

DIOXIN-INDUCED DEREGULATION OF NEUTROPHIL RECRUITMENT TO THE
LUNGS OF MICE INFECTED WITH INFLUENZA VIRUS

By

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Abstract

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The pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; dioxin) has the highest binding affinity for an orphan transcriptional regulator called the aryl hydrocarbon receptor (AhR). Immune modulation is one of the most sensitive adverse health effects observed in rodents treated with the AhR ligand TCDD, and numerous studies have shown that exposure to TCDD severely suppresses adaptive immunity. In contrast, a limited number of studies have examined the effects of TCDD on the inflammatory response. Studies conducted by us and others have shown that exposure to TCDD enhances inflammation, characterized by increased number of neutrophils at the site of antigen challenge. However, the mechanism underlying this exacerbated neutrophilia remains to be elucidated.

The overall goal of this dissertation was to determine the mechanism that underlies the enhanced number of neutrophils in the lungs of TCDD-treated, influenza virus-infected mice. In these studies, we demonstrated that AhR activation directly mediates the excess number of neutrophils in lungs of TCDD-exposed, infected mice. Furthermore, we determined that *in vivo* depletion of neutrophils in TCDD-treated mice significantly improved survival following infection with influenza virus. However, our data indicate that exposure to TCDD did not elevate

levels of soluble neutrophil-chemoattractants in the lung, up-regulate expression of adhesion molecules on pulmonary neutrophils, or delay pulmonary neutrophil apoptosis in infected mice. Furthermore, treatment with TCDD did not increase pulmonary vascular permeability, leading to leakage of neutrophils from the blood stream to the lungs.

Additionally, we have shown that exposure to TCDD did not systemically enhance the number of neutrophils. In the absence of a direct effect of TCDD on pulmonary neutrophils and given our finding that the excess neutrophilia was restricted to the site of antigen challenge (i.e., the lung), we determined whether AhR-mediated events within or external to the immune system underlie the exacerbated neutrophilia. We found that TCDD-treated, infected CD45.2AhR^{-/-} → CD45.1AhR^{+/+} bone marrow chimeric mice had an excess of pulmonary neutrophils, indicating that AhR-driven events external to the immune system mediate the enhanced neutrophilia. These novel findings indicate that AhR-driven events external to the immune system underlie deregulated neutrophil recruitment to the lungs of mice treated with TCDD.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS.....	iii
ABSTRACT.....	vi
LIST OF TABLES.....	xi
LIST OF FIGURES.....	xii
CHAPTER	
1. INTRODUCTION	
Polyhalogenated aromatic hydrocarbons	1
Sources of polyhalogenated hydrocarbons.....	2
Mechanism of toxicity.....	6
Toxic effects of exposure to PHAHs in humans, domestic animals, and wildlife.....	14
Toxicity in laboratory animals.....	17
TCDD immunotoxicity.....	21
Chronic inflammatory lung diseases in humans and excess number of neutrophils.....	31
Objectives and hypotheses.....	32
2. ACTIVATION OF THE ARYL HYDROCARBON RECEPTOR INCREASES PULMONARY NEUTROPHILIA AND DIMINISHES HOST RESISTANCE TO INFLUENZA A VIRUS	
Abstract.....	35

Introduction.....	36
Materials and Methods.....	40
Results.....	48
Discussion.....	66
3. EXPOSURE TO TCDD DOES NOT ENHANCE VASCULAR PERMEABILITY IN THE LUNGS OF MICE INFECTED WITH INFLUENZA A VIRUS.....	72
4. RECRUITMENT OF EXCESS NEUTROPHILS DURING RESPIRATORY VIRAL INFECTION IS DEPENDENT UPON ARYL HYDROHARBON RECEPTOR (AHR)-MEDIATED EVENTS WITHIN THE LUNG AND DOES NOT REQUIRE AHR IN THE HEMATOPOIETIC SYSTEM	
Abstract.....	79
Introduction.....	80
Materials and Methods.....	84
Results.....	92
Discussion.....	110
5. SUMMARY AND FUTURE DIRECTIONS	
Summary.....	115
Future Directions.....	117

APPENDICES

Appendix A – Interferon-(IFN) γ	127
Appendix B – Toll-like receptor-(TLR)-4.....	129
Appendix C – Surfactant protein-(SP)-A.....	131
Appendix D – Tissue inhibitor of matrix metalloproteinase-(TIMP)-1.....	133
Appendix E – Vascular endothelial growth factor (VEGF).....	135
Appendix F – Selectins and cellular adhesion molecules (CAMs).....	137
LIST OF REFERENCES.....	141

LIST OF TABLES

3.1	Exposure to TCDD does not increase infection-associated pulmonary edema.....	75
3.2	Protein levels in lung lavage fluid are not altered by treatment with TCDD.....	76
3.3	Exposure to TCDD does not enhance the concentration of lactate dehydrogenase (LDH) in lung lavage fluid in mice infected with influenza A virus.....	77
4.1	TCDD-mediated excess pulmonary neutrophilia occurs in several strains of mice in response to infection with either influenza virus X-31 or Mem-72.....	93
4.2	Position of dioxin response elements (DREs) in the upstream regulatory region of ICAM-1 and PECAM-1.....	106

LIST OF FIGURES

1.1	Structure of PHAHs.....	1
1.2	Mechanism of PHAH-mediated gene induction.....	10
1.3	Neutrophil recruitment to the lung during respiratory viral infection.....	33
2.1	Exposure to TCDD leads to excess pulmonary neutrophilia, decreased host resistance to influenza virus, and severe bronchopneumonia.....	50
2.2	Activation of the AhR mediates the enhanced neutrophilia in the lungs of TCDD-exposed mice.....	52
2.3	Pulmonary neutrophils from vehicle- and TCDD-treated mice are functionally equivalent.....	54
2.4	Kinetics of chemokine induction in response to infection with influenza virus.....	56
2.5	Treatment with TCDD does not enhance the levels of LIX in BAL fluid of infected mice.....	57
2.6	Levels of IL-6 are elevated in lung lavage fluid throughout infection with influenza virus, however, exposure to TCDD does not increase the levels of IL-6.....	58
2.7	C5a is constitutively present in BAL fluid.....	59
2.8	An equivalent number of neutrophils migrates toward BAL fluid from vehicle- and TCDD-exposed mice.....	61
2.9	Expression profile of adhesion molecules on pulmonary neutrophils in response to infection with influenza virus.....	63

2.10	Exposure to TCDD does not affect pulmonary neutrophil apoptosis and necrosis.....	65
4.1	Exposure to TCDD does not increase the percentage or number of neutrophils in the bone marrow and blood of infected mice.....	95
4.2	Treatment with TCDD does not enhance the number of neutrophils in the spleen or liver	97
4.3	AhR ^{-/-} bone marrow cells successfully reconstitute the immune system of lethally irradiated AhR ^{+/+} mice, generating immunocompetent CD45.2AhR ^{-/-} →CD45.1AhR ^{+/+} bone marrow chimeric mice.....	100
4.4	Excess number of neutrophils in the lungs of TCDD-exposed, infected mice is mediated by AhR-driven events external to the immune system.....	102
4.5	The suppressed T cell response of CD8 ⁺ T cells in TCDD-treated, infected mice requires AhR-mediated events within the immune system.....	104
4.6	Infection with influenza virus slightly enhances expression of VCAM-1 in the lungs of vehicle- and TCDD-treated mice.....	107
4.7	PECAM-1 is constitutively expressed in the lungs of vehicle- and TCDD-exposed mice.....	108
4.8	Infection with influenza virus significantly induces expression of ICAM-1 in the lungs of vehicle- and TCDD-treated mice.....	109
6.1	TCDD-treated, infected IFN γ -deficient mice have excess number of pulmonary neutrophils.....	128
6.2	Exposure to TCDD slightly increases the number of neutrophils in the lungs of virus-infected C3HHe/N and C3H/HeJ mice.....	130

6.3	Infection with influenza virus does not increase levels of SP-A in the lungs of TCDD-treated mice.....	132
6.4	Infection with influenza virus strongly increases levels of TIMP-1 in the lungs of vehicle- and TCDD-exposed mice.....	134
6.5	Exposure to TCDD does not elevate levels of VEGF in the lungs of infected mice.....	136
6.6	Treatment with TCDD does not increase levels of E-selectin, P-selectin, ICAM-1, and VCAM-1 in the lungs of infected mice.....	139

Dedication

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In Memory of
my grandmother Lydia Minuth.

CHAPTER ONE

Introduction

Polyhalogenated aromatic hydrocarbons

Polyhalogenated aromatic hydrocarbons (PHAHs) constitute a large family of structurally-related, cyclic compounds, which include dioxins, dibenzofurans, and biphenyls (Figure 1.1). Specifically, among the 419 polychlorinated congeners of the PHAH family 75 congeners are dibenzo-*p*-dioxins, 135 are dibenzofurans, and 209 are biphenyls (PCBs; Gilpin *et al.*, 2003). In addition to chlorinated congeners, PHAHs also include brominated and mixed brominated/chlorinated congeners. Congeners of the PHAH family with chlorine, bromine, or mixed chlorine/bromine substitutions at the 2,3,7,8 positions of the benzene ring are extremely resistant to biodegradation and metabolism by cytochrome P450 enzymes.

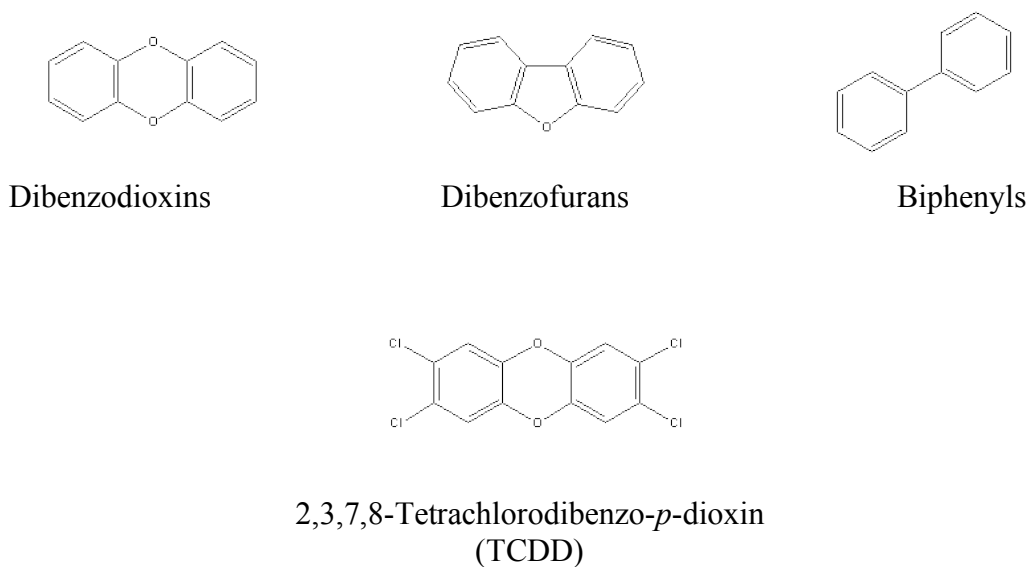


Figure 1.1 Structure of PHAHs

Adapted from ChemFinder.com

Among the 2,3,7,8-substituted PHAHs, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; dioxin) exerts the greatest resistance to biodegradation and metabolism, and is, therefore considered the most toxic member of the PHAH family.

In the past, polychlorinated and polybrominated biphenyls were produced for use in transformers and capacitors; in contrast, TCDD and many other PHAHs were generated inadvertently as by-products of many industrial processes and waste burning (Gilpin *et al.*, 2003). This has made PHAHs ubiquitous environmental contaminants. As a result of their ubiquitous nature and resistance to degradation, PHAHs and in particular TCDD have been the focus of many research studies since the late 1970s. Among the myriad of adverse health effects from exposure to TCDD, alteration of the immune response in rodents constitutes one of the most sensitive toxic endpoints reported to date. Despite extensive research in the field of immunotoxicology since the 1970s, many facets of the immunomodulatory effects of exposure to TCDD remain to be elucidated. The goals of the forthcoming Chapters of this dissertation are to provide background on PHAHs and then to describe studies from our laboratory to further characterize some of the mechanisms by which exposure to TCDD adversely affects the immune system.

