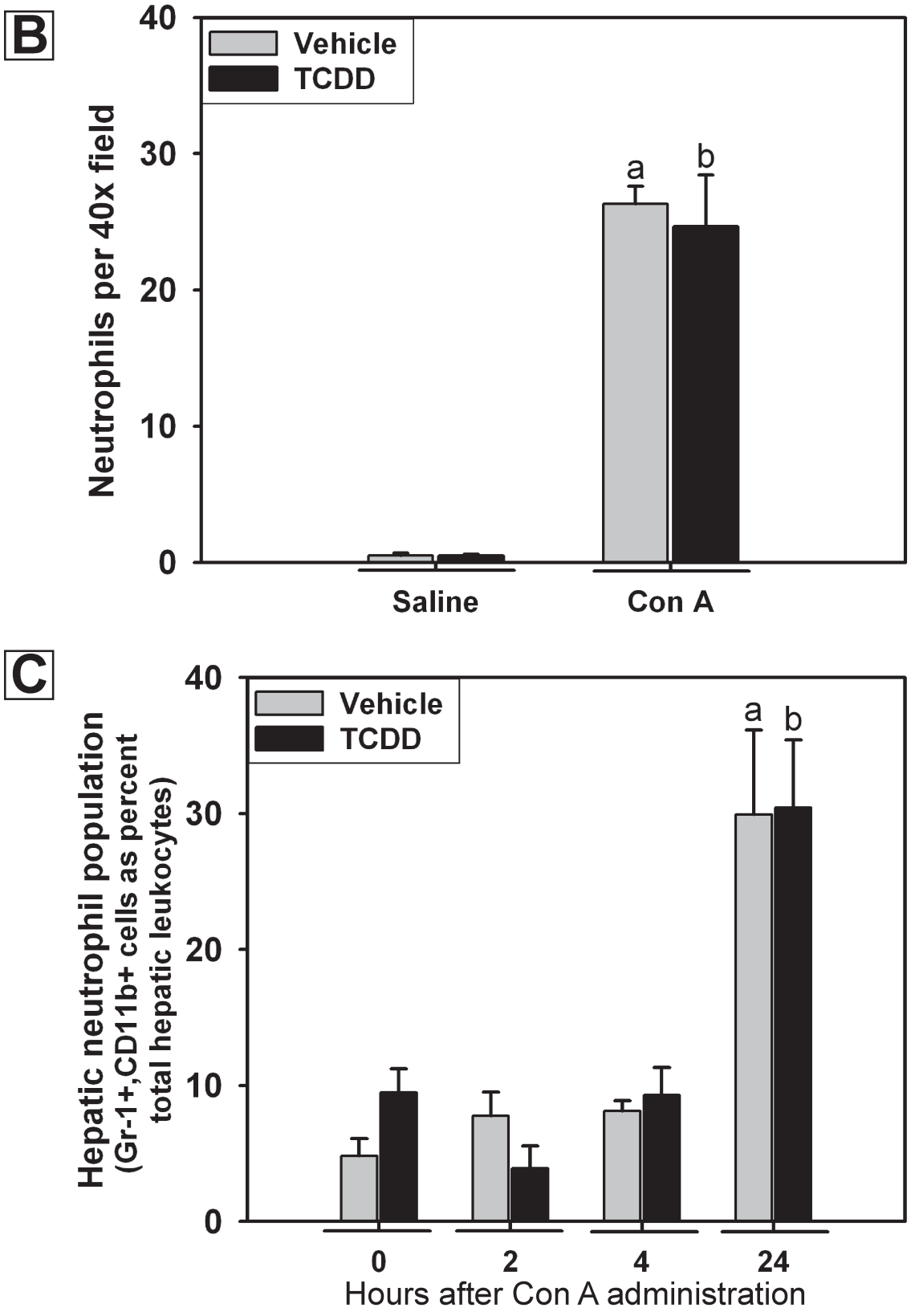


Figure 15: Hepatic neutrophil accumulation after Con A administration. Mice were treated as described in the legend to Fig. 14 with vehicle/saline, TCDD/saline, vehicle/Con A or TCDD/Con A. (A) Mice were euthanized 4 h after saline or Con A administration. Paraffin-embedded liver sections were stained for neutrophils. Representative liver sections were photographed at 40x magnification. Examples of positive neutrophil staining are indicated by arrows. (B) Immunohistochemical staining of neutrophils in the livers at 4 h was quantified as described in 3.3 *Materials and Methods*. (C) Hepatic leukocytes were isolated from mice at various times after Con A, and the percentage of neutrophils (Gr-1$^+$, CD11b$^+$ cells) in the intrahepatic leukocyte

Figure 15 (cont'd)

population was determined by flow cytometry analysis as described in section 3.3 *Materials and Methods*. a, $p<0.05$ vehicle/Con A versus vehicle/saline. b, $p<0.05$ TCDD/Con A versus TCDD/saline. Data represent the mean ± SEM of 3-4 independent replicates per treatment group.

Figure 15 (cont'd)



Role of hepatic macrophages in TCDD/Con A-induced liver injury

To evaluate the role of hepatic macrophages in the development of TCDD/Con A-induced liver injury, we isolated intrahepatic leukocytes after Con A administration. The percentage of monocytes/macrophages (F4-80$^+$ cells) in the population of intrahepatic leukocytes was evaluated by flow cytometry at times before the development of liver injury. Compared to vehicle, TCDD-pretreatment 4 days earlier did not alter the percentage of macrophages measured in the liver at 0 h (Fig. 16A). The percentage of macrophages observed was not affected by Con A administration in either vehicle or TCDD-pretreated mice.

In addition to the quantification of macrophages by flow cytometry, the effect of TCDD pretreatment on the production of macrophage-derived cytokines in response to Con A was evaluated. The plasma concentration of IL-12 was measured at times before the development and at the peak of liver injury. Con A administration resulted in an increased concentration of IL-12 in plasma at 3, 6, and 8 h after treatment (Fig. 16B). TCDD-pretreatment decreased the concentration of IL-12 in plasma measured 8 h after Con A administration. The hepatic expression of IL-12 and IL-27 mRNA was assessed by real-time PCR. Expression of both was increased in Con A-treated mice at 3 and 6 h after administration, respectively, but expression was not affected by pretreatment with TCDD (Fig. 16C and D).

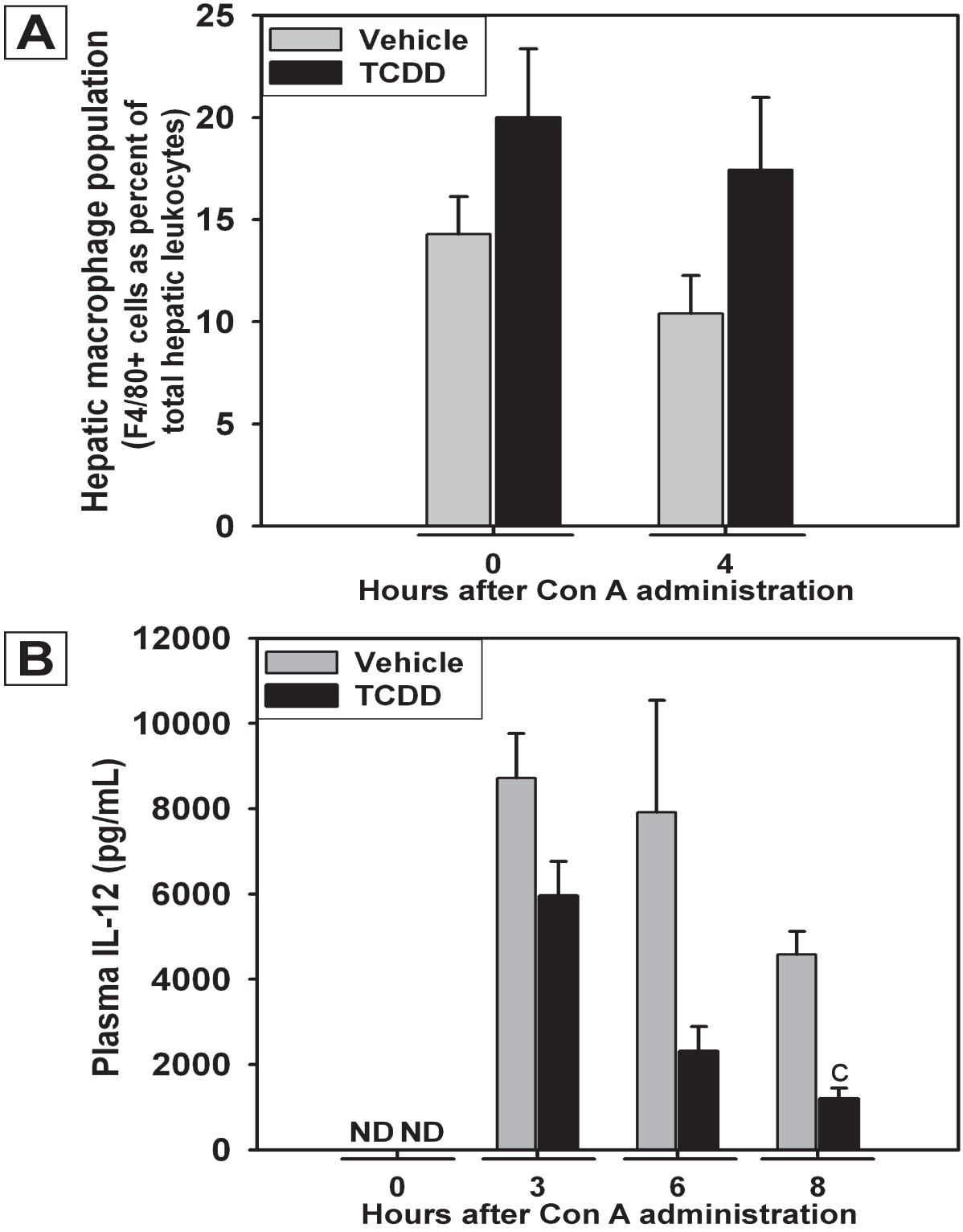
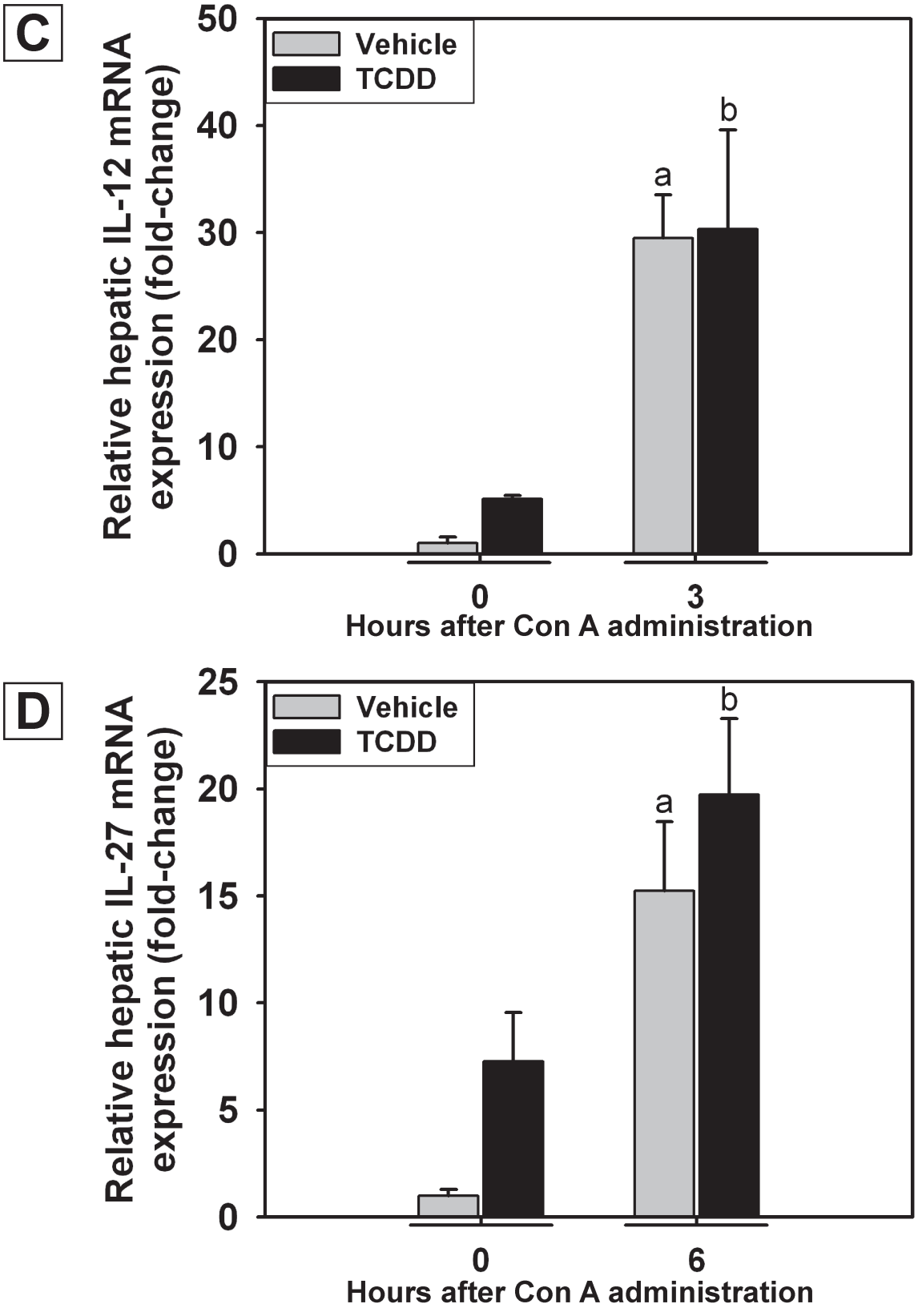


Figure 16: Hepatic macrophages and macrophage-associated cytokines after Con A administration. Mice were treated as described in the legend to Fig. 14 with

Figure 16 (cont'd)

vehicle/Con A (gray bars) or TCDD/Con A (black bars). (A) percentage of F4/80-positive hepatic leukocytes. (B) concentration of IL-12 in plasma, and hepatic expression of (C) IL-12 and (D) IL-27 mRNA after Con A administration. a, $p<0.05$ versus vehicle at 0 h. b, $p<0.05$ versus TCDD at 0 h. c, $p<0.05$ TCDD/Con A versus vehicle/Con A at the same time point. ND = not detected (value below the limit of detection; 3.2 pg/mL). Data represent the mean ± SEM of 4-6 independent replicates per treatment group. Data were combined from at least 2 separate experiments.

Figure 16 (cont'd)



NKT and T cells in TCDD-induced sensitization of mice to Con A hepatotoxicity

Both NKT and conventional $CD4^+$ T cells have been implicated as major effector cells in the development of Con A-induced liver injury, and the requirement for these cell types in the sensitization to Con A hepatotoxicity induced by TCDD pretreatment was evaluated. TCDD treatment alone did not cause liver injury in NKT cell deficient B6.129S6-Cd1d1/Cd1d2^{tm1Spb}/J (CD1d KO) mice, B6.129S7-*Rag1*^{tm1Mom}/J (RAG1 KO) mice lacking mature T cells, or C57Bl/6J (wild-type) mice (data not shown). TCDD-pretreated wild-type mice given Con A had increased plasma ALT activity compared to vehicle-pretreated, wild-type mice (Fig. 17A). Plasma ALT activity was reduced in CD1d KO mice regardless of TCDD treatment; however, ALT activity in CD1d KO mice treated with TCDD/Con A was reduced only to the level in wild-type mice treated with Con A. RAG1 KO mice were completely protected from liver injury induced by either vehicle/Con A or TCDD/Con A treatments.

In vehicle/Con A-treated mice, the plasma concentration of IFNγ was similar in wild-type and CD1d KO mice but undetectable in RAG1 KO mice (Fig. 17B). In mice treated with Con A, TCDD-pretreated, wild-type mice had increased concentrations of IFNγ compared to wild-type mice given Con A alone. The plasma concentration of IFNγ in TCDD/Con A-treated CD1d KO mice was not different from wild-type mice given the same treatment or from vehicle/Con A-treated CD1d KO mice. In RAG1 KO mice treated with TCDD/Con A, IFNγ was not detectable in plasma.

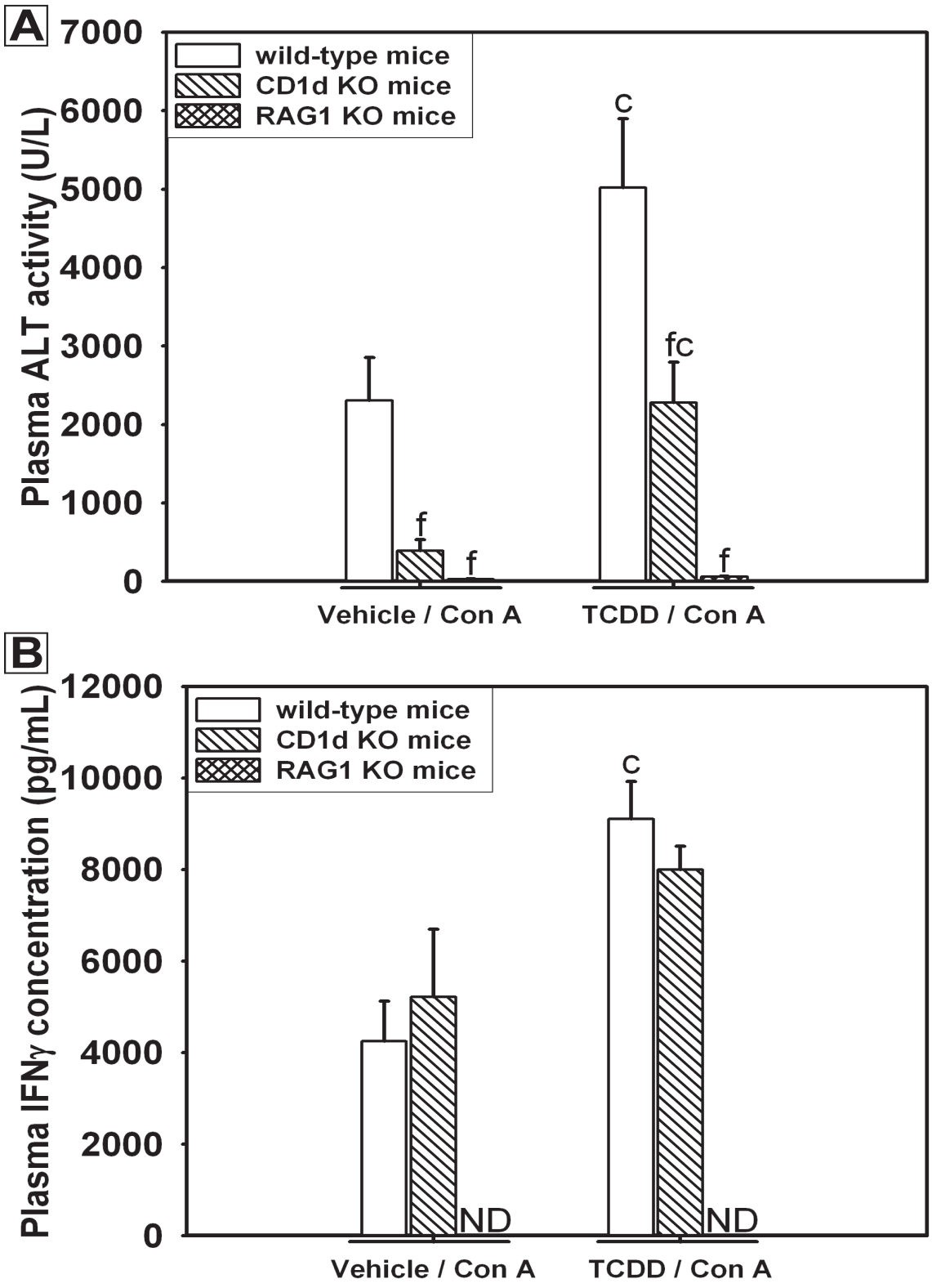


Figure 17: Liver injury (A) and plasma IFNγ concentration (B) after TCDD/Con A treatment in CD1d KO and RAG1 KO mice. Wild-type (open bars), B6.129S6-

Figure 17 (cont'd)

Cd1d1/Cd1d2^{tm1Spb}/J (CD1d KO) (striped bars), and B6.129S7-*Rag1*^{tm1Mom}/J (RAG1 KO) (cross-hatched bars) mice were treated as described in the legend to Fig. 14 with vehicle/Con A or TCDD/Con A. ALT activity in plasma was determined 8 h after Con A administration. c, $p<0.05$ TCDD/Con A versus vehicle/Con A in the same mouse genotype. f, $p<0.05$ versus the same treatment in wild-type mice. ND = not detected (value below the limit of detection). Data represent the mean ± SEM of 4-10 independent replicates per treatment group. Data were combined from at least 2 separate experiments.