Sources of polyhalogenated aromatic hydrocarbons

In contrast to industrial processes (e.g., paper bleaching, metal processing, pesticide/herbicide production) as main sources of PHAHs in the past, burning processes are currently the primary sources of PHAHs in developed countries (Fiedler, 1996). Among these burning processes, incineration of municipal and medical waste, as well as backyard burning lead to release of PHAHs into the environment (Fiedler, 1996; Thornton *et al.*, 1996). In fact,

backyard burning is considered an emerging source of PHAHs, with profound increase in the release of PHAHs from approximately four percent in 1987 to 19 percent in 1995 (Gilpin *et al.*, 2003).

Once released into the environment, some PHAHs are extremely resistant to degradation and are, hence, termed persistent environmental contaminants. The resistance to degradation of PHAHs is due to their planar cyclic structure. As a result of the planar structure, PHAHs fit poorly into the substrate binding pocket of xenobiotic-metabolizing cytochrome P450 enzymes (Denison and Nagy, 2003). Additionally, the number of halogen substitutes, as well as the position of halogens on the benzene ring, further enhances resistance to degradation. PHAHs with halogens at the 2,3,7,8 positions of the benzene ring are most resistant to degradation and, consequently, have the longest half-life in the environment. For example, TCDD has a half-life of approximately six to eleven years in humans, making it the most persistent and toxic PHAH (Martinez *et al.*, 2003).

In addition to the planar structure of some PHAHs, the lipophilic nature of PHAHs contributes to the long half-life of these compounds. Due to their lipophilic nature, PHAHs bioaccumulate in adipose tissue. In humans, greater than 95 percent of exposure to PHAHs occurs via the food chain (Parzefall, 2002; Charnley and Doull, 2005). Exposure to PHAHs via the food chain takes place as follows: PHAHs are released into the air, and ultimately deposit on soil and vegetation grown on this soil. Both wild and farm animals feed on PHAH-contaminated vegetation (Lohman and Seigneur, 2001). Upon ingestion of this vegetation, PHAHs accumulate in adipose tissue of these animals. Human exposure occurs by consumption of food products derived from HAH-exposed wild and farm animals. Animal products with high fat content (e.g., eggs, milk, dairy products, and fatty meat/fish products) lead to higher level of exposure to

PHAHs than products with low fat content (e.g., fruits/vegetables and lean meat/fish products) (Schechter *et al.*, 2001).

To account for the varying toxic potencies of PHAHs, toxic equivalency factors (TEFs) have been developed (Schechter *et al.*, 2001). TEFs list the toxicity of each PHAH in relation to TCDD, which is the most toxic member of the PHAH family, and is assigned a TEF of 1. Thereby, a PHAH with a TEF close to 1 is more toxic compared to a PHAH with a smaller TEF. To determine the cumulative toxic equivalency (TEQ) of a PHAH mixture, the sum of the individual TEF multiplied by the concentration of the appropriate PHAH is calculated. With regard to human health, in 1998 the World Health Organization (WHO) has recommended the TEF for PHAHs between 1 and 10 pg TEF/kg body weight/day (Greene *et al.*, 2003).

In addition to daily low level of exposure to PHAHs via the food chain, there have been some accidents in the past that led to larger scale exposure to PHAHs via the food chain. The first of these accidents occurred in Yusho, Japan in 1968, in which rice oil was contaminated with Kanechlor-400, a mixture of PCBs (Masuda, 2003). This accident afflicted almost 2000 people, who suffered from a range of diverse adverse health effects, including skin abnormalities, ocular discharge, alopecia, fatigue, and headaches (Masuda, 2003). In 1979, a similar accident took place in Yucheng, Taiwan (Guo *et al.*, 2003). Again, rice oil contaminated with Kanechlor-400 and 500 gained entry into the food chain and exposed a similar number of people as the previous incident in Japan (Guo *et al.*, 2003).

A more recent accident occurred in the late 1990s in Belgium. This incident is commonly referred to as the “Belgium dioxin poisoning” although the PHAHs present in the Belgium incident consisted of a mixture of PCBs. About 40 to 50 kg of PCB-contaminated mineral oil was mixed with animal fat, which was used to produce animal feed (van Larebeke *et al.*, 2001).

The contaminated animal feed was fed to poultry, pigs, and cattle. Among these animals, poultry were highly susceptible to adverse health effects and succumbed to a syndrome known as “chick edema disease.” Chick edema disease first occurred in the Southeastern U.S. in 1957 upon exposure of poultry to PHAH-contaminated food (Firestone, 1973). Chick edema disease is characterized by a multitude of symptoms, including ascites, subcutaneous edema, ataxia, muscular degenerative changes, and ultimately death (Bernard *et al.*, 2002). The occurrence of this disease in Belgium made authorities suspicious about potential dioxin/PCB exposure in poultry. Adverse health consequences of the Belgium incident were poultry death throughout Belgium and adjacent countries, as well as contamination of the human food chain (van Larebeke *et al.*, 2001; Bernard *et al.*, 2002). Eggs, pork, beef, and dairy products were food products with high levels of PCB contamination. The use of these PCB-contaminated meat and dairy products led to contamination of various other food products, including the prominent Belgium chocolate (van Larebeke *et al.*, 2001).

In addition to these large-scale PHAH exposure accidents involving the food chain, two other accidents not involving the food chain have resulted in large scale PHAH exposure in humans: The use of the TCDD-contaminated herbicide Agent Orange as a defoliant during the Vietnam War and an explosion at a chemical plant in Italy. Agent Orange, named after its orange-labeled storage containers, was produced by mixing 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) with 2,4-dichlorophenoxyacetic acid (2,4-D; Stellman *et al.*, 2003). However, during this process TCDD was inadvertently produced. The U.S. Air Force sprayed this TCDD-contaminated herbicide over Vietnamese territory during the Vietnam War in 1961 to 1972 to defoliate the dense vegetation, and make the terrain more accessible for U.S. ground troops. This aerial spraying of Agent Orange exposed U.S. and Vietnamese soldiers, as well as Vietnamese

civilians to TCDD. In 1991, the Agent Orange Act was formed by the U.S. government to monitor Vietnam War Veterans for adverse health effects due to TCDD exposure (Stellman *et al.*, 2003).

An explosion at the herbicide manufacturing Givaudan-Hoffmann-LaRoche plant in Seveso, Italy in 1976 constitutes the second hallmark example of large-scale human exposure to PHAHs, including TCDD (Bertazzi and Domenico, 2003). The explosion at the Givaudan-Hoffmann-LaRoche plant occurred during the manufacture of the herbicide 2,4,5-trichlorophenol (TCP), leading to the inadvertent production of TCDD as a by-product. Approximately 600 g of TCDD was produced and released into the environment, exposing approximately 10,000 residents living in the vicinity of chemical plant (Bertazzi and Domenico, 2003). To this date, the residents of the Seveso area have been the subjects of several epidemiological studies documenting the adverse health effects of this accident.

In summary, these examples illustrate that exposure to PHAHs occurs not only at low levels on a daily basis via the food chain, but has occurred at higher levels via accidental exposure. Given these diverse exposure routes, ubiquitous nature, and resistance to degradation make it imperative to understand the mechanism of toxicity of PHAHs. Understanding the mechanism of toxicity will improve risk assessment, which will allow us to better predict adverse health effects resulting from exposure to PHAHs.

Mechanism of toxicity

The crucial step in the mechanism of action of the 400+ compounds of the PHAH family involves binding and activation of the aryl hydrocarbon receptor (AhR) by the PHAH. This fact has been collaboratively established from studies using mice with allelic variants of the AhR,

using mice deficient in the AhR, and PHAH ligands with different binding affinities for the AhR.

Initially, studies using mice with allelic variants of the AhR established the pivotal role of binding of PHAHs to the AhR with regard to the mechanism of toxicity. Mice carry four allelic variants of the AhR: the Ah^{b-1}, Ah^{b-2}, Ah^{b-3}, and Ah^d alleles (Poland *et al.*, 1994). Mice with *b* alleles of the AhR (e.g., C57BL, C3H, and BALB/c strains) encode a high ligand-binding affinity variant of the AhR, whereas mice with *d* alleles (e.g., DBA, AKR, and 129 strains) encode a low ligand-binding affinity variant of the AhR (Poland *et al.*, 1994). For example, C57BL mice carry the high ligand-binding affinity AhR (AhR^{bb} genotype) and are, therefore, extremely sensitive to even minute doses of exposure to PHAHs (Birnbaum *et al.*, 1990). In contrast to PHAH-high responsive C57BL mice, DBA mice carry the low ligand-binding affinity AhR (AhR^{dd} genotype; Birnbaum *et al.*, 1990). A single amino acid mutation (Ala³⁷⁵→Val³⁷⁵) leads to lower PHAH responsiveness in DBA mice compared to C57BL mice (Ema *et al.*, 1994). DBA mice (ED₅₀ ≥ 10 nmol/kg body weight) are approximately ten-fold less sensitive to PHAHs than C57BL mice (ED₅₀ = 1 nmol/kg body weight; Poland and Glover, 1980). Consequently, to observe the same toxic effects in DBA than in C57BL mice, 10-fold higher doses of PHAHs have to be administered to DBA mice.

Second, studies using AhR-deficient mice have demonstrated that the toxic effects of exposure to PHAHs are directly mediated by activation of the AhR (Fernandez-Salguero *et al.*, 1996; Schmidt and Bradfield, 1996). These studies illustrate that AhR-deficient mice are protected from the adverse health effects observed in PHAH-exposed wildtype mice. Additionally, structure-activity studies have shown that binding affinities of PHAHs to the AhR correlate with toxic effects. PHAHs are considered classical AhR ligands and generally exert a higher binding affinity for the AhR (pM to nM) than non-classical ligands (nM to μM; Denison

and Nagy, 2003). In contrast to structurally-related PHAHs, non-classical low-affinity AhR ligands exhibit a diverse chemical structure (Denison and Nagy, 2003). Many of these non-classical AhR ligands have been recently identified and are naturally occurring compounds (Jeuken *et al.*, 2003). The following are some examples of naturally occurring AhR ligands to which we are exposed via the diet: tryptophan metabolites, indole-3-carbinol, polyphenolics, carotenoids, and flavonoids (Heath-Pagliuso *et al.*, 1998; Jeuken *et al.*, 2003). For example, consuming Brussels sprouts, broccoli, and cauliflower leads to exposure to the non-classical AhR ligand indole-3-carbinol (Jeuken *et al.*, 2003). Contrary to the fairly well-characterized toxic effects of exposure to classical AhR ligands, the effects of long-term exposure to non-classical, naturally occurring AhR ligands remain to be determined.

Although the AhR and the binding properties of AhR ligands have been well-characterized using various rodent models, the expression of this receptor does not exclusively occur in the rodents. In fact, expression of the AhR has been detected in various mammals (e.g., dogs, horses, sheep, monkeys, minks, beluga whales, seals, guinea pigs, and humans). Furthermore, the AhR is expressed in non-mammalian species, such as birds, fish, amphibians, mollusks, fruitflies, and nematodes (Hahn, 2003). Interestingly, the amino acid sequence of the AhR is highly conserved among this diverse group of species. For example, the AhR in the nematode *C. elegans* exhibits 38 percent amino acid sequence homology with the human AhR, and the amino acid sequence of the AhR in the fruitfly *D. melanogaster* is 71 percent identical to the human AhR (Hahn, 2003). This sequence homology makes the AhR an evolutionarily highly conserved protein. Furthermore, the evolutionarily highly conserved sequence of the AhR among different species suggests physiological importance of this receptor.

Additionally, the AhR is member of a large family of basic helix-loop-helix, Per-ARNT-Sim (bHLH-PAS) proteins. bHLH-PAS proteins regulate a diverse spectrum of intricate biological processes, such as circadian rhythms, hypoxia response, cell cycle progression (Nie *et al.*, 2001; Puga *et al.*, 2002). Given this fact, in combination with the evolutionarily conserved sequence of the AhR, and the recent findings that many naturally occurring compounds with completely different chemical structures than traditional AhR ligands (e.g., PHAHs) bind and activate the AhR suggests that the AhR likely plays a role beyond currently known AhR-mediated mechanisms of toxicity. Taken together, these findings have led to a paradigm shift in the classification of AhR from a true orphan receptor with unknown physiological role to a rather unique receptor with potentially many diverse physiological functions.

While many facets of AhR-mediated physiology still appear rather enigmatic, AhR activation in hepatocytes has been extensively characterized. The mechanism of activation of the AhR by PHAHs in hepatocytes encompasses a well-coordinated process, which cumulates in the induction of xenobiotic-metabolizing cytochrome P450 enzymes (illustrated in Figure 1.2):

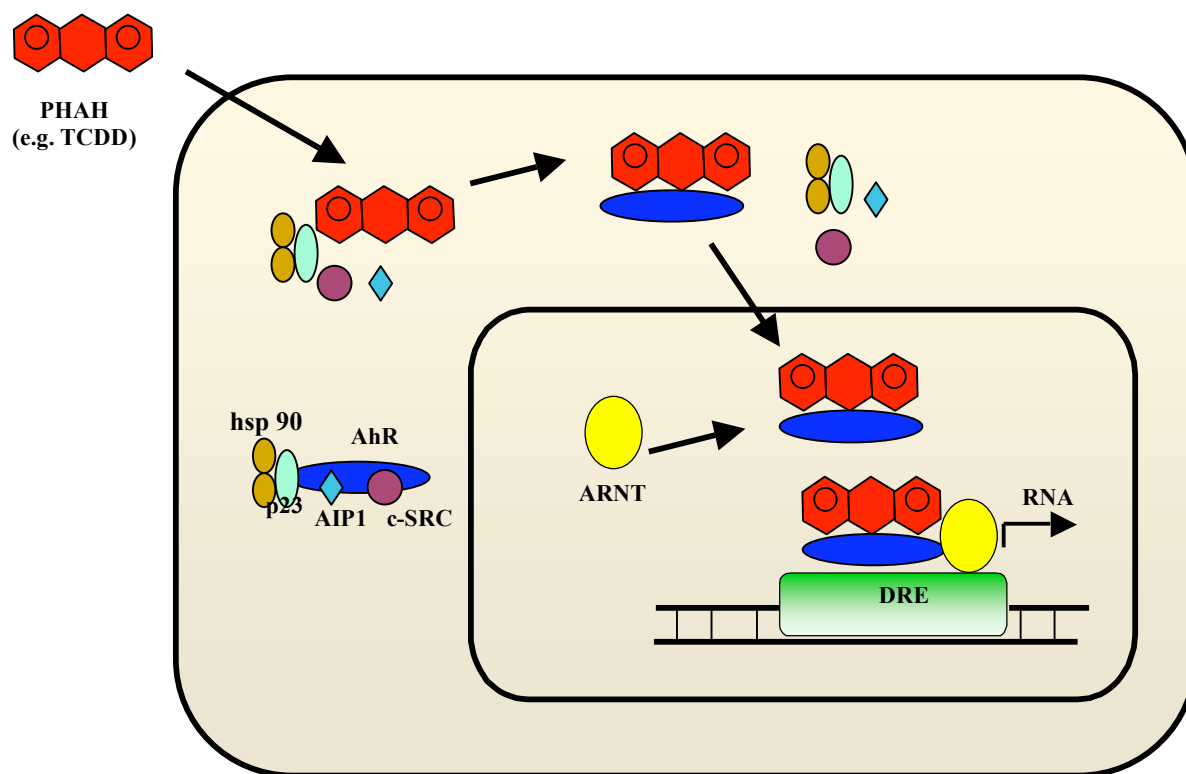


Figure 1.2 Mechanism of PHAH-mediated gene induction (e.g., xenobiotic-metabolizing cytochrome P450 enzymes)

PHAHs (e.g., TCDD) are highly lipophilic compounds, which can easily transverse the lipid bilayer of the plasma membrane. After entering the cell, PHAHs bind to the inactive AhR, which is located in the cytoplasm (Martinez *et al.*, 2003). The inactive form of the AhR is complexed with two heat-shock protein 90 molecules (hsp 90), a c-SRC protein kinase (Enan and Matsumura, 1996), an immunophilin-related protein1 (AIP1; Ma and Whitlock, 1997), and co-chaperone protein p23, which binds hsp 90 proteins (Kazlauskas *et al.*, 1999). Upon ligand binding, these chaperone proteins dissociate from the AhR. The AhR-ligand complex then translocates from the cytoplasm into the nucleus. Once in the nucleus, the receptor-ligand

complex dimerizes with a protein called aryl hydrocarbon nuclear translocator (ARNT) prior to binding to response elements in the DNA. The response elements to which the AhR-ligand-ARNT heterodimer binds contain the 5mer core 5'-GCGTG-3', and are known as dioxin (DRE), xenobiotic (XRE), or aryl hydrocarbon (AhRE) response elements (Whitlock, 1999). Binding of the AhR-ligand-ARNT complex to DREs in the upstream regulatory region of genes leads to conformational changes in DNA. These conformational changes in DNA can lead to unwinding of DNA in the upstream regulatory region, making the promoter more accessible for transcription factors, which leads to induction of gene transcription. The hallmark targets of AhR ligand-mediated gene induction are xenobiotic-metabolizing cytochrome P450 cyp1A1, cyp1A2, and cyp1B1 enzymes (Whitlock *et al.*, 1997). All three cytochrome P450 isoforms contain functional DREs in their promoter region and are readily induced by exposure to TCDD and other PHAHs.

In addition to functional DREs in the upstream regulatory region of xenobiotic-metabolizing cytochrome P450 genes, a functional DRE in the murine upstream regulatory region of the gene encoding the cytokine interleukin-(IL)-2 has been identified (Jeon and Esser, 2000). Likewise, using a murine B cell lymphoma cell line, Sulentic *et al.* (2000) identified two functional DREs in the immunoglobulin (Ig) μ heavy chain, which encodes the heavy chain of the IgM antibody. Furthermore, studies using software programs to analyze upstream regulatory regions of genes have identified several putative DREs. For example, Lai *et al.* (1996) identified putative DREs in the murine 5' upstream regulatory region of the following cytokine genes: IL-1 β , IL-5, IL-6, IL-10, and transforming growth factor-(TGF)- β . Among the upstream regulatory regions of approximately 18,000 human and 12,000 murine genes that were searched for DREs by Sun *et al.* (2004), 5489 putative DREs were identified in the human and 3182 in the murine

gene sequences. Ongoing research efforts in this area will elucidate which of these putative DREs are actually functional DREs. Identification of further functional DREs in the upstream regulatory region of genes encoding for products other than cytochrome P450 enzymes, IL-2, and the IgM heavy chain will advance our understanding of AhR-mediated mechanisms.

The AhR does not exclusively act as a ligand-activated transcription factor, which binds to DREs in the upstream regulatory region of genes, but can further exhibit three alternative mechanisms of action. As the first alternative mechanism, it is postulated that the AhR functions as repressor of proteins regulated by c-SRC (Enan and Matsumura, 1996). c-SRC is a tyrosine kinase, which phosphorylates tyrosine residues on target proteins. In the absence of bound ligand, some c-SRC is complexed with the AhR, preventing its mode of action. Upon ligand binding to the AhR, c-SRC dissociates from the receptor into the cytoplasm and can undergo phosphorylation of target proteins. The latter results in activation of kinase-mediated signaling pathways.

Second, it is hypothesized that the AhR interferes with the endogenous function of ARNT (Nie *et al.*, 2001). Once the AhR is bound by a ligand, the receptor-ligand complex forms a heterodimer with the nuclear protein ARNT. This diminishes the pool of free ARNT present in the nucleus. ARNT is a member of the bHLH-PAS family and is involved in the hypoxia signaling pathway by binding to the hypoxia inducible factor-(HIF)-1. Prasch *et al.* (2004) have shown that induction of hypoxia in zebra fish decreased TCDD-mediated up-regulation of cytochrome P450 cyp1A1. This finding suggests that activation of the AhR and the hypoxia pathway leads to competition for a common factor, and induction of one pathway inhibits the other pathway.

Third, the AhR can interfere with transcription factors, such as estrogen and NFκB. With regard to estrogen, AhR activation by PHAHs is generally thought to be anti-estrogenic, since AhR activation interferes with the actions of this hormone. This can be beneficial by counteracting the growth of estrogen-dependent tumors (Safe *et al.*, 1998; Safe and Wormke, 2003). In contrast, the role of AhR activation and NFκB function remains controversial (reviewed in Tian *et al.*, 2002). On the one hand, AhR activation is believed to enhance gene expression of NFκB-regulated genes (Kim *et al.*, 2000). In this case, the AhR is thought to bind the p65 subunit of NFκB. This heterodimer then binds to κB sites in the DNA, activating transcription of NFκB-controlled genes. On the other hand, activation of the AhR is thought to antagonize NFκB-regulated genes (Ke *et al.*, 2001). In this case, binding of the AhR to the p65 subunit of NFκB diminishes the pool of free p65. As a result, less p65 can form homodimers with p65 or heterodimers with p50, another NFκB subunit. Consequently, less p65-p65 homodimers or p65-p50 heterodimers can bind to κB sites in the DNA and activate transcription of NFκB-regulated genes.

Although the aforementioned alternative AhR-driven mechanisms are less characterized than traditional AhR-mediated mechanisms (i.e., induction of xenobiotic-metabolizing cytochrome P450 enzymes), these mechanisms clearly demonstrate that the AhR is a unique receptor with a diverse mode of action. PHAH-mediated activation of AhR can lead to direct alteration of gene transcription by binding to DREs in the upstream regulatory gene region. Alternatively, activation of the AhR can lead to indirect effects by interfering with the role of other regulatory factors, such as c-SRC, ARNT, estrogen, and NFκB. The complexity of AhR-mediated mechanisms of toxicity potentially leads to the wide array of adverse health effects that are observed upon exposure to PHAHs.

Toxic effects of exposure to PHAHs in humans, domestic animals, and wildlife

Acute exposure to PHAHs in humans leads to dermal toxicities, such as discolorations and dysplasia of finger/toe nails, teeth, ocular discharge, and chloracne (Lu and Wong, 1984). Among these adverse dermal health effects, chloracne is the most prominent visible effect in humans. Unlike regular acne, which is characterized by hyperactive sebaceous glands leading to formation of pustules and inflamed nodules, individuals afflicted with chloracne have hypoactive sebaceous glands and develop yellow cysts (Tindall, 1985). Furthermore, chloracne does not preferentially afflict adolescents, but instead affects every age group equally (Sterling and Hanke, 2005). Lastly, compared to regular acne, chloracne is less manageable with drug treatment and often does not improve for many years after exposure to PHAHs has occurred. In fact, some of the chloracne-induced cysts are completely non-responsive to drug treatment and require surgical removal (Sterling and Hanke, 2005), leading to disfigurement of afflicted patients. Chloracne was prevalent in residents exposed to PHAH-contaminated rice oil in Japan and Taiwan in the 1960/70s (Guo *et al.*, 2003; Masuda, 2003). More recently, in late 2004, the Ukrainian president Victor Yushchenko was afflicted with chloracne following a dioxin poisoning (Sterling and Hanke, 2005). Yushchenko's blood dioxin levels exceeded background levels by thousand-fold at the time of the incident (CNN, 2004; Fox, 2004). Although Victor Yushchenko survived this assassination attempt, it will only be apparent with time whether he will suffer any long-term, dioxin exposure-related adverse health effects.

PHAHs not only cause visible dermal toxicities in humans, but have known carcinogenic properties. In 1997, TCDD was classified as a type I carcinogen, which is defined as a “known human carcinogen” by the International Agency for Research on Cancer (IARC, 1997). In fact, Vietnam War Veterans and residents near the 1976 herbicide plant explosion in Seveso, Italy showed increased incidence of lung, liver, rectal, hematopoietic and lymphatic cancers (Teeguarden and Walker, 2003; Baccarelli *et al.*, 2004). Although these studies show that exposure to TCDD does not lead to one particular type of cancer, they provide compelling epidemiological evidence that exposure to TCDD increases the incidence of a variety of cancers.

In addition to carcinogenic properties, exposure to PHAHs has been shown to lower immunity, resulting in increased infections. Increased incidence of respiratory infections and otitis media has been documented in the Canadian Inuit population (Dewailly *et al.*, 2000; Dallaire *et al.*, 2004). This population primarily consumes high fat content fish and whale products, leading to elevated PHAH exposure compared to the general population. Reproductive toxicity constitutes another example of PHAH-mediated toxicity. Increased spontaneous abortions and low birth weights of offspring were observed in Japan and Taiwan in the 1960s/70s, where residents were exposed to PHAHs in two separate incidents via consumption of contaminated rice oil (Theobald *et al.*, 2003). Altered birth ratios, leading to preferentially female offspring, were observed in Seveso, Italy following the explosion of the herbicide plant (Kogevinas, 2001). Lastly, increased incidence of diabetes is another adverse health effect, which has been reported in many different study populations including residents of Seveso, Italy and Vietnam War Veterans (Michalek *et al.*, 1999; Baccarelli *et al.*, 2004).