Effect of TCDD pretreatment on in vivo and ex vivo activation of hepatic lymphocytes by Con A

Lymphocytes were isolated from livers of mice pretreated with vehicle or TCDD 2 h after administration of either saline or Con A. Expression of the early lymphocyte activation marker CD69 was evaluated by flow cytometry. TCDD pretreatment alone increased the percentage of activated CD69-positive NK ($NK1.1^+$, $CD3\varepsilon^-$) cells compared to vehicle pretreatment (Fig. 18A). Con A administration also increased the percentage of CD69-positive NK cells, and this response was further increased by TCDD pretreatment. An increase in the percentage of CD69-positive NKT ($NK1.1^+$, $CD3\varepsilon^+$) cells was only observed in the TCDD/Con A group. Con A administration increased the percentage of CD69-positive T ($NK1.1^-$, $CD3\varepsilon^+$) cells, but this response was not altered by TCDD-pretreatment.

The effect of TCDD pretreatment on ex vivo activation of lymphocytes by Con A was also evaluated. Hepatic leukocytes were isolated from mice treated 4 days earlier with TCDD or saline. They were exposed in culture to PBS (vehicle) or to Con A (7.5 μg/mL) for 5 h and analyzed by flow cytometry. A greater percentage of NK cells isolated from TCDD-pretreated mice were CD69-positive compared to those isolated from vehicle-pretreated mice (Fig. 18B). Con A stimulation of NK cells isolated from TCDD-pretreated mice, but not vehicle-pretreated mice, resulted in significantly increased percentage of CD69-positive cells. Con A activation increased the percentage of CD69-positive NKT cells, and pretreatment with TCDD increased the percentage of CD69-positive NKT cells in the absence and presence of Con A

stimulation. Exposure of T cells isolated from vehicle-pretreated mice to Con A led to an increased percentage of CD69-positive T cells, and this response was further increased in T cells isolated from TCDD-pretreated mice.

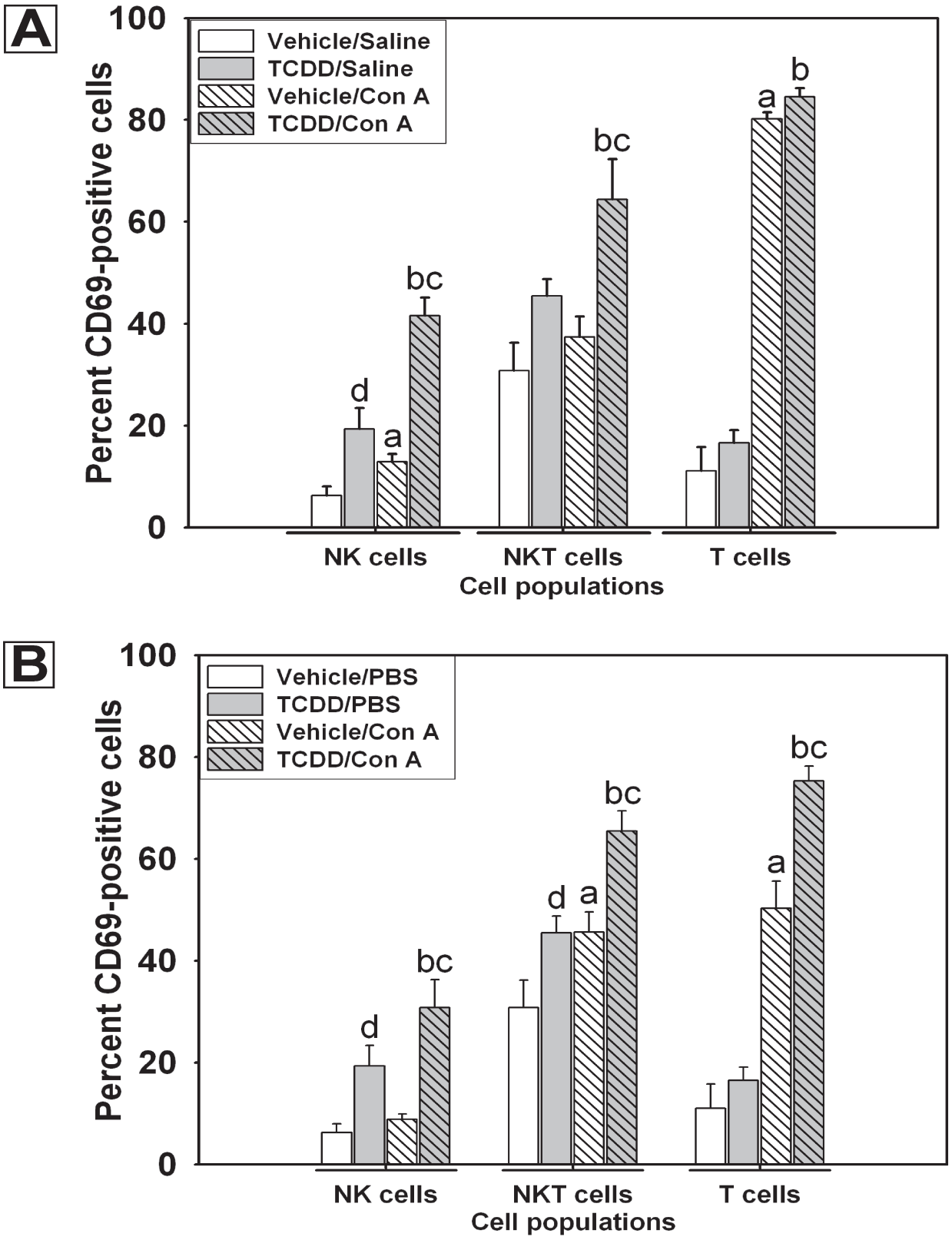


Figure 18: Effect of pretreatment with TCDD on in vivo and ex vivo activation of hepatic lymphocytes by Con A. (A) Mice were pretreated with vehicle (white bars) or

Figure 18 (cont'd)

TCDD (gray bars) as described in the legend to Fig. 14, and intrahepatic leukocytes were isolated 2 h after saline (open bars) or Con A (striped bars) administration. Cells were stained for expression of activation marker CD69 and analyzed by flow cytometry. NK cells were identified as ($NK1.1^+$,$CD3\varepsilon^-$), NKT cells as ($NK1.1^+$,$CD3\varepsilon^+$) and T cells as ($NK1.1^-$,$CD3\varepsilon^+$). (B) Intrahepatic leukocytes were isolated from mice treated 4 days earlier with vehicle (white bars) or 30 μg/kg TCDD (gray bars) and were treated ex-vivo with either PBS (open bars) or 7.5 μg/mL Con A (striped bars) in culture for 5 h. NK cells, NKT cells and T cells were stained for the lymphocyte activation marker CD69 and analyzed by flow cytometry. a, $p<0.05$ vehicle/Con A versus vehicle/saline in the same cell type. b, $p<0.05$ TCDD/Con A versus TCDD/saline in the same cell type. c, $p<0.05$ TCDD/Con A versus vehicle/Con A in the same cell type. d, $p<0.05$ TCDD/saline versus vehicle/saline in the same cell type. Data represent the mean ± SEM of 6-7 independent replicates per treatment group. Data were combined from 3 separate experiments.

Activation of NK cells in TCDD/Con A-induced liver injury

Flow cytometry was used to develop a more extensive time course of the activation of NK cells after TCDD/Con A treatment. Hepatic lymphocytes were isolated from mice at 0, 2, 3, 4 or 8 h after Con A administration. In vehicle/Con A-treated mice, increased percentages of CD69-positive NK cells were detected at 3, 4 and 8 h (Fig. 19A and B). TCDD pretreatment increased the percentage of CD69-positive NK cells compared to vehicle pretreatment at all times evaluated. The percentage of NK cells expressing the activating receptor NKG2d was increased 3 h after Con A administration (Fig. 19C). This response to Con A was not altered by pretreatment with TCDD.

The percentage of CD69-positive NK cells was decreased in RAG1 KO mice compared to wild-type or CD1d KO mice in both vehicle/Con A and TCDD/Con A treatments at 8 h (Fig. 20). In each genotype, TCDD/Con A treatment significantly increased the percentage of CD69-positive NK cells compared to vehicle/Con A treatment.

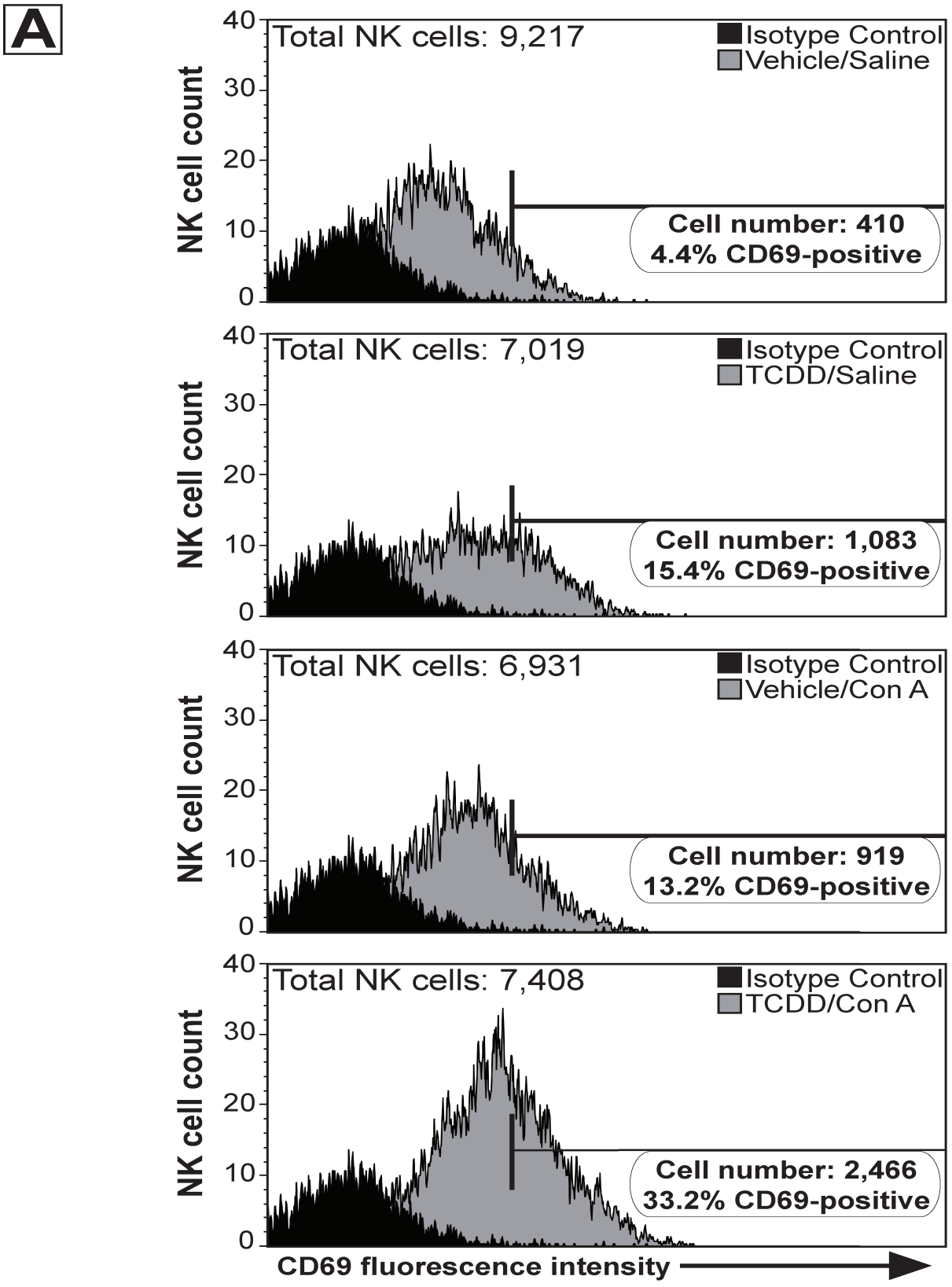
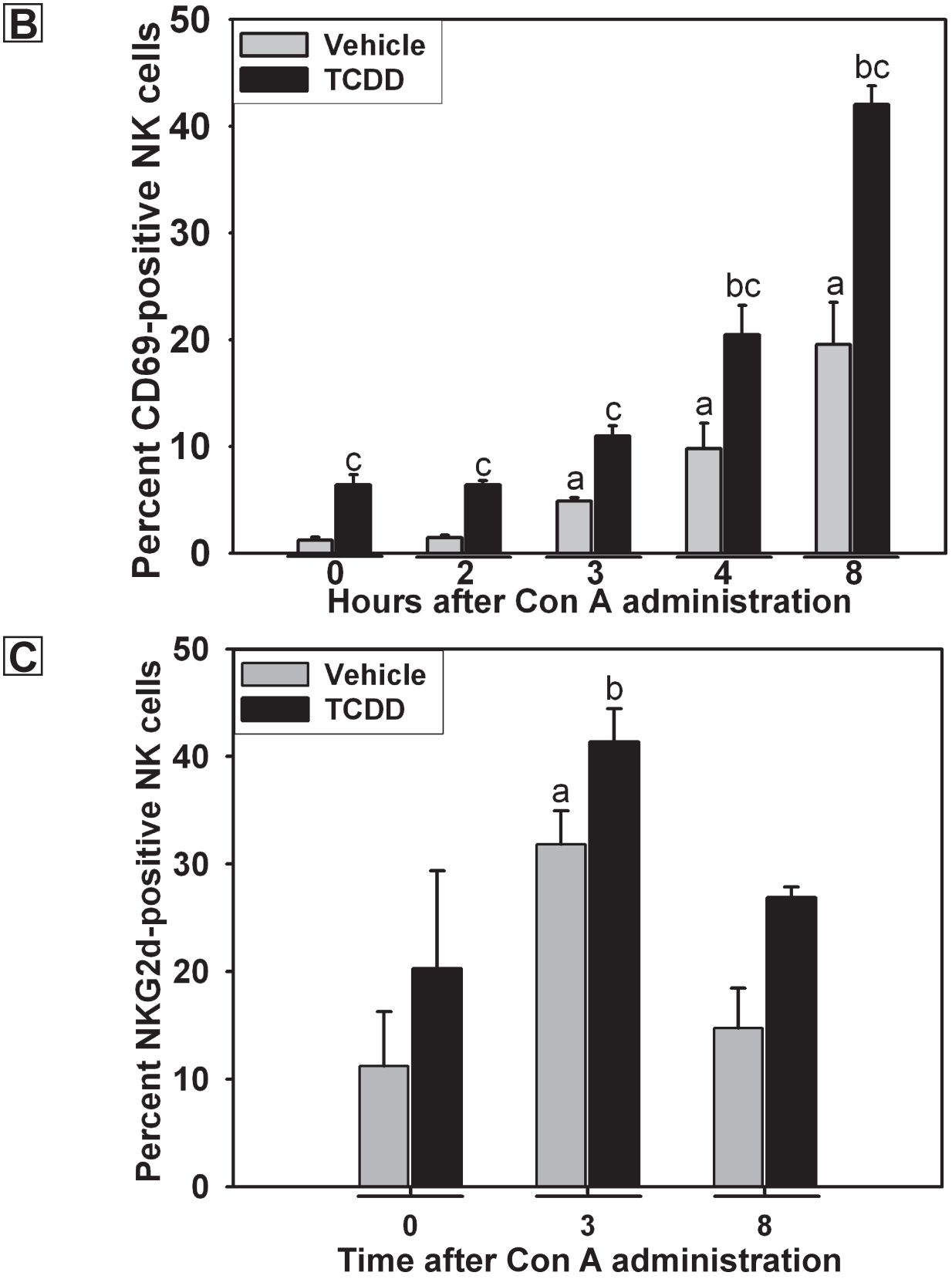


Figure 19: Activation of NK cells after TCDD/Con A treatment. (A) Representative

Figure 19 (cont'd)

histograms showing fluorescence intensity of CD69 staining on NK cells 4 h after Con A or saline administration. Treatments are indicated in the panels. The horizontal bar represents the area of positive staining based on the isotype control. (B) The percentage of NK cells staining positive for CD69 at 0, 2, 3, 4 and 8 h after Con A administration. a, $p<0.05$ versus vehicle pretreatment at 0 h. b, $p<0.05$ versus TCDD pretreatment at 0 h. c, $p<0.05$ TCDD/Con A versus vehicle/Con A at the same time point. Data represent the mean ± SEM of 4-7 independent replicates per treatment group. Data were collected from at least 2 separate experiments. (C) The percentage of NK cells staining positive for NKG2d at 0, 3 and 8 h after Con A or saline administration. a, $p<0.05$ versus vehicle pretreatment at 0 h. b, $p<0.05$ versus TCDD pretreatment at 0 h. Data represent the mean ± SEM of 3-5 independent replicates per treatment group. Data were combined from 2 separate experiments.

Figure 19 (cont'd)