The devastating adverse health effects following acute exposure to PHAHs are not limited to humans, but also affect animals. A well-publicized example of toxic effects of acute

PHAH exposure in animals occurred in Times Beach, Missouri in 1971. In an effort to control dust, waste oil was sprayed around a horse track in Times Beach. However, this waste oil was contaminated with TCDD, leading to death of race horses (Carter *et al.*, 1975). In addition to the death of race horses, farm animals from surrounding farms died following exposure to this TCDD-contaminated waste oil (Carter *et al.*, 1975).

Mink of the Great Lakes region provide another example of animals adversely affected from exposure to PHAHs. Lower number of mink has been observed in the Great Lake region (Giesy *et al.*, 1994). This reduced number of mink has been attributed to mink feeding on PHAH-contaminated fish species present in the Great Lakes (Giesy *et al.*, 1994). Among the fish species, carp in the Great Lakes contain one of the highest concentrations of PHAHs (Heaton *et al.*, 1995). Likewise to mink, birds of the Great Lakes region are exposed to PHAHs by feeding on PHAH-contaminated fish. Among the adverse effects observed in these fish-feeding birds are thinning of the eggshell and increased mortality and deformities in offspring bird (Gilbertson *et al.*, 1991). The latter condition is known as Great Lakes embryo mortality, edema, and deformities syndrome (GLEMEDS; Gilbertson *et al.*, 1991). Similar to the Great Lakes region, high levels of PHAHs are present in the oil refinery and shipping region of Houston and Galveston, Texas (Frank *et al.*, 2001). Although a toxic syndrome, such as GLEMEDS, has not been detected in birds or other wildlife species in this area, the proximity of birds of the Houston and Galveston area to oil refineries directly correlated with PHAH concentration detected in eggs from these birds (Frank *et al.*, 2001).

In summary, these studies illustrate that the adverse health effects from exposure to PHAHs are very diverse and affect humans, domestic animals, and wildlife. At higher exposures more visible adverse effects (e.g., chloracne in humans and deformities in animals) predominate,

while at intermediate to lower exposure levels less visible health effects occur. To link the latter group of adverse health effects to exposure to PHAHs it is imperative to collect adequate test samples and have accurate detection methods to analyze these samples. In the past, this was often hindered by not including appropriate control samples or collecting adequate samples, as well as by the limit of analytical detection techniques. Recently, detection methods have greatly improved allowing for accurate detection of PHAHs in humans and animals.

Toxicity in laboratory animals

The majority of our knowledge of PHAH-toxicity, and in particular TCDD-mediated toxicity, is derived from studies using laboratory animals. In contrast to the limited number of human epidemiological studies, numerous research reports involving laboratory animals have thoroughly characterized PHAH-toxicity in various organ systems. Among the different animal species used in these studies, the guinea pig is the most sensitive species to the toxic effects of exposure to TCDD with an acute LD₅₀ of 0.6 to 2.1 µg/kg (Schwetz *et al.*, 1973). The least sensitive species are hamsters, with an acute LD₅₀ of 3000 to 5000 µg/kg (Olson *et al.*, 1980; Umbreit *et al.*, 1989). Compared to guinea pigs and hamsters, mice exhibit intermediate sensitivity to TCDD, and depending on the strain of mice, the acute LD₅₀ is generally 130 to 300 µg/kg (Martinez *et al.*, 2003). Regardless of the dose of exposure, the half-life of TCDD is approximately 10-11 days in mice (Van den Berg *et al.*, 1994).

Treatment with TCDD causes a wide spectrum of adverse health effects in laboratory animals. The following are examples of some of these adverse effects, presented in order of effects occurring at high doses to those effects occurring at lower exposure levels:

Wasting syndrome

At high exposure levels to TCDD, mice suffer from a severe wasting syndrome or cachexia, which can be lethal. The progressive weight loss characteristic of this toxic effect is due to reduced food intake, which leads to depletion of adipose and muscle tissues in mice (Martinez *et al.*, 2003). Although the precise mechanism underlying this TCDD-induced wasting syndrome is not known, some evidence exists implicating tumor necrosis factor (TNF; Taylor *et al.*, 1992), as well as the regulatory kinase c-SRC (Vogel *et al.*, 2003) in the underlying mechanism.

Thymic atrophy

In addition to wasting syndrome, thymic atrophy, characterized by reduced number of T cells in the thymus, constitutes another hallmark effect of TCDD toxicity. The thymus is the anatomical site for the generation of mature T cells, which are produced by lymphoid progenitors in the bone marrow (Haynes *et al.*, 2000). However, the thymus does not exert an active role in adult immunity (Haynes *et al.*, 2000). The mechanism underlying thymic atrophy remains controversial. For example, using a co-culture system, Greenlee *et al.* (1985) document that thymic epithelial cells from TCDD-treated mice reduced maturation of murine thymocytes, suggesting that thymic epithelial cells are direct upstream targets of TCDD-mediated thymic toxicity. Likewise, Kamath *et al.* (1999) have shown that exposure to TCDD induced thymocyte apoptosis via induction of the Fas-FasLigand cascade. In contrast, using CD45.2AhR^{-/-} → CD45.1AhR^{+/+} bone marrow chimeric mice, Staples *et al.* (1998b) have shown that hematopoietic (e.g., lymphocytes) and not non-hematopoietic cells (e.g., epithelial cells) in the thymus were targets of TCDD-mediated thymic toxicity. In summary, the findings from these

studies illustrate that the effects of exposure to TCDD on thymocytes are not uniform, and vary among different model systems.

Tumor promotion and tumor suppression

TCDD has been classified as a type I human carcinogen (IARC, 1997). In rodents, studies have shown that TCDD can act either as a tumor promoter or as a tumor suppressor. *In vivo* exposure to TCDD has been shown to act as a tumor promoter at multiple anatomical sites, including the liver (Pitot *et al.*, 1980), lung (Beebe *et al.*, 1995), and skin (Wyde *et al.*, 2004). In contrast, TCDD's tumor suppressive effects are thought to be mediated by TCDD's anti-estrogenic properties (Safe and Wormke, 2003). For example, *in vitro* exposure to TCDD impaired estradiol-mediated up-regulation of the estrogen receptor in human breast cancer cells (Kietz *et al.*, 2004), making these breast cancer cells less responsive to estrogen. Likewise, long-term dietary exposure to TCDD reduced spontaneous age-dependent mammary and uterine tumor formation in rats (Kociba *et al.*, 1977). In summary, the anti-estrogenic properties of AhR ligands have evoked interest in these compounds as potential therapeutic agents for treatment of estrogen-dependent cancers (Safe *et al.*, 1999). This illustrates that AhR ligands are unique compounds, which not only exert a wide range of toxic effects, but potentially prove to be beneficial if used to target specific cancers.

Reproductive and endocrine toxicity

Exposure to TCDD adversely affects both the male and female reproductive system. The effects of *in utero* or lactational exposure to TCDD lead to decreased prostate size, altered prostate morphogenesis, diminished sperm counts, and increased sperm abnormalities in male offspring (Theobald and Peterson, 1997; Ko *et al.*, 2002). Decreased uterine weights and altered uterine morphology have been observed in female offspring following postnatal exposure to TCDD (Gallo *et al.*, 1986). Administration of estradiol ablated this diminished uterine weight in TCDD-treated female mice in the study performed by Gallo *et al.* (1986), suggesting that TCDD-mediated endocrine disruption is implicated in uterine abnormalities. In addition to a potential antagonistic interaction of estradiol and TCDD, *in vivo* exposure to TCDD decreased levels of thyroid hormone in male and female mice offspring (Nishimura *et al.*, 2005). Furthermore, *in vitro* treatment of rat pancreatic islet cells with TCDD diminished insulin secretion (Novelli *et al.*, 2005). Although the precise mechanism of exposure to TCDD on hormone homeostasis remains to be elucidated, the findings from these studies nevertheless demonstrate that TCDD exerts the potential to interfere with hormone homeostasis.

Teratogenicity and developmental toxicity

Cleft palate, hydronephrosis, altered dentition, and neurobehavioral defects are examples of teratogenic effects in offspring of TCDD-exposed rodents. In offspring of TCDD-treated mice palatal epithelial cells failed to fuse together, resulting in cleft palate (Abbott *et al.*, 1994; Yoon *et al.*, 2000). Hyperplasia of the ureteric epithelium leads to obstruction of the ureters and concomitant hydronephrosis, which is characterized by retention of water in kidneys of afflicted animals (Abbott *et al.*, 1987). Altered dentition, characterized by impaired enamel maturation

and mineralization, leading to dental dysplasia and discoloration, was observed in rats and rhesus monkeys exposed to TCDD *in utero* and lactationally (Gao *et al.*, 2004; Yasuda *et al.*, 2005). In addition to these physical defects, exposure to TCDD has been shown to lead to various behavioral abnormalities in rodent offspring (reviewed in Wormley *et al.*, 2004). Furthermore, treatment with TCDD affects organ development in adult animals, such as the mammary gland differentiation in pregnant dams (Fenton *et al.*, 2002; Vorderstrasse *et al.*, 2004b). The findings from these studies show that exposure to TCDD adversely affects development of many different organs, indicating that TCDD is a potent teratogen.

TCDD Immunotoxicity

Immune alteration constitutes another toxic endpoint of exposure to TCDD. In fact, among the plethora of toxic effects of exposure to TCDD, immune modulation is considered one of the most toxic effects in rodent models. Treatment with TCDD alters both adaptive and innate immunity (Vos *et al.*, 1997; Kerkvliet, 2003), thereby adversely affecting both arms of the immune system. Consequently, TCDD-mediated immunomodulation diminishes host resistance to numerous pathogens. The following provides a summary of the detrimental effects of treatment with TCDD on immune function and host resistance:

I. TCDD and ADAPTIVE IMMUNITY

Effects of TCDD on T cells

T cells are among the prime target cells adversely affected by treatment with TCDD. Numerous studies by our laboratory (Lawrence *et al.*, 2000; Warren *et al.*, 2000; Vorderstrasse *et al.*, 2003a) and others (Lundberg *et al.*, 1992; Prell *et al.*, 1995; Kerkvliet *et al.*, 1996; Nohara *et al.*, 2000; Shepherd *et al.*, 2000; Funatake *et al.*, 2004) have shown that exposure to TCDD profoundly decreased the number of T cells upon antigen stimulation. The adverse effects of treatment with TCDD not only affect T cell number, but T cell function as well. Diminished differentiation of CD8⁺ T cells into cytotoxic T cells (CTLs), which express the CD44^{hi}CD62L^{lo} phenotype and destroy infected host cells, provides one example of TCDD-mediated suppression of T cell function (Clark *et al.*, 1983a; Kerkvliet *et al.*, 1996; Warren *et al.*, 2000; Mitchell and Lawrence, 2003; Neff-LaFord *et al.*, 2003). Reduced cytokine production by T cells is another example of decreased T cell function. Findings from our (Warren *et al.*, 2000; Mitchell and Lawrence, 2003) and other laboratories (Prell *et al.*, 1995; Kerkvliet *et al.*, 1996; Nohara *et al.*, 2000; Shepherd *et al.*, 2000) reported decreased levels of interleukin-(IL)-2 and interferon-(IFN) γ in response to treatment with TCDD.

Although these effects on T cells have been extensively characterized, the mechanism underlying TCDD-mediated T cell dysfunction remains to be elucidated. For example, data from our laboratory suggest that exposure to TCDD in the context of infection with influenza virus rendered T cells anergic (Mitchell and Lawrence, 2003). Funatake *et al.* (2005) recently showed that treatment with TCDD increased the number of regulatory CD4⁺ T cells, which are suppressive, and could potentially lead to suppression of T cell-mediated immunity. In addition to anergy and increased number of suppressive T regulatory cells, enhanced T cell apoptosis has

been proposed to underlie TCDD-induced T cell suppression (Camacho *et al.*, 2001). These findings illustrate that TCDD-mediated mechanisms of toxicity vary greatly among different model systems and likely depend on parameters, such as dose of TCDD, type of antigenic stimulation, and target tissues. In order to completely understand how exposure to TCDD alters T cell-mediated immune responses, future studies are undoubtedly warranted.

Effects of TCDD on B cells

During a humoral immune response activated B cells differentiate into plasma cells, which secrete antibodies (Ollila and Vihinen, 2005). Antibodies neutralize antigen, opsonize infected cells for phagocytosis, activate the complement cascade, and mediate antibody-dependent cellular cytotoxicity (ADCC; Ollila and Vihinen, 2005). All of the latter lead to elimination of antigen, assisting the adaptive immune response to overcome an infection. Several studies have characterized the immunosuppressive effects of treatment with TCDD on humoral immunity. For example, *in vivo* exposure to TCDD suppressed B cell function by reducing antibody production by B cells (Tucker *et al.*, 1986; Kerkvliet *et al.*, 1996; Warren *et al.*, 2000; Ito *et al.*, 2002; Vorderstrasse *et al.*, 2003a). Likewise, Luster *et al.* (1988) and Karras *et al.* (1996) showed that treatment with TCDD reduced production of the antibody isotype IgM *in vitro*. Sulentic *et al.* (2000) recently identified two DREs in the immunoglobulin (Ig) μ heavy chain, which encodes the heavy chain protein for the IgM antibody. Taken together, these findings suggest that B cells are potentially a direct target of TCDD-mediated suppression of humoral immunity.

Effects of TCDD on antigen presenting cells (APC)

In contrast to the plethora of studies characterizing the effects of exposure to TCDD on T and B cells, few studies have characterized the effects of TCDD on APCs. During the adaptive immune response, APCs uptake and process antigen for presentation to naïve T cells via major histocompatibility complex (MHC) molecules and induce cytokine production (Moll, 2003). Furthermore, APCs up-regulate expression of costimulatory molecules, such as CD80 (B7-1), CD86 (B7-2), and CD40, to activate naïve T cells (Moll, 2003). Among APCs, dendritic cells (DCs) constitute the prototypic APC (Moll, 2003). The effects of TCDD on DCs are of interest since inadequate presentation of antigen to naïve T cells by DCs or failure to up-regulate costimulatory molecules on DCs could underlie TCDD-mediated suppression of T cell-mediated immune responses. Vorderstrasse *et al.* (2001) showed that treatment with TCDD decreased the number of splenic DCs in mice compared to the number of splenic DCs in control-treated mice. Although the number of splenic DCs was diminished in TCDD-exposed mice, expression of costimulatory molecules by splenic DCs was not decreased upon treatment with TCDD (Vorderstrasse and Kerkvliet, 2001; Vorderstrasse *et al.*, 2003b). In fact, expression of CD40 and CD86 were perplexingly up-regulated on splenic DCs from TCDD-treated mice (Vorderstrasse and Kerkvliet, 2001; Vorderstrasse *et al.*, 2003b). Consistent with the latter two studies (Vorderstrasse and Kerkvliet, 2001; Vorderstrasse *et al.*, 2003b), Shepherd *et al.* (2001) have shown that treatment with TCDD did not overtly affect expression of costimulatory molecules on splenic DCs from P815 tumor cell-challenged mice, suggesting that DCs are not a direct target of TCDD-mediated toxicity. In summary, these findings suggest that suppression of adaptive immunity by exposure to TCDD is likely not due to inadequate DC function and/or activation.

Effects of TCDD on host resistance

The coordinated interplay of DC, T, and B cells, as well as cytokines during an adaptive immune response are crucial to successfully fight infections. Suppression of adaptive immunity by exposure to TCDD has been attributed to reduced host resistance to variety of infectious diseases. For example, Tucker *et al.* (1986) have shown that exposure to a single dose of TCDD diminished host resistance in a dose-dependent manner to *Plasmodium yoeli*, a nonlethal strain of the malaria parasite. TCDD-treated mice showed significantly higher numbers of the blood-born parasite *P. yoeli* compared to vehicle control-treated mice. TCDD-mediated suppression of humoral immunity has been thought to underlie the decreased clearance of *P. yoeli* from TCDD-treated mice (Tucker *et al.*, 1986). Likewise, TCDD-exposed mice exhibited lower host resistance to a lethal challenge with *Herpes simplex* type II virus compared to vehicle control-treated, infected mice (Clark *et al.*, 1983b). A diminished CTL response is thought to underlie the reduced survival of TCDD-treated, *H. simplex*-infected mice (Clark *et al.*, 1983b). Luebke *et al.* (1994) reported decreased host resistance in mice treated with a single dose of TCDD following challenge with the nematode *Trichinella spiralis*. The study demonstrated that the clearance rate of *T. spiralis* in TCDD-treated mice was significantly lower than in vehicle control-treated mice (Luebke *et al.*, 1994).

In addition to adversely affecting host resistance to these pathogens, treatment with TCDD has been shown to decrease host resistance in mice challenged with influenza virus. In fact, Burleson *et al.* (1996) have shown that mice treated with a single, minute dose of TCDD (0.01 µg/kg body weight) had a significant decrease in survival in response to influenza virus infection compared to vehicle-treated mice. To date, this TCDD-mediated decrease in survival is considered one of the most sensitive adverse endpoints of TCDD-induced immunomodulation in

mice. Similar to the findings by Burleson *et al.* (1996), our laboratory (Lawrence *et al.*, 2000; Warren *et al.*, 2000; Vorderstrasse *et al.*, 2003a) and others (House *et al.*, 1990; Luebke *et al.*, 2002) have reported that exposure to a single dose of TCDD (0.1 to 10 $\mu\text{g}/\text{kg}$ body weight) severely diminished survival following infection with influenza virus. This decrease in survival was dose-dependent, as mice treated with ‘higher’ doses of TCDD (e.g., 10 $\mu\text{g}/\text{kg}$ body weight) had lower survival following infection with influenza virus (Burleson *et al.*, 1996; Warren *et al.*, 2000; Luebke *et al.*, 2002; Vorderstrasse *et al.*, 2003a). Collectively, the findings from these reports illustrate that exposure to TCDD not only affects specific immune cell types, but also adversely alters host resistance to a wide variety of antigens. Furthermore, the diverse nature of antigens (e.g., different viruses and parasites) used in these studies, illustrates TCDD’s wide spectrum of immunotoxicity. Given TCDD’s detrimental effects on host resistance makes it pertinent to understand the mechanism of TCDD-induced immune alteration.

II. TCDD and INNATE IMMUNITY

In a primary infection, the innate immune response restrains the spread of infection prior to the delayed, adaptive immune response. Cells important for the innate immune response are macrophages, natural killer (NK) cells, and neutrophils (Tosi, 2005). These cells synthesize and secrete several mediators, which counteract the spread of infections. Among the mediators synthesized and secreted by cells of the innate immune system are cytotoxic mediators. These cytotoxic mediators include reactive oxygen (ROS) and nitrogen species (RNS), and various degradative enzymes all of which target and destroy infected cells (Tosi, 2005). In addition to cytotoxic mediators, cytokines produced by innate immune cells stimulate and recruit additional

immune cells to the site of infection (Tosi, 2005). This allows the host to keep an infection under control until the adaptive immune response has been generated.

In order for a host to successfully combat an infection, it is crucial for the adaptive and innate arm of the immune system to act coordinately. The preceding sections have described the well-characterized effects of exposure to TCDD on adaptive immunity. The forthcoming section describes the limited number of studies that have documented the effects of treatment with TCDD on innate immunity. In particular, the effects of exposure to TCDD on macrophage, NK cell, and neutrophil number and function during the innate immune response are summarized below:

Effects of TCDD on macrophages

The effects of exposure to TCDD on number of macrophages are not uniform, but instead vary in different experimental model systems. For example, an elevated number of macrophages in the peritoneal cavity of TCDD-treated mice have been observed in a model system using antigenic challenge with sheep erythrocytes (Kerkvliet and Oughton, 1993). In contrast, TCDD treatment in the absence of antigenic challenge resulted in lower number of macrophages in the peritoneal cavity of mice (Mantovani *et al.*, 1980). In the context of intranasal challenge with influenza virus, our laboratory (Warren *et al.*, 2000) and others (Luebke *et al.*, 2002) did not detect an alteration in the number of alveolar macrophages in mice exposed to TCDD.

Contrary to the divergent effects of TCDD on macrophage number, exposure to TCDD did not alter macrophage function. House *et al.* (1990) assessed the following parameters of macrophage function in peritoneal macrophages from control- and TCDD-treated mice: cytostasis and cytolysis against macrophage-specific target cells, phagocytosis of fluorescent

beads, and production of the cytokine interleukin-(IL)-1 and the ROS hydrogen peroxide. Treatment with TCDD did not alter any of these parameters compared to the control group. Likewise, Mantivani *et al.* (1980) showed that peritoneal macrophages from TCDD-treated mice had similar cytolytic and cytostatic activity against macrophage-specific target cells compared to peritoneal macrophages from control-treated mice. Furthermore, Kerkvliet *et al.* (1993) showed that treatment with TCDD did not increase macrophage activation, as evidenced by lack of increased expression of the activation markers F4/80 and MHCII on peritoneal exudate macrophages.

Effects of TCDD on natural killer (NK) cells

Similar to macrophages, the effects of exposure to TCDD on NK cell cytolytic function vary with species and anatomical source of the NK cells. For example, Funseth *et al.* (1992) reported increased splenic and blood NK cell activity in mice exposed to TCDD. Yang *et al.* (1994) showed that NK cell activity against NK cell-specific YAC-1 tumor cells was decreased in the lungs of TCDD-treated, influenza virus-infected rats, yet was unaltered in the spleens of the same rats. Findings from our laboratory showed an increase in the number of NK cells in the lungs of TCDD-exposed, influenza virus-infected mice (Neff-LaFord *et al.*, 2003), however, *in vitro* cytolytic activity against NK cell-specific target cells was not elevated in pulmonary NK cells from TCDD-treated, infected mice compared to NK cells from vehicle-treated, infected mice. The observation from our laboratory is consistent with earlier studies by House *et al.* (1990) and Mantovani *et al.* (1980).

Effects of TCDD on neutrophils

Several studies jointly report that antigen challenge in TCDD-treated rodents results in excess number of neutrophils at the site of antigen challenge. For example, Kerkvliet and Oughton (1993) and Moos *et al.* (1994) detected increased number of neutrophils in the peritoneal cavity of TCDD-treated mice following antigenic challenge with sheep erythrocytes. A study by Choi *et al.* (2003) observed enhanced number of neutrophils in the spleens of TCDD-exposed mice challenged with allogeneic P815 tumor cells. Our laboratory typically observes a 2-fold increase in the number of neutrophils in the lungs of TCDD-exposed, infected mice (Warren *et al.*, 2000; Vorderstrasse *et al.*, 2003a). Likewise, Luebke *et al.* (2002) documented a significant increase in number of neutrophils in the lungs of TCDD-treated mice following infection with influenza A virus.

Similar to the effects of exposure to TCDD on NK cell cytolytic activity, the effects of exposure to TCDD on neutrophil function vary with the model system. For example, neutrophil-mediated Yac-1 tumor cells lysis was decreased in splenic, peritoneal exudate, and blood neutrophils from TCDD-exposed mice (Ackermann *et al.*, 1989; Choi *et al.*, 2003). In addition to YAC-1 tumor cell lysis, production of ROS is another indicator of neutrophil function. With regard to ROS production by neutrophils, Choi *et al.* (2003) have shown that *in vitro* production of superoxide anion by splenic and blood neutrophils from TCDD-exposed, P815 tumor cell-challenged mice was significantly enhanced compared to superoxide anion production by neutrophils from control-treated, tumor cell-challenged mice. In contrast, Ackermann *et al.* (1989) reported that *in vitro* production of superoxide anion and hydrogen peroxide by peritoneal neutrophils from TCDD-treated mice was not altered compared to ROS production by neutrophils from control-treated mice.

The effects of exposure to TCDD on neutrophils are of particular interest given their role during infection. Unlike macrophages, which are constitutively present in all tissues of the body, neutrophils are restrained to the bloodstream in the absence of infection. Directional migration of neutrophils to the site of infection is governed by an increasing gradient of soluble neutrophil chemoattractants at the site of infection and by a coordinated expression of adhesion molecules on the neutrophil surface, vascular endothelial, and epithelial cells (Wagner and Roth, 2000). Neutrophils are terminally differentiated cells with a short half-life of approximately six to ten hours (Dancey *et al.*, 1976; Savill *et al.*, 1989). Apoptotic neutrophils are removed via phagocytosis by macrophages (Fannig *et al.*, 1999). To replenish apoptotic neutrophils, neutrophils are continuously produced by the bone marrow and released into the bloodstream (Fannig *et al.*, 1999).