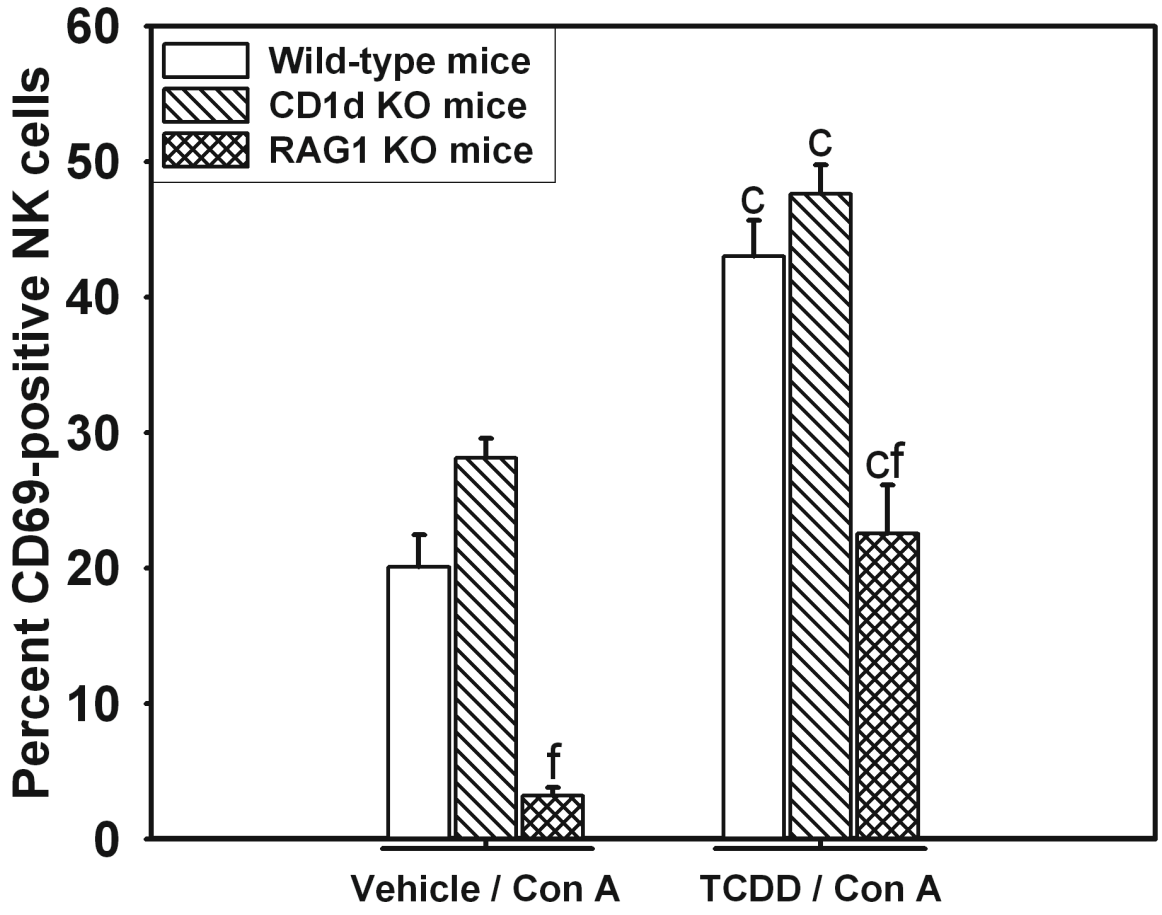


Figure 20: Activation of NK cells in CD1d KO and RAG1 KO mice after TCDD/Con A treatment. Wild-type (open bars), B6.129S6-Cd1d1/Cd1d2^{tm1Spb}/J (CD1d KO) (striped bars), and B6.129S7-*Rag1*^{tm1Mom}/J (RAG1 KO) (cross-hatched bars) mice were treated as described in the legend to Fig. 14 with vehicle/Con A or TCDD/Con A. Intrahepatic leukocytes were isolated from mice 8 h after Con A administration, and NK cells were stained for CD69 expression. c $p<0.05$ TCDD/Con A versus vehicle/Con A in the same mouse genotype. f $p<0.05$ versus the same treatment in wild-type. Data represent the mean ± SEM of 3-5 independent replicates per treatment group.

Role of NK cells in exacerbation of Con A hepatotoxicity by TCDD pretreatment

NK cells are not considered to be important effectors of liver injury caused by large doses (15-25 mg/kg) of Con A (Toyabe *et al.*, 1997; Takeda *et al.*, 2000); however, the TCDD-mediated activation of NK cells prompted the investigation of the importance of these cells in the development of liver injury after TCDD/Con A treatment. Pretreatment with TCDD led to increased plasma ALT activity in Con A (6 mg/kg)-treated mice given control serum (Fig. 21A). Anti-asialoGM1 depletion of NK cells significantly reduced injury in vehicle- and TCDD-pretreated mice administered 6 mg/kg Con A. In these experiments a separate group of mice was pretreated with vehicle and then given a larger dose (20 mg/kg) of Con A to compare the effects of anti-asialoGM1 treatment in our studies with results previously reported in the literature (Toyabe *et al.*, 1997). The plasma ALT activity in control serum-treated mice given this larger dose of Con A was comparable to ALT activity in TCDD/Con A-treated mice given control serum. Anti-asialoGM1 treatment did not protect against injury in mice given 20 mg/kg Con A. Compared to mice treated with control serum, anti-asialoGM1 treatment significantly reduced the concentration of IFNγ in plasma 8 h after Con A administration in all treatment groups (Fig. 21B).

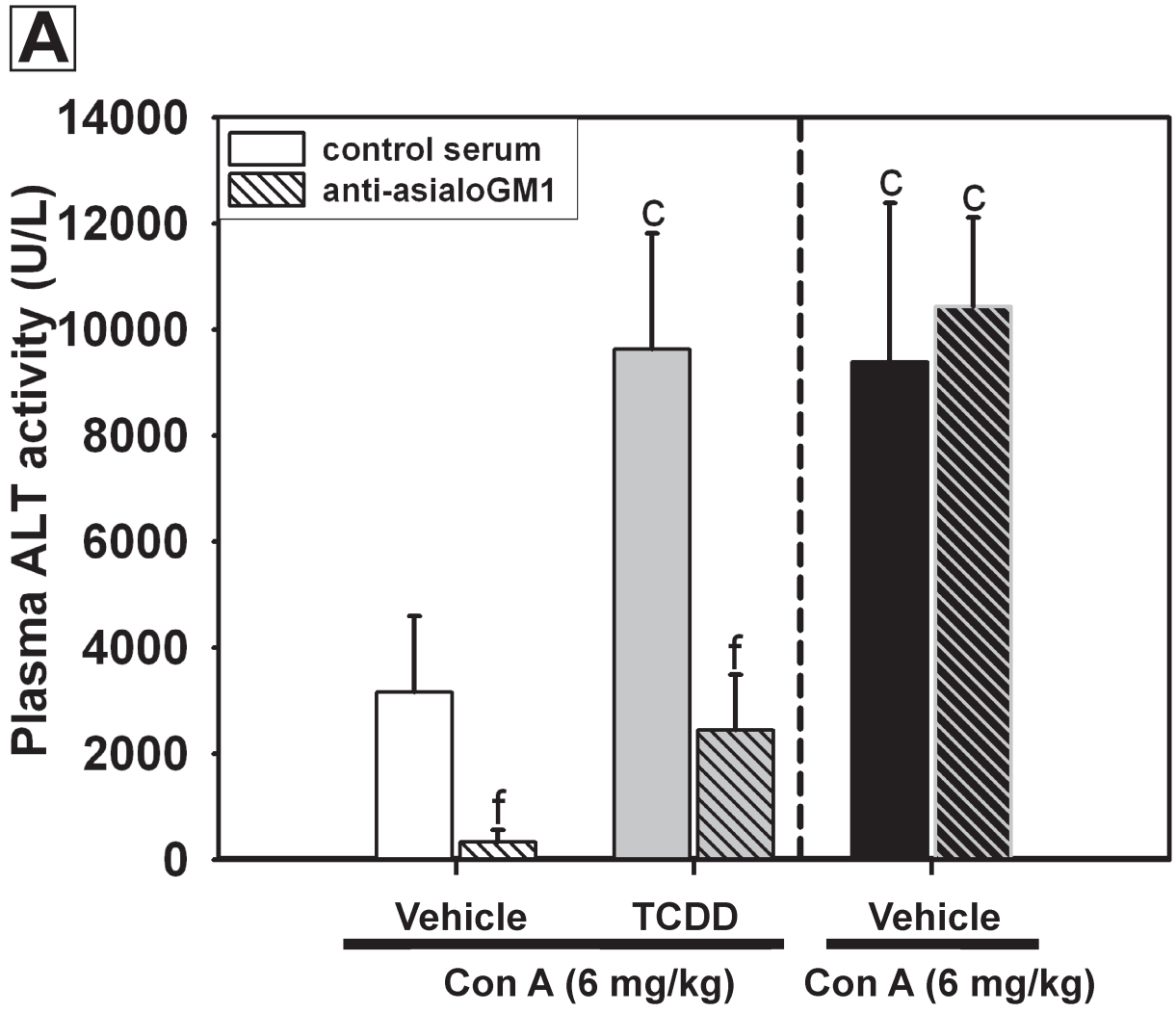
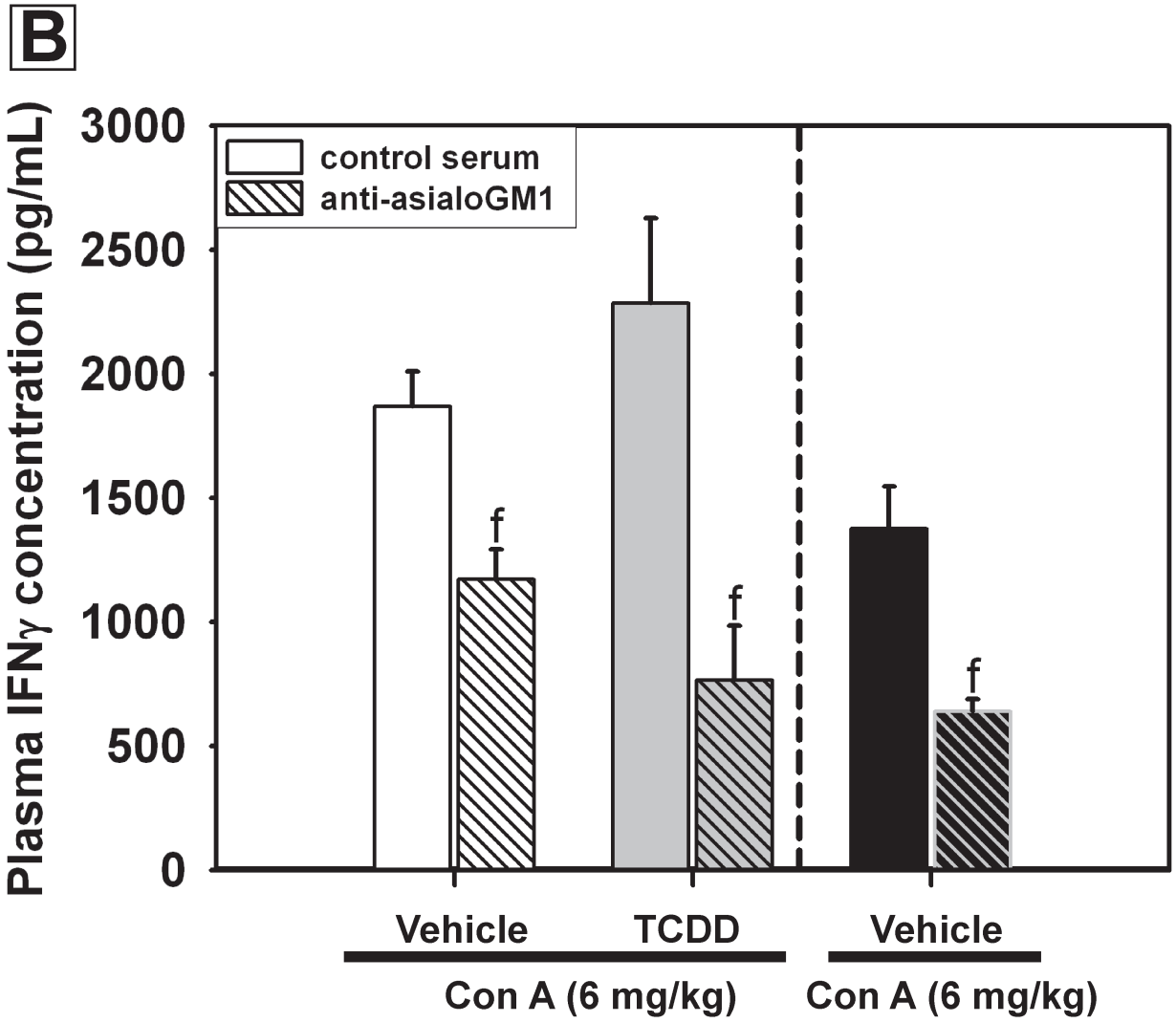


Figure 21: The effect of NK cell depletion on TCDD/Con A-mediated liver injury (A) and IFNγ production (B). Mice were treated as described in the legend to Fig. 14 with vehicle (white bars) or TCDD (gray bars) on day 0 then treated with either normal rabbit serum (open bars) or rabbit anti mouse/rat asialoGM1 polyclonal antibody (striped bars), as described in 3.3 *Materials and Methods,* 18 h prior to the administration of 6 mg/kg (white and gray bars) or 20 mg/kg (black bars) Con A. c, $p<0.05$ versus vehicle/6 mg/kg Con A in the same control serum or anti-asialo treatment. f, $p<0.05$ versus the same treatment with control serum. Data represent the mean ± SEM of 7-11

Figure 21 (cont'd)

independent replicates for all groups treated with 6 mg/kg Con A and 3-4 independent replicates for all groups treated with 20 mg/kg Con A. Data were combined from 2 separate experiments.



3.5 Discussion

We previously determined that TCDD increased the sensitivity of mice to liver injury in a model of autoimmune hepatitis induced by the administration of Con A (Fullerton *et al.*, 2013). Here we present data demonstrating that a 10-fold smaller dose of TCDD produced an equivalent degree of liver injury in Con A-treated mice (Fig. 13A). Decreasing the TCDD dose to 0.3 μg/kg resulted in loss of the TCDD-induced sensitization. These results suggest that the threshold dose for the exacerbation of Con A-induced liver injury is between 0.3 and 3 μg/kg TCDD.

Toxicokinetic studies revealed that accumulation of TCDD in the liver peaks 4 days following treatment, and the elimination half-life of TCDD is approximately 8 days in mice (Birnbaum, 1986). TCDD exacerbated the hepatotoxic response to Con A when given 4, 7, or 10 days earlier (Fig. 13B), and the greatest response was seen with Con A administration 7 days after TCDD-pretreatment. Since liver concentration decreases from 4 to 7 days after a single TCDD administration (Birnbaum, 1986; Kopec *et al.*, 2008) our results suggest that the degree of injury in TCDD/Con A-treated mice is not simply related to the hepatic concentration of TCDD at the time of Con A administration. Nonetheless, the TCDD-induced sensitization to Con A is a persistent effect.

A number of chemokines are responsible for the recruitment of inflammatory cells into the liver during the development of injury. KC and MIP-2 both belong to the CXC type chemokine family and act on CXC receptor 2. These chemokines are synthesized by activated tissue macrophages. KC and MIP-2 perform similar functions to increase neutrophil egress from the bone marrow and mediate transmigration of these cells into the peripheral tissues (Lee *et al.*, 1995; De Filippo *et al.*, 2008; Sadik *et al.*, 2011).

MCP-1 is a CCL type chemokine responsible for the recruitment of monocytes to sites of inflammation (Zimmermann *et al.*, 2012). TCDD treatment enhanced the expression of MCP-1 and KC and increased the recruitment of neutrophils and monocytes to sites of tissue injury (Vogel *et al.*, 2007). Con A treatment induced the production of MIP-2 in a TNFα-dependent manner, and neutralization of MIP-2 decreased the accumulation of neutrophils in the liver and reduced hepatocellular injury resulting from Con A administration (Nakamura *et al.*, 2001). In the studies presented here, administration of Con A induced KC, MIP-2 and MCP-1 production. Although TCDD treatment alone did not affect the plasma concentration of these chemokines, in Con A-treated mice TCDD increased the concentration of MIP-2 in plasma (Fig 14A-C).

Neutrophils contribute to the development of injury and to the production of IFNγ by lymphocytes in Con A-treated mice (Hatada *et al.*, 2005), and neutrophil depletion reduced the severity of liver injury (Bonder *et al.*, 2004). Despite the increased concentration of MIP-2 in plasma in TCDD/Con A-treated mice, TCDD pretreatment did not alter the accumulation of neutrophils in the liver in response to Con A (Fig. 15). Based on these results, it is unlikely that neutrophils play an important role in the exacerbation of Con A-induced liver injury by TCDD.

Macrophages are another hepatic immune cell known to play an accessory role in hepatitis and liver damage induced by large doses of Con A. Hepatic macrophages contribute to injury via the production of inflammatory mediators such as TNFα, which can induce hepatic parenchymal cell death (Gantner *et al.*, 1996; Schumann *et al.*, 2000). In addition, stimulated macrophages produce IL-12 and IL-27, which activate hepatic lymphocytes and enhance cytolytic activity of NK and NKT cells while promoting

Th1 polarization of T cells. IL-12 and IL-27 also increase the production of IFNγ from NK and $CD4^+$ T cells (Pflanz *et al.*, 2002; Vignali and Kuchroo, 2012). IFNγ and TNFα can act synergistically to kill hepatic parenchymal cells (Adamson and Billings, 1993). In studies presented here, TCDD-pretreatment of mice given Con A did not change the percentage of macrophages (F4/80-positive cells) recovered from the liver (Fig. 16A). In addition, there was no difference in plasma concentration of IL-12 in TCDD-pretreated and vehicle-pretreated mice at 3 h (Fig. 16B), a time before the development of liver injury (Fullerton *et al.*, 2013). Furthermore, TCDD pretreatment decreased IL-12 production induced by Con A. TCDD pretreatment also did not alter hepatic mRNA expression of IL-12 or IL-27 (Fig. 16C and D). Collectively, these results suggest that while macrophages are likely involved in the development of injury, they do not play an important role in the increased sensitivity to Con A observed in TCDD-pretreated mice.

The importance of NKT cells and conventional $CD4^+$ T cells has been well documented in the development of Con A-induced liver injury. In particular, NKT cells are required for the development of injury and directly contribute to the killing of hepatic parenchymal cells via expression of cytolytic effectors such as Fas ligand (FasL) (Seino *et al.*, 1997; Tagawa *et al.*, 1998). A number of studies have demonstrated protection from Con A-induced liver injury in mice deficient in NKT cells and in Rag1 KO mice lacking mature T cells (Kaneko *et al.*, 2000; Takeda *et al.*, 2000). Using a smaller dose of Con A, we saw similar results (Fig. 17A). TCDD/Con A-induced liver injury was abolished in RAG1 KO mice, confirming the essential role of T cells in the pathogenesis. Pretreatment with TCDD increases the activation of NKT cells and promotes expression of FasL in these cells after Con A administration (Fullerton *et al.*, 2013). However,

CD1d KO mice were only partially protected from TCDD/Con A-induced liver injury. In fact, ALT activity in the plasma of TCDD/Con A-treated CD1d KO mice was comparable to the ALT activity measured in the plasma of vehicle/Con A-treated wild-type mice. These results suggested that a cell type in addition to NKT cells contributes to TCDD-induced sensitization to Con A hepatotoxicity.

To identify this cell type, the activation of hepatic lymphocytes was assessed. TCDD/Con A treatment in vivo resulted in a greater percentage of activated NK and NKT cells than either treatment alone (Fig. 18A). Interestingly, this response did not require exposure to Con A in vivo; a greater percentage of NK and NKT cells isolated from TCDD-treated mice than from vehicle-treated mice became activated upon exposure to Con A ex vivo (Fig. 18B). The ex vivo treatment eliminates the possibility that the increased activation results from danger signals released from damaged parenchymal cells in TCDD/Con A-treated mice. These results suggest that the increased activation of lymphocytes in TCDD-pretreated mice is the result of the direct action of Con A on hepatic lymphocytes or a response to inflammatory mediators released from Con A-activated leukocytes.

One interesting result was that TCDD alone increased activation of NK cells (Fig. 18). Upon further investigation, it was observed that TCDD pretreatment increased the activation of NK cells at all times examined (0-8 h) after Con A administration (Fig 19A and B). These results were unexpected because the role of NK cells as effectors in Con A-induced liver injury has been discounted (Kaneko *et al.*, 2000; Takeda *et al.*, 2000; Dong *et al.*, 2007). Despite not being associated with Con A-induced liver injury, NK cells are known to play important roles in human autoimmune disease and

inflammatory liver injury (Schleinitz *et al.*, 2010). For example, NK cell activation is increased in the livers of patients with primary biliary cirrhosis (Chuang *et al.*, 2006; Shimoda *et al.*, 2011). In addition, a clear role for NK cells has been demonstrated in other animal models of immune-mediated liver injury. The administration of alpha-galactoceramide causes liver injury that is mediated by both NK and NKT cells (Trobonjaca *et al.*, 2002). In alpha-galactoceramide-induced liver injury, NKT cells are responsible for activating NK cells by producing IFNγ (Carnaud *et al.*, 1999; Eberl and MacDonald, 2000). In studies presented here, there was no difference in the percentage of CD69-positive NK cells detected after TCDD/Con A treatment of wild-type and CD1d KO mice (Fig. 20). This result indicates that increased NK cell activation is independent of the presence of NKT cells in this model. Interestingly, after either vehicle/Con A or TCDD/Con A treatment the percentage of CD69 positive NK cells in RAG1 KO mice was decreased compared to wild-type and CD1d KO mice indicating a role for conventional T cells in the activation of NK cells following TCDD/Con A administration.

To determine if increased NK cell activity could be a contributor to injury in TCDD/Con A treated mice, NK cells were depleted with anti-asialoGM1 prior to the administration of Con A. As previously mentioned, NK cell activity is reported to be inconsequential in the development of hepatitis from large doses of Con A (Toyabe *et al.*, 1997). Our results are consistent with this finding (Fig. 21A): anti-asialoGM1 did not diminish injury in mice treated with 20 mg/kg Con A. This treatment resulted in injury comparable to TCDD-pretreated mice given only 6 mg/kg Con A. However, in vehicle- or TCDD-pretreated mice administered 6 mg/kg Con A, NK cell depletion by anti-

asialoGM1 significantly protected against the development of injury. The reduction in hepatotoxicity was accompanied by a decrease in the concentration of IFNγ (Fig. 21B) which is critical to the development of liver injury.