Deregulations in this directional recruitment process results in excess neutrophils at the site of infection. Neutrophils play a pertinent role at the site of infection by releasing cytotoxic mediators and other chemokines, which lead to the destruction of infected cells and recruitment of other immune cells. However, enhanced number of neutrophils leads to elevated levels of cytotoxic mediators, which damage both infected as well as healthy host cells. Hence, the balance between neutrophil-mediated host-protective and host-detrimental effects is very delicate. Studies conducted by our laboratory (Warren *et al.*, 2000; Vorderstrasse *et al.*, 2003a) and others (Kerkvliet and Oughton, 1993; Moos, 1994; Luebke *et al.*, 2002; Choi *et al.*, 2003) have uniformly shown that exposure to TCDD significantly increases the number of neutrophils at the site of antigen challenge. These findings and the known contribution of excess neutrophils to pathology make it compelling to further investigate the effects of TCDD on neutrophil trafficking and function. Furthermore, the findings from the studies presented in this dissertation

illustrate that the effects of TCDD on immune function are not exclusively immunosuppressive, but rather dichotomous: exposure to TCDD decreases adaptive immunity, yet increases certain facets of innate immunity (i.e., enhanced number of neutrophils).

Chronic inflammatory lung diseases in humans and excess number of neutrophils

Chronic inflammatory lung diseases, including chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS), and asthma, are examples of diseases in which excess neutrophils contribute to disease-associated lung pathologies. In COPD (Pettersen and Adler, 2002; Barnes, 2004) and ARDS (Weiland *et al.*, 1986; Ware and Matthay, 2000) elevated number of neutrophils lead to alveolar damage via the release of cytotoxic mediators. Alveolar damage leads to fluid accumulation in the alveoli, resulting in pulmonary edema, which, in turn, decreases respiratory function (Ware and Matthay, 2000). With regard to asthma, in addition to the well-characterized role of eosinophils as allergic mediators in eosinophilic asthma, neutrophils are the main mediators of pathology in neutrophilic asthma (Jatakanon *et al.*, 1999).

Although the etiology of these chronic respiratory diseases is not completely known, preliminary evidence exists that suggests a link between exposure to environmental contaminants and chronic lung inflammatory diseases. For example, epidemiological evidence shows increased incidence of COPD in women exposed to TCDD during the chemical plant explosion in Seveso, Italy in the late 1970s (Baccarelli *et al.*, 2004). Likewise, exposure to diesel exhaust particles has been shown to increase respiratory syncytial infection in rodents (Harrod *et al.*, 2003) and lead to enhanced lung inflammation in humans (Salvi *et al.*, 1999; Diaz-Sanchez *et al.*, 2000). In order to understand the precise mechanism by which exposure to pollutants leads to the onset and/or

exacerbation of chronic respiratory diseases, it is pivotal to elucidate the events underlying deregulated neutrophil recruitment. Given the ubiquitous nature of environmental contaminants and the dramatic increase in chronic lung inflammatory diseases worldwide (Hurd and Pauwels, 2002), the studies presented in this dissertation are not only relevant to the field of TCDD immunotoxicology, but also extend our knowledge how exposure to pollutants can deregulate neutrophil recruitment in the context of respiratory viral infection. The wide prevalence of lung inflammatory diseases makes the latter relevant to human health.

Objectives and Hypotheses

The overall goal of the studies presented in this dissertation was to determine the mechanism underlying the deregulated recruitment of neutrophils to the lungs of TCDD-treated, infected mice. Prior to investigating this mechanism, we characterized the following three parameters in Chapter 2. First, using AhR-deficient mice, we assessed whether the elevated number of neutrophils in the lungs of TCDD-treated, infected mice is mediated by activation of the AhR. Second, we *in vivo* depleted neutrophils to examine the effect the excess number of pulmonary neutrophils exerts on host resistance in response to infection with influenza virus. Third, we measured *ex vivo* function of pulmonary neutrophils by measuring levels of hydrogen peroxide, superoxide anion, and myeloperoxidase. As described in Chapters 2, 3, and 4, we then examined several mechanisms that could underlie the exacerbated pulmonary neutrophilia in mice exposed to TCDD. Specifically, in Chapter 2 we investigated whether treatment with TCDD elevates levels of stable, soluble neutrophil chemoattractants in the lung, up-regulates

expression of adhesion molecules on pulmonary neutrophils, or delays apoptosis/necrosis of neutrophils in the lungs of virus-infected mice (illustrated in Figure 1.3).

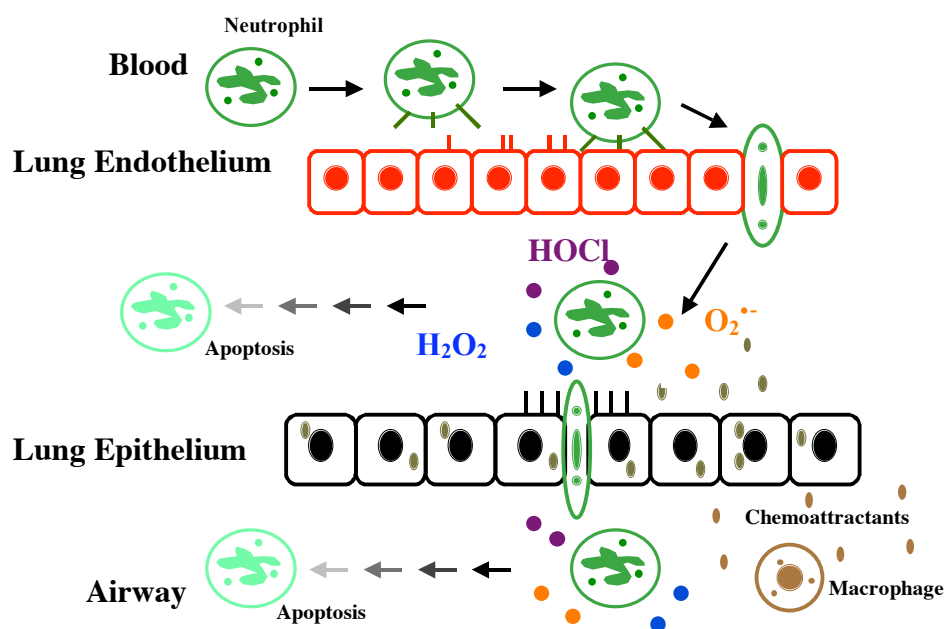


Figure 1.3 Neutrophil recruitment to the lung during respiratory viral infection

Furthermore, as described in Chapter 3, we examined whether treatment with TCDD enhances pulmonary vascular permeability in influenza virus-infected mice, leading to leakage of excess neutrophils to the lungs of TCDD-exposed, infected mice. In Chapter 4, we determined whether exposure to TCDD elevates the number of circulating neutrophils in infected mice, resulting in increased number of neutrophils in the lungs of virus-infected mice. Additionally, using CD45.2AhR^{-/-}→CD45.1AhR^{+/+} chimeric mice, we discerned whether the excess number of

neutrophils in TCDD-treated, infected mice is due to AhR-mediated events within or external to the immune system (Chapter 4). Lastly in Chapter 4, we examined the effects of exposure to TCDD on the expression of adhesion molecules on lung endothelium and epithelium of infected mice. The results from Chapters 2, 3, and 4 are summarized in Chapter 5 of this dissertation. Additionally, future directions for investigating TCDD-induced recruitment of excess neutrophils to the lungs of influenza virus-infected mice are provided in Chapter 5. The appendix summarizes findings from several other studies that we have conducted as part of our goal to delineate the mechanism underlying the exacerbated pulmonary neutrophilia in TCDD-treated, infected mice.

CHAPTER TWO

Activation of the aryl hydrocarbon receptor by TCDD increases pulmonary neutrophilia and diminishes host resistance to influenza A virus¹

ABSTRACT

Unlike their role in bacterial infection, less is known about the role of neutrophils during pulmonary viral infection. Exposure to pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) results in excess neutrophils in the lungs of mice infected with influenza A virus. TCDD is the most potent agonist for the aryl hydrocarbon receptor (AhR), and exposure to AhR ligands has been correlated with exacerbated inflammatory lung diseases. However, knowledge of the effects of AhR agonists on neutrophils is limited. Likewise, the factors that regulate neutrophil responses during respiratory viral infections are not well characterized. To address these knowledge gaps, we determined the *in vivo* levels of KC, MIP-1 α , MIP-2, LIX, IL-6, and C5a in the lungs of infected mice. Our data show that these neutrophil chemoattractants are generally produced transiently in the lung within 12-24 hours of infection. We also report that expression of CD11a, CD11b, CD49d, CD31 and CD38 is increased on pulmonary neutrophils in response to influenza virus. Using AhR-deficient mice, we demonstrate that excess neutrophilia in the lung is mediated by activation of the AhR, and that this enhanced neutrophilia correlates directly with decreased survival in TCDD-exposed mice.

¹With the exception of the data shown in Figure 2.8, all other data were published in the following manuscript: Teske, S., Bohn, A. A., Regal, J. F., Neumiller, J. J., and Lawrence, B. P. (2005). Activation of the aryl hydrocarbon receptor increases pulmonary neutrophilia and diminishes host resistance to influenza A virus. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 289:L111-L124.

While AhR activation results in more neutrophils in the lungs, we show that this is not mediated by deregulation in levels of common neutrophil chemoattractants, expression of adhesion molecules on pulmonary neutrophils, or delayed death of neutrophils. Likewise, exposure to TCDD did not enhance pulmonary neutrophil function. This study provides an important first step in elucidating the mechanisms by which AhR agonists exacerbate pulmonary inflammatory responses.

INTRODUCTION

In spite of advances in health care, morbidity and mortality from respiratory pathogens have not declined in the past 20 years, and the incidence of chronic inflammatory lung diseases has increased. According to the World Health Organization (WHO), respiratory diseases are among the top ten leading causes of illness and death, resulting in approximately four million deaths annually (WHO, 2003b). Among lower respiratory tract infections, infection with influenza virus constitutes one of the major factors in this morbidity and mortality (WHO, 2003a). In addition to infectious agents, chronic inflammatory disorders of the lung further contribute to the burden caused by respiratory diseases. For example, chronic obstructive pulmonary disease (COPD) is a group of diseases of unknown etiology characterized by airflow obstruction and inflammation. COPD is the third leading cause of mortality in elderly adults, and in the United States alone, current estimates indicate that about 31.3 million people have been diagnosed with this disease (Mannino *et al.*, 2002; Anderson and Smith, 2003).

Although it is not clear why certain individuals are more susceptible to chronic inflammatory diseases of the lung, one etiologic factor is infectious disease. In particular, defects in the ability to fight respiratory infections correlate with enhanced incidence and severity of

chronic inflammatory disorders of the lower respiratory tract (Schwarze *et al.*, 1999; Konstantinos and Sheridan, 2001). In addition, increasing evidence exists that exposure to pollutants increases inflammatory responses in the lung (Diaz-Sanchez *et al.*, 2000; Gilmour *et al.*, 2001; Hao *et al.*, 2003; Harrod *et al.*, 2003; Hogg *et al.*, 2004). The mechanisms by which infectious agents or pollutants increase pathology in the lung are not entirely understood, but knowledge of the effects of pollutants on the pulmonary immune system is an important consideration in our efforts to reduce the incidence and severity of pulmonary inflammatory diseases.

One category of pollutants known to affect the lung are ligands for the aryl hydrocarbon receptor (AhR). The AhR is an orphan nuclear receptor that is widely expressed in mammalian tissue, including the lung and cells of the immune system (Lang *et al.*, 1998; Gasiewicz and Park, 2003). Activation of AhR by exogenous compounds is of interest for several reasons. First, ligands for the AhR include an extensive list of exogenous compounds, many of which are very abundant and persistent airborne pollutants. In particular, agonists for the AhR include dioxin-like compounds and polyaromatic hydrocarbons (PAHs) found in cigarette smoke and diesel exhaust. Second, the AhR is a member of the per-arnt-sim (PAS) protein family of basic helix-loop-helix (bHLH) transcription regulators. This family of proteins controls a wide spectrum of complex biological processes that includes toxin metabolism, circadian rhythms, the response to hypoxia, cell cycle progression and cell lineage commitment (Nie *et al.*, 2001; Puga *et al.*, 2002; Denison and Nagy, 2003). Therefore, inappropriate AhR activation affects numerous biological processes. Finally, many AhR ligands have well-characterized immunomodulatory properties (Burns *et al.*, 1994; Kerkvliet and Burleson, 1994; Tryphonas, 1994; Geng *et al.*, 1995; Burchiel and Luster, 2001; Kerkvliet, 2003).