These results clearly demonstrate a role for increased NK cell activation by TCDD pretreatment in the development of hepatotoxicity from TCDD/Con A administration. Although the mechanisms underlying this response are not yet determined, a number of possibilities exist. TCDD treatment of mice increases the activity of NK cells in the spleen and blood (Funseth and Ilback, 1992). In studies of human cohorts exposed occupationally to TCDD, an increase in the number of NK cells was observed in peripheral blood. In addition, TCDD treatment alters the expression of numerous immune-related genes that include coregulatory NK cell receptors (Sun *et al.*, 2004; Kerkvliet, 2009). TCDD pretreatment did not alter the expression of the stimulatory receptor NKG2d in NK cells after Con A treatment (Fig. 19C), but there are many other coregulatory receptors that were not investigated in this model. In addition, TCDD pretreatment did not alter the production of IL-12 after Con A administration; however, TCDD can increase the expression of IL-12 receptor β1 and might increase the sensitivity of NK cells to the stimulatory effects of IL-12 via that mechanism (Kerkvliet, 2009).

In summary, the results presented here demonstrate that pretreatment with TCDD exacerbates liver injury in a model of autoimmune hepatitis induced by Con A administration. Furthermore, TCDD pretreatment increased the activation of NK cells by Con A, and NK cells play an important role in the development of TCDD/Con A-induced liver injury. This mechanism is distinctly different from the development of

comparable injury obtained by administration of a larger dose of Con A. As such, the enhanced immune response induced by TCDD treatment warrants further investigation into mechanisms of NK cell activation and the larger role that exposure to TCDD and other environmental xenobiotics that influence AhR signaling might play in the development of autoimmune liver disease.

CHAPTER 4

4.1 Summary and conclusions

My dissertation research primarily focuses on two major issues relevant to the evaluation of public health and environmental safety. The first addresses the idea that, among other environmental factors, AhR ligands such as TCDD may be important contributors to the risk of developing autoimmune hepatitis. The second further characterizes the effects of TCDD on the hepatic immune system and compares these effects within the microenvironment of the liver to the wealth of information available regarding immunotoxic effects of TCDD in other organs. The research presented in this dissertation was conducted to test the hypothesis that TCDD pretreatment enhances the response of intrahepatic innate immune cells to the inflammatory stimulus Con A, resulting in increased hepatotoxicity in a mouse model of autoimmune liver disease.

Initial experiments indicated that pretreatment with TCDD sensitizes mice to Con A and exacerbates immune-mediated liver injury. This response was observed at multiple doses of Con A (Fig. 3A), including those that were not hepatotoxic alone (4 mg/kg), as well as doses that caused mild injury when given alone (6-10 mg/kg) indicating that the effect of TCDD not only decreased the threshold for toxicity, but also exacerbated existing mild to moderate hepatotoxicity induced by Con A alone. In addition to the effect on the sensitivity to Con A, pretreatment with TCDD altered the time course for development of Con A-induced hepatitis. In mice pretreated with TCDD, Con A-induced liver injury was evident at earlier time points (4 h after Con A administration) than in vehicle/Con-treated mice (6 h after Con A administration). Furthermore, even though liver injury in both treatment groups peaked 8 h after Con A administration, the ALT activity in plasma remained elevated at peak levels as late as

24 h after TCDD/Con A treatment while plasma ALT activity in vehicle/Con A-treated mice was reduced by 24 h (Fig. 3B). These results indicate that pretreatment with TCDD accelerated the development of injury and increased the duration of Con A-induced hepatitis. In addition, the TCDD-induced sensitization to Con A was observed with multiple doses of TCDD (Fig. 13A) and sensitization was a persistent response that affected injury as late as 10 days after pretreatment (Fig. 13B). Interestingly, 10 days is longer than one half-life of elimination for TCDD in the liver (Birnbaum, 1986; Kopec *et al.*, 2008). The ability of TCDD to exacerbate Con A-mediated liver injury while not requiring particularly exacting dose-response relationships or precise timing between pretreatment and Con A administration implies that this relationship is quite robust and could be broadly applicable to other instances of TCDD exposure and immune-mediated liver injury. The results also suggest that the TCDD-mediated effects in this liver injury model are not particularly acute, and a similar response in humans could persist for some time following TCDD exposure.

The balance of inflammatory and anti-inflammatory cytokines plays an important and well-characterized role in the development of immune-mediated liver injury. This is certainly true for Con A-induced liver injury. In my attempts to discern a mechanism for TCDD-mediated exacerbation of Con A-induced hepatotoxicity I characterized the involvement of a number of cytokines. During this process some interesting discrepancies arose between the typical cytokine profiles reported in the literature for liver injury induced by large doses (ex. 20 mg/kg) of Con A alone (Sass *et al.*, 2002) and the cytokine profile of TCDD/Con A-induced liver injury. Of particular interest was the result that, of the major cytokines reported to contribute to Con A-induced hepatitis

including TNFα, IL-2, IL-4, IL-12 and IFNγ, only IFNγ was significantly increased by pretreatment with TCDD (Fig. 5).

IFNγ is not generally considered a potent, direct inducer of hepatocellular death, however through activation of janus-kinase and signal transducer and activator of transcription (JAK-STAT) signaling IFNγ can activate leukocytes and interact with downstream signaling from cytokines such as TNFα to enhance the induction of apoptosis in liver parenchymal cells (Tura *et al.*, 2001). Therefore, despite the lack of alterations to the induction of other inflammatory cytokines, increased IFNγ represents an important finding requiring further investigation. Subsequent experiments demonstrated that IFNγ is necessary for the development of TCDD/Con A-induced liver injury as indicated by protection observed in IFNγ KO mice (Fig 7). This is consistent with reports in the literature indicating that the mechanism of injury after treatment with Con A alone is heavily biased towards the role of IFNγ (Kusters *et al.*, 1996; Jaruga *et al.*, 2004).

Pretreatment with TCDD decreased the expression of IL-4, which is an important contributor to autocrine signaling involved in the activation of NKT cells (Fig 5). In addition, TCDD pretreatment also reduced the plasma concentration of IL-2 after Con A (Fig 5). The decreased production of IL-2 and IL-4 by TCDD treatment is consistent with the well established role of AhR signaling in the inhibition of Th1 and Th2 T cell differentiation (Kerkvliet, 2009).

Different cytokine profiles were reported in response to administration of nonhepatotoxic (3 mg/kg) versus hepatotoxic (15 mg/kg) doses of Con A (Xu *et al.*, 2006). Smaller doses of Con A are biased towards the production of anti-inflammatory

cytokines IL-6 and IL-10. A preliminary hypothesis was that pretreatment with TCDD could enhance the development of liver injury by decreasing the Con A-induced production of these protective cytokines. However, my results indicated this was not the case. Pretreatment with TCDD did not alter the production of either IL-6 or IL-10 by Con A administration (Fig. 5). Furthermore I investigated a possible role for IL-17 and IL-22 in the development of TCDD/Con A-induced liver injury. Both cytokines are induced by AhR activation and are reported to influence the development of Con A-induced liver injury as well as alter T cell differentiation (Zenewicz *et al.*, 2007; Martin *et al.*, 2009; Alam *et al.*, 2010; Hammerich *et al.*, 2011; Xu *et al.*, 2011). However, at the time points I investigated, Con A-induced hepatic mRNA expression of IL-17 and IL-22 was not altered by pretreatment with TCDD (Fig 6), indicating that these cytokines do not play a role in the increased sensitivity to Con A induced by pretreatment with TCDD.

Pretreatment with TCDD significantly increased a number of chemotactic factors induced by Con A administration. The plasma concentration of MIP-2, a chemokine known to contribute to the development of injury in Con A-induced hepatitis, was increased by pretreatment with TCDD (Fig.14) (Nakamura *et al.*, 2001). In addition, TCDD increased the hepatic mRNA expression of the adhesion molecule ICAM-1 (Fig. 10), which plays an important role in leukocyte extravasation into the hepatic parenchyma. The importance of leukocyte extravasation in TCDD/Con A-induced liver injury was demonstrated by the protection provided by pretreatment with CD18 antiserum which prevents binding of integrins to ICAM-1 and inhibits extravasation of leukocytes from the vasculature into the liver (Fig. 10.) Despite the findings that pretreatment with TCDD increased chemokine production and adhesion molecule

expression in the liver, I did not detect a TCDD-mediated increase in the accumulation of inflammatory immune cells in the liver. There were no significant differences in the accumulation in the liver, or relative percentages of lymphocytes (Fig. 8) neutrophils (Fig. 15) or hepatic macrophages (Fig. 16) that could account for the increased liver injury in TCDD-pretreated mice. However, evaluation of the expression of activation markers CD69 and CD25 on intrahepatic lymphocyte populations revealed that pretreatment with TCDD increased the activation of NKT cells, a cell type well characterized as a main effector cell in the development of Con A-induced liver injury (Toyabe *et al.*, 1997; Takeda *et al.*, 2000). The ability of TCDD to alter the activation of NKT cells by Con A or any other stimulus has not been well described in the literature and represents an important finding given the importance of this cell type to the pathophysiology of numerous immune-mediated diseases (Smyth *et al.*, 2002a; Dennert and Aswad, 2006; Yamamura *et al.*, 2007; Gao *et al.*, 2009).

IFNγ increases lymphocyte expression of cytolytic effectors such as FasL (Boselli *et al.*, 2007) and pretreatment with TCDD increased the percentage of NKT cells expressing FasL after Con A administration (Fig. 11). The cytolytic action of FasL on hepatic parenchymal cells is a major contributor to Con A-induced liver injury (Seino *et al.*, 1997; Takeda *et al.*, 2000). The importance of FasL to the development of TCDD/Con A-induced liver injury was demonstrated by results demonstrating that *gld/gld* (FasL KO) mice are protected (Fig. 12). These results indicate that pretreatment with TCDD enhanced both the activation and cytolytic potential of NKT cells in Con A-induced liver injury. I then evaluated the development TCDD/Con A-mediated liver injury in NKT-cell deficient CD1d KO mice. The results were particularly intriguing.