Of all the AhR ligands identified to date, the pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin) has the highest affinity for the receptor, and has therefore been used for many studies characterizing the toxic and biological effects of AhR activation. In addition to being a prototypic AhR ligand, TCDD is among the 33 chemicals listed as priority air toxics in the U.S. Environmental Protection Agency's Integrated Urban Air Toxics Strategy. The potent suppressive effects of TCDD on the cell-mediated and humoral immune responses to a large variety of pathogenic and non-pathogenic antigens have been particularly well characterized (Kerkvliet, 2002). Furthermore, studies using AhR ligands that vary in receptor affinity and using AhR-deficient mice indicate that impaired function of the adaptive immune system is mediated by and dependent upon activation of the AhR (Kerkvliet and Burleson, 1994; Kerkvliet *et al.*, 2002; Yamamoto *et al.*, 2004).

In contrast to suppression of adaptive immune responses, our laboratory (Warren *et al.*, 2000; Vorderstrasse *et al.*, 2003a) and others (Kerkvliet and Oughton, 1993; Moos, 1994; Luebke *et al.*, 2002; Choi *et al.*, 2003) have reported that exposure to TCDD enhances inflammatory responses. Although the levels of inflammatory cells and molecules affected by exposure to TCDD vary among experimental systems, this exacerbated inflammatory response is consistently characterized by an increase in the number of neutrophils at the site of antigen challenge. With regard to the lung, exposure to TCDD results in twice as many neutrophils in airways of infected mice compared to vehicle control-treated, infected mice (Warren *et al.*, 2000; Luebke *et al.*, 2002; Vorderstrasse *et al.*, 2003a). This effect of TCDD on neutrophils is observed exclusively in the context of infection, suggesting that exposure to TCDD alters the immunoregulatory balance in the lung, such that upon infection, adaptive responses are suppressed and inflammatory responses are enhanced.

Enhanced inflammatory responses characterized by excess number of neutrophils constitute an important component of the pathology associated with inflammatory lung diseases. Neutrophils produce cytotoxic molecules, such as reactive oxygen species and degradative enzymes. While controlled production of these cytotoxic mediators aids the host and helps to control infection, excess levels are detrimental (McGuire *et al.*, 1996; Aaron *et al.*, 2001; Suliman *et al.*, 2001). Thus, enhanced number or function of neutrophils lead to increased production of cytotoxic mediators, which ultimately contribute to the pathology observed in inflammatory lung diseases (Kim and Lee, 1996; Aaron *et al.*, 2001; Pettersen and Adler, 2002; Cataldo *et al.*, 2003; Qiu *et al.*, 2003; Hogg *et al.*, 2004).

We have shown that infected mice treated with TCDD exhibit a dose-dependent decrease in host resistance following infection with a sublethal dose of virus (Warren *et al.*, 2000; Vorderstrasse *et al.*, 2003a). Viral titer assays have shown that these mice do not die from excess viral burden. In fact, TCDD-treated mice clear the virus from their lungs with kinetics similar to vehicle-treated mice (Lawrence *et al.*, 2000; Neff-LaFord *et al.*, 2003). Moreover, although CD8⁺ T cells are fewer in number, influenza virus is cleared by a CD8⁺ T cell-dependent mechanism (Neff-LaFord *et al.*, 2003). Given that exposure to TCDD does not impair viral clearance mechanisms, another mechanism likely accounts for the impaired host resistance. We believe that, after infection with influenza virus, increased number of pulmonary neutrophils are detrimental the host.

Knowledge of the effects of AhR agonists on neutrophils or on factors that control neutrophil migration is highly limited. Likewise, there is relatively little known regarding the *in vivo* levels of neutrophil chemoattractants in the mouse lung during infection with influenza A virus. To address these gaps in knowledge, we conducted experiments to characterize the levels

of common neutrophil chemoattractants in the lung over the course of infection with influenza virus. In the presented studies, we also determined whether there is a positive correlation between enhanced neutrophil number in the lung and decreased survival following infection observed in TCDD-treated mice, and whether the exacerbated pulmonary neutrophilia occurs through an AhR-dependent mechanism. We then sought to examine whether exposure to TCDD enhances the level of neutrophil chemoattractants in the lung, alters neutrophil function, or alters the expression of adhesion molecules on pulmonary neutrophils. In addition to examining the relationship between AhR activation and deregulation of multiple components of the pulmonary innate immune response, our findings provide novel information regarding the *in vivo* kinetics and levels of common neutrophil chemoattractants in the lungs of mice infected with influenza A virus.

MATERIALS AND METHODS

Mice

Female C57BL/6 wild-type mice (6-8 weeks of age) were purchased from The Jackson Laboratory (Bar Harbor, ME) or NCI-Frederick (Frederick, MD). Breeding pairs of C57BL/6-AhR-deficient (AhR^{-/-}) mice (deficient for exon 2 of AhR; Schmidt and Bradfield, 1996) were purchased from The Jackson Laboratory. The female AhR-deficient mice used in this study were obtained by breeding AhR-heterozygous (AhR^{+/-}) females with AhR^{+/-} males. We extracted DNA from ear punches (Benedict *et al.*, 2000) and used PCR to determine AhR status of offspring using the following primers: CAG TGG GAA TAA GGC AAG AGT GA and AGG GAG ATG AAG TAT GTG TAT GTA (Qiagen, Alameda, CA). Samples from an AhR^{-/-} mouse yield a 260-bp product, whereas DNA from an AhR^{+/-} mouse yields a 300-bp product. AhR^{+/-} mice have

both the 260- and 300-bp products. The PCR conditions were as follows: 95°C for 5 min; 30 cycles at 95°C for 30 sec; 30 cycles at 60°C for 30 sec; 30 cycles at 72°C for 30 sec; 72°C for 5 min. Gel electrophoresis of 20 µl of PCR products was performed on a 2.5% NuSieve 3:1 agarose gel (Cambrex Bio Science Rockland Inc., Rockland, ME).

All mice were housed under pathogen-free conditions in microisolator units (three to five mice per cage) and provided with water and food *ad libitum*. All animal treatment was performed in accordance with protocols approved by the Institutional Animal Care and Use Committee.

Animal treatment

TCDD ($\geq 99\%$ pure, Cambridge Isotope Laboratories, Inc., Woburn, MA) was dissolved in anisole and diluted in peanut oil to 1 µg/ml. Mice were gavaged with a single dose of TCDD (10 µg/kg body weight). Vehicle-control mice received a single dose of peanut oil-anisole vehicle. One day following exposure to vehicle or TCDD, mice were infected intranasally (i.n.) with murine-adapted human influenza A virus, strain HKx31 (H3N2). Mice received 120 hemagglutinating units (HAU) influenza virus in a final volume of 25 µl endotoxin-tested PBS under general anesthesia (Avertin, 2,2,2-tribromoethanol, Aldrich, Milwaukee, WI). Infection with 120 HAU does not usually cause mortality in vehicle-treated control mice (Lawrence *et al.*, 2000; Warren *et al.*, 2000). Mock-infected mice were treated with vehicle or TCDD and given 25 µl endotoxin-tested PBS (i.n.). Data from these mice are defined in figures at the zero time points. Mice were sacrificed on multiple days after infection by either anesthetic overdose or CO₂ asphyxiation. Depending on the experiment, immune cells were collected from the airways only, or from both airways and interstitial spaces.

Bronchoalveolar lavage (BAL) cells and fluid

Lungs were lavaged as previously described (Warren *et al.*, 2000). Briefly, three sequential washes with serum-free RPMI 1640 media containing 1% BSA and 10 mM HEPES were performed. The first wash was collected separately (BAL fluid). BAL cells were separated from BAL fluid by centrifugation. BAL fluid was stored at -80°C until further analysis. BAL cells from all washes were pooled and enumerated using a Coulter counter (Beckman Coulter Corp., Miami, FL).

Total lung-derived immune cells

To obtain total lung-derived immune cells, lungs were digested with collagenase (Legge and Braciale, 2003). Lungs were incubated for 25 min at 37°C, 5% CO₂ with RPMI 1640 media containing 2.5% FBS (Hyclone, Logan, UT), 1 mg/ml collagenase type 2 (Worthington Biochemical Corp., Lakewood, NJ), and 30 µg/ml deoxyribonuclease I (Sigma-Aldrich Co., St. Louis, MO). Following digestion with collagenase, the lung cell suspension was layered over Lympholyte-M (Cedarlane Laboratories Limited, Hornby, Ontario, Canada) and centrifuged (900 x g) for 20 min at room temperature to separate immune cells from parenchymal cells, dead cells, and erythrocytes. Lung-derived immune cells were counted using a Coulter counter.

Lung homogenates

Lavaged lungs were homogenized in 1.5 ml ice-cold sucrose buffer containing Tris and protease inhibitors (0.25 M sucrose, 10 mM Tris, pH 7.4, with 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 10 µg/ml leupeptin). PMSF, aprotinin and leupeptin were purchased from Sigma-Aldrich. After the homogenization, debris was removed by centrifugation

(8000 x g) for 10 min at 4°C. The protein concentration of the lung homogenates was determined using the Pierce BCA assay (Pierce Biotechnology, Rockford, IL).

***In vivo* depletion of neutrophils**

To deplete neutrophils (Gr-1⁺ cells) *in vivo*, we used a rat monoclonal antibody Gr-1 (RB6-8C5; Ly-6G), which targets the neutrophil surface antigen Gr-1 (Tateda *et al.*, 2001; Choi *et al.*, 2003). This antibody was generously provided by Drs. Robert Coffman (Dynavax Technol. Corp., Berkeley, CA) and Nancy Kerkvliet (Oregon State Univ., Corvallis, OR). Preliminary studies were conducted to define an effective dosing regimen of the anti-Gr-1 antibody. Mice were treated with vehicle or TCDD one day prior to intraperitoneal (i.p.) administration of 300 µg anti-Gr-1 or rat IgG control (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Two hours after antibody administration, mice were infected (i.n.) with 120 HAU influenza virus. Mice received a second 300 µg dose of anti-Gr-1 or rat IgG control four days after infection. Depletion efficacy was determined by flow cytometry and differential cell counts, and was greater than 80 percent.

Lung histology

Vehicle- or TCDD-treated mice given the anti-Gr-1 antibody or rat IgG control were sacrificed seven days after infection with influenza virus. Lungs were tracheally perfused with 10% buffered formalin, excised, and placed into the fixative. After fixation, the lungs were paraffin embedded and 5 µm tissue slices were cut, mounted, and stained with hematoxylin and eosin at the Washington Animal Disease Diagnostic Laboratory and Center for Reproductive Biology (Washington State University, Pullman, WA).

Immunophenotypic analyses

Cells were stained with the different combinations of the following monoclonal antibodies purchased from BD Pharmingen (San Diego, CA) or Caltag Laboratories (Burlingame, CA): biotinylated anti-CD11a (LFA-1), FITC-labeled anti-CD11b (Mac-1), FITC-labeled anti-CD31 (PECAM-1), biotinylated anti-CD38, biotinylated anti-CD49d (VLA-4), FITC-labeled anti-Gr-1, or PE-labeled anti-Gr-1. SpectralRed (SPRD)-conjugated streptavidin (Southern Biotechnology Associates, Inc., Birmingham, AL) was used as the secondary reagent for the biotinylated antibodies. Appropriately labeled, isotype-matched antibodies were used as negative controls for nonspecific fluorescence. Data from 20,000 to 50,000 cells were collected by listmode acquisition, using a FACSort flow cytometer (Becton Dickinson, San Jose, CA). Data analyses were performed using WinList software (Verity Software, Topsham, ME).