Consistent with reports in the literature, vehicle/Con A-treated CD1d KO mice were protected from liver injury, whereas in TCDD/Con A-treated CD1d KO mice injury was only partially attenuated (Fig. 17). Interestingly, TCDD/Con A-treated CD1d KO mice developed injury equivalent to that of vehicle/Con A-treated wild-type mice indicating that an additional cell type was likely to be contributing to the increased injury in TCDD/Con A treatment.

Upon further investigation I determined that pretreatment with TCDD enhanced the activation of NK cells in the presence and absence of Con A (Fig. 18). At every time point examined after the administration of Con A, mice-pretreated with TCDD had increased percentages of activated NK cells. In addition, pretreatment with TCDD resulted in increased percentages of CD69-positive NK cells in CD1d KO and in Rag1 KO mice. Accordingly, treatment with TCDD increases NK cell activation in the absence of NKT and/or T cells (Fig. 20) to provide co-stimulatory signals. These results indicate that TCDD alone is sufficient to induce NK cell activation and pretreatment with TCDD increases Con A-induced activation of NK cells. The literature on Con A-induced hepatitis suggests that NK cells do not play a major role in the development of liver injury (Toyabe *et al.*, 1997). This conclusion was consistent with our finding that depletion of NK cells did not protect against a large dose (20 mg/kg) of Con A (Fig. 21). In contrast, NK cell depletion in our model significantly attenuated TCDD/Con A-induced liver injury (Fig. 21). Taken together, the data indicate that both NKT and NK cells are significant contributors to the development of TCDD/Con A-induced liver injury.

To summarize the major findings, pretreatment with TCDD exacerbated the development of Con A-induced liver injury (Fig. 3). Pretreatment with TCDD increased

liver injury by increasing the production of IFNγ (Fig. 5), an inflammatory cytokine required for the development of liver injury (Fig. 6). IFNγ is capable of mediating a number of effects on both immune and parenchymal cells. TCDD pretreatment also increased the activation (Fig. 9) and cytolytic potential (Fig. 11) of NKT cells, a main effector cell in Con A-induced liver injury. Furthermore, TCDD/Con A-treatment induced liver injury in CD1d KO mice (Fig. 17) through the increased activation of NK cells (Fig. 19) that were determined to be important contributors to TCDD/Con A-induced liver injury (Fig. 21). Based on these results, a proposed scheme of the mechanism through which TCDD exacerbates Con A-induced liver injury is outlined in Figure 22.

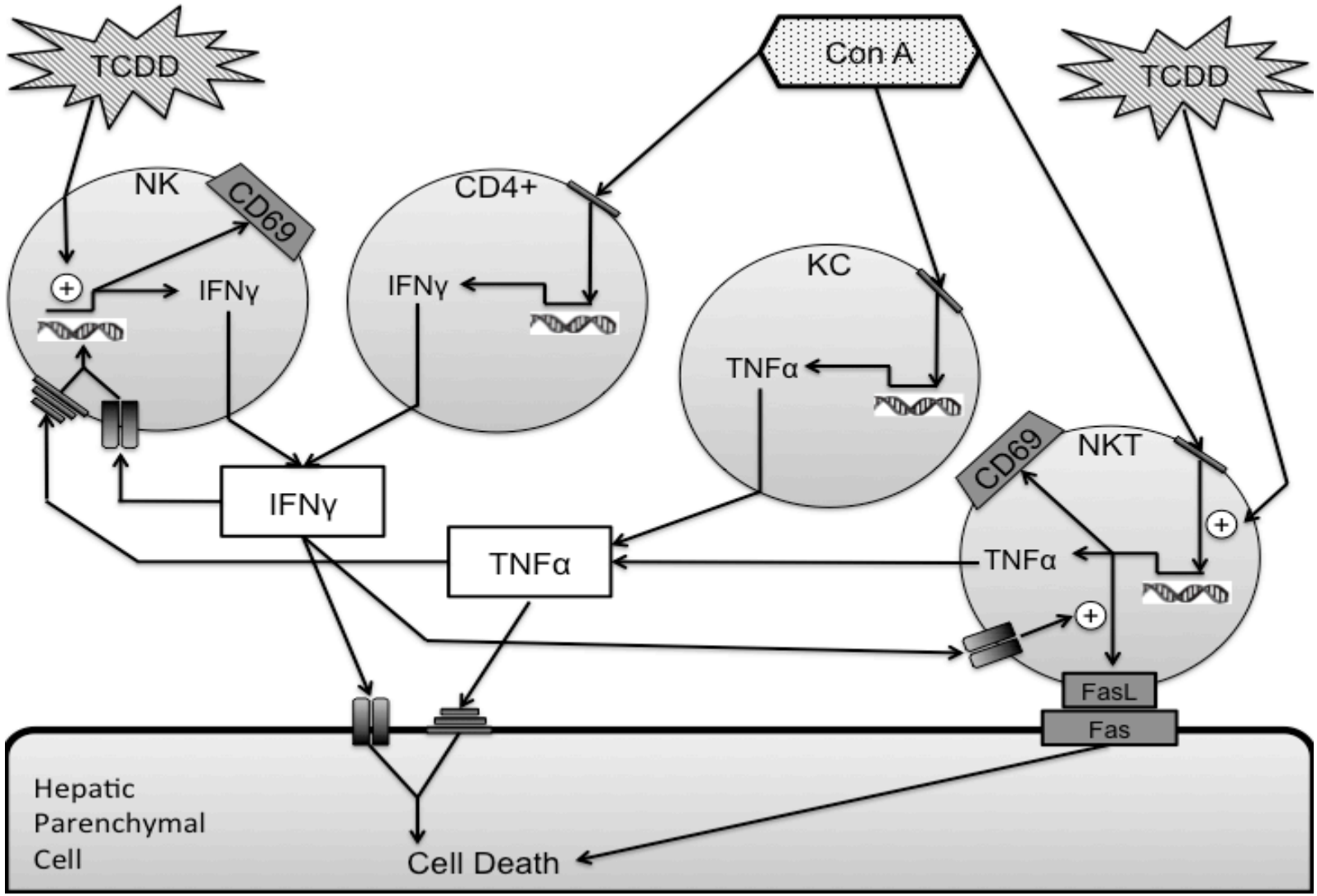


Figure 22: Proposed pathway by which TCDD-pretreatment exacerbates Con A-induced liver injury. Con A activates NKT, CD4+ T-cells and Kupffer cells, increasing production of cytokines such as IFNγ and TNFα as well as

Figure 22 (cont'd)

expression of cytolytic molecules such as Fas ligand. These mediators directly kill hepatic parenchymal cells. TCDD pretreatment increased NKT cell activation and FasL expression while also activating NK cells and increasing IFNγ production resulting in increased HPC death. Also depicted are positive feedback loops for IFNγ and TNFα. Con A: Concanavalin A, TCDD: 2,3,7,8 tetrachlorodibenzo-para-dioxin, CD4+: conventional CD4+ T cells, NKT: natural killer T cells, NK: natural killer cells, KC: Kupffer cells, CD69: early lymphocyte activation marker, IFNγ: interferon gamma, TNFα: tumor necrosis factor alpha.

The studies presented in this dissertation attempt to characterize the effects of TCDD on immune-mediated liver injury. This category of liver injury including viral and autoimmune hepatitis accounts for approximately 15 percent of acute liver failure cases in the United States (Lee *et al.*, 2008), and factors that alter the response of hepatic immune cells and contribute to the pathophysiology of these diseases are an important public health concern. As the liver is a major target organ for TCDD toxicity as well as a primary site of distribution in bodily tissues following exposure, it is important to thoroughly understand the effects of TCDD on the activation and response of intrahepatic immune cells to various stimuli. In this study, pretreatment with doses of TCDD that achieve hepatic concentrations comparable to reported tissue concentrations in human exposure studies was sufficient to induce persistent sensitivity to Con A-mediated liver injury that lasted as long as one half-life of TCDD elimination from the liver. If this response can be translated to comparable human toxicokinetics, exposure to TCDD might result in the increased sensitivity of individuals to immune-mediated liver injury that persists for as long as one half-life of TCDD elimination in humans (5-11 years).

The results presented here demonstrate that TCDD is capable of exacerbating liver injury by enhancing the activation and function of hepatic immune cells. While a wealth of information exists regarding the hepatotoxicity of TCDD, the vast majority of data available on the effects of TCDD on immune cell function is based on immune cells of the spleen, thymus, lymph nodes, and peripheral circulation. While that information is instructive for purposes of comparing results from the studies presented here and to suggest possible mechanisms of TCDD toxicity, there is insufficient data on whether

immune cells existing within the unique microenvironment of the liver can be expected to respond to TCDD in a manner similar to what has been reported for immune cells in other organs. As previously described in the Introduction (section 1.3), the intrahepatic immune cells possess phenotypic qualities that render them functionally distinct from immune cells of other organs and as such, the information provided in this dissertation will contribute to comparisons of TCDD-mediated immune effects between cells of the liver and those of other organs. The effects of TCDD on NK and NKT cells are not well characterized in the literature and the effects on hepatic NK and NKT cells are almost completely unknown. This dissertation presents the novel finding that TCDD exacerbates injury in a model of autoimmune hepatitis by enhancing the activation of these immune cell types and increasing the cytolytic killing of parenchymal cells. Furthermore, it is of particular interest that in this instance, TCDD not only exacerbated established mechanisms of Con A-induced liver injury (NKT cell activation and IFNγ production) but also altered the pathophysiology of liver injury to include the activity of NK cells.

These results suggest that TCDD has a multitude of effects on the response of hepatic immune cells. In this model, TCDD appears to enhance their response to stimulus. In addition, activation of AhR signaling by exposure to TCDD or other ligands may be a significant contributor to the development or exacerbation of autoimmune liver injury and potentially play a role in the development of other types of immune-mediated liver injury.

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