Reactive oxygen species (ROS) detection

Detection of ROS levels in neutrophils was assessed as described previously (Lawrence *et al.*, 1999). Briefly, BAL cells were incubated with either 6-carboxy-2'7'-dichlorofluorescein diacetate (DCFH-DA) or dihydroethidine (HE; both from Molecular Probes, Eugene, OR) for 15 min at 37°C in the dark. Excess dye was removed by sequential washes with PAF (endotoxin-tested PBS containing 0.02% sodium azide and 4% FBS). The BAL cells were stained with PE-labeled anti-Gr-1 for the DCF staining set or FITC-labeled anti-Gr-1 for the HE staining set, and analyzed using a FACSort flow cytometer.

Myeloperoxidase (MPO) activity

MPO activity was assessed according to LeVine *et al.* (2002). Briefly, we used a neutrophil-specific gradient isolation media, Mono-Poly resolving media (ICN Biomedicals, Aurora, OH) to obtain neutrophils from BAL cells. For the vehicle and TCDD treatment group, the neutrophil-specific fraction contained 50 percent neutrophils. Neutrophils were suspended in a final volume of 50 μ l hexadecyltrimethylammonium bromide (HETAB) and incubated for 1 hr at 37°C to allow for release of MPO. Following the incubation, 100 μ l of freshly prepared assay buffer containing 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide was added to each well. Absorbance readings (OD) were taken immediately on a microplate reader for a period of 4 min in intervals of 12 sec.

Cytokine and chemokine analyses

Cytokines and chemokines were analyzed using matched antibody pairs in sandwich enzyme-linked immunosorbant assays (ELISAs). ELISA reagents for MIP-1 α , MIP-2, and KC were supplied by R&D Systems (Minneapolis, MN). IL-6 ELISA reagents were purchased from Pierce Biotechnology. ELISAs were performed according to the manufacturers' recommended protocols. The limits of detection were 125 pg/ml for MIP-1 α , 31 pg/ml for MIP-2 and KC, and 250 pg/ml for IL-6. The LIX-specific ELISA was performed in the laboratory of Dr. Daniel Remick (Univ. of Michigan, Ann Arbor, MI). The limit of detection for the LIX ELISA was 20 pg/ml.

Complement split product C5a analysis

BAL fluid was collected by lavaging mice once with 1 ml endotoxin-tested PBS. Levels of C5a were measured in BAL fluid by Western blotting (Maeno *et al.*, 1992), which was modified from previous studies for guinea pig C3a (Regal and Klos, 2000). C5a in BAL fluid was separated from intact C5 by SDS-PAGE under denaturing conditions using a 20% acrylamide gel (Laemmli, 1970). Proteins were electrophoretically transferred to a 0.2 μ m nitrocellulose membrane (BA-S 83, Schleicher & Schuel, Keene, NH). The primary antibody used for immunodetection was the IgG fraction of a rabbit polyclonal antibody to the 15 carboxy-terminal amino acids of murine C5a. The nitrocellulose blot was incubated in 3% BSA overnight at 4°C and probed with anti-C5a-peptide antibody at 1:2500 dilution for two hours at 25°C followed by goat anti-rabbit IgG coupled to horseradish peroxidase at 1:10,000 dilution (Pierce Biotechnology). ECL Plus reagents were used to develop the blot (Amersham Biosciences UK Ltd., Buckinghamshire, UK). Images of light emission were recorded on x-ray film, digitized and quantified by densitometric analysis using Scion Image for Windows (public domain NIH Image program, developed at the U.S. National Institutes of Health). A standard pool of yeast-activated complement (YAC) was prepared by activating pooled mouse serum with yeast cell walls. A dilution series of YAC was used to construct a standard curve and regression equation for each Western blot. Relative amounts of C5a in each sample are expressed as YAC equivalents, based on the signal intensity of YAC dilution.

***Ex vivo* neutrophil chemotaxis**

Migration of neutrophils from mice treated with thioglycollate (VWR, San Francisco, CA) toward BAL fluid obtained from vehicle- and TCDD-treated, infected mice was assessed using a fluorimetric 96-well transwell migration kit (3 μm pore size; Chemicon Intl., Temecula, CA). The 3 μm pore size allows for migration of neutrophils but not macrophages (Cataisson *et al.*, 2005). To obtain neutrophils, untreated mice were injected (i.p.) with 0.5 ml thioglycollate medium, and three hours after injection, neutrophils were collected by peritoneal lavage. Peritoneal lavage cells (2.0×10^5) containing approximately 50% neutrophils (determined by differential cell counts) were resuspended in 100 μl serum-free RPMI 1640 media, containing 1% BSA and 10 mM HEPES and placed in the upper compartment of the transwell assay. BAL fluid (150 μl) derived from vehicle- and TCDD-treated mice was placed into the lower compartment. After 45 min at 37°C, 5% CO₂, migration of peritoneal neutrophils from the upper to the lower compartments was determined using a fluorescence plate reader. To quantitate the number of neutrophils, we generated a standard curve by incubating known numbers (0 to 140,000) of peritoneal neutrophils with the fluorescent CyQuant GR[®] dye provided with the kit.

Neutrophil apoptosis and necrosis

To identify neutrophils, cells were stained with biotinylated anti-Gr-1 followed by APC-conjugated streptavidin (BD Pharmingen). Cells were then stained with FITC-labeled annexin V and 7-amino-actinomycin (7-AAD) to assess apoptosis and necrosis (BD Pharmingen; (Muppidi *et al.*, 2004). For the TUNEL staining method, the in situ cell death detection kit was purchased from Roche Diagnostics (Indianapolis, IN). To identify neutrophils using the TUNEL staining method, cells were stained with biotinylated anti-Gr-1 followed by SPRD-conjugated

streptavidin (BD Pharmingen; Southern Biotechnology Associates, Inc.), and Gr-1⁺ TUNEL⁺ cells were enumerated by flow cytometry.

Statistical analyses

All statistical analyses were performed using StatView statistical software (SAS, Cary, NC). Significant treatment effects were determined by one-way analysis of variance (ANOVA), followed by a Fisher's PLSD post hoc test, to compare the mean values from each treatment group at a specific point in time. To examine infection-associated changes, the mean values in a treatment group were compared over time within that group. Values of $p \leq 0.05$ serve as the basis for designation of statistical significance.

RESULTS

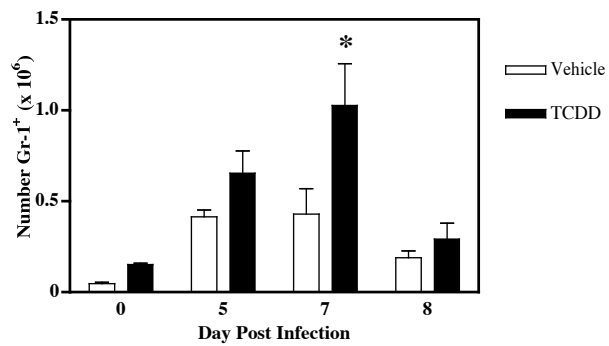
In vivo depletion of neutrophils improves the survival of TCDD-treated mice infected with influenza A virus.

In previous studies, we have found that neutrophil numbers are increased in the airways of TCDD-treated mice infected with influenza virus (Warren *et al.*, 2000; Vorderstrasse *et al.*, 2003a), and that this excess neutrophilia peaks about seven days after infection (Warren *et al.*, 2000). The same phenomenon was observed when we examined the number of neutrophils in total lung-derived immune cells (i.e., airways and interstitial spaces). That is, exposure to TCDD increases the number of neutrophils in the interstitial spaces as well as the airways, and this increase peaks 7 days after infection (Figure 2.1A).

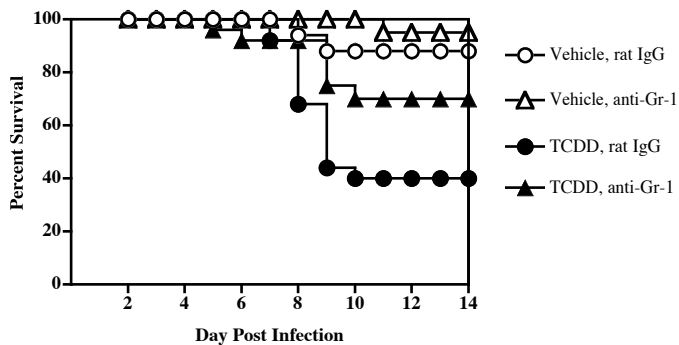
Concomitant with the elevated neutrophilia in the lungs of TCDD-treated mice, we have repeatedly observed a decrease in survival following a sublethal infection with influenza virus compared to vehicle-exposed mice (Lawrence *et al.*, 2000; Warren *et al.*, 2000; Vorderstrasse *et al.*, 2003a). This observation prompted us to investigate whether a relationship exists between excess number of neutrophils and impaired survival after infection with influenza virus. To accomplish this, we depleted neutrophils in mice treated with vehicle or TCDD using the monoclonal antibody anti-Gr-1, and monitored survival for 14 days after infection. Interestingly, *in vivo* depletion of neutrophils did not detrimentally affect survival in vehicle-treated mice (Figure 2.1B; open triangles). Consistent with our previous observations (Lawrence *et al.*, 2000; Warren *et al.*, 2000; Vorderstrasse *et al.*, 2003a), survival was severely compromised in mice treated with TCDD (Figure 2.1B; closed circles). In contrast, depletion of neutrophils improved survival from 40 percent in the TCDD-treated, rat IgG group to 70 percent in the TCDD-treated, neutrophil-depleted group (Figure 2.1B; closed triangles). These data strongly suggest that the excess number of neutrophils in the lungs of mice exposed to TCDD impairs survival following infection with influenza virus.

In addition to monitoring host resistance, we compared lung tissue from mice treated with vehicle or TCDD to investigate whether neutrophil depletion reduces lung pathology (Figure 2.1C). We examined lungs on day 7, the peak day of enhanced neutrophilia in TCDD-treated mice. Bronchointerstitial pneumonia was present in all treatment groups. Administration of anti-Gr-1 ablated the severity of the cellular infiltrate observed in TCDD-treated, infected mice relative to those given the rat IgG control (Figure 2.1C).

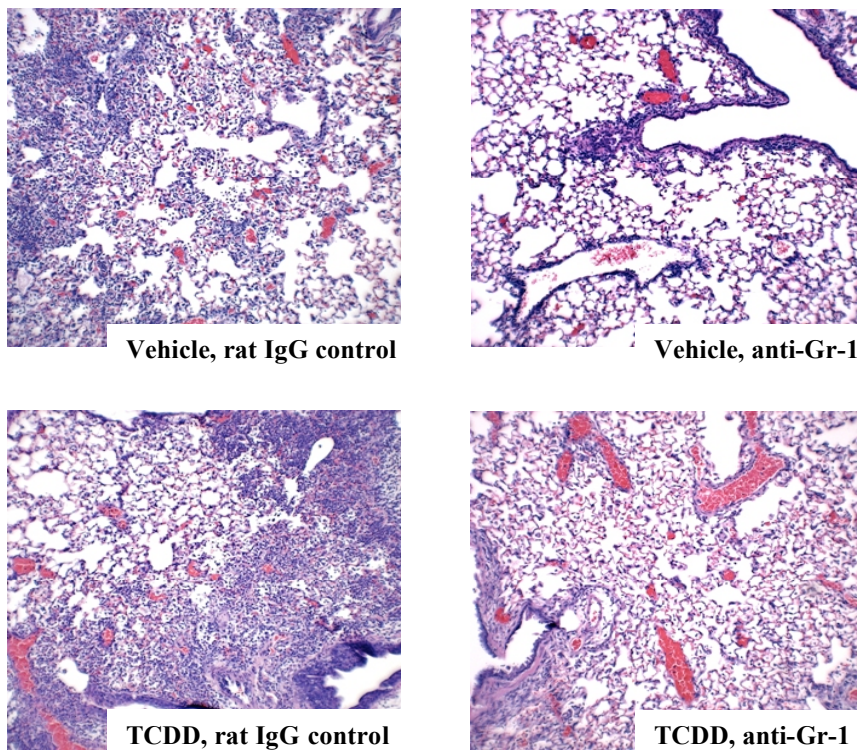
A



B



C



Day 7 Post Infection

Figure 2.1 Exposure to TCDD leads to excess pulmonary neutrophilia, decreased host resistance to influenza virus, and severe bronchopneumonia. (A) Mice were treated with peanut oil vehicle or 10 µg/kg of TCDD one day prior to i.n. infection with 120 HAU influenza virus (A/HKx31). Mice were sacrificed on the indicated days relative to infection, and lungs were digested with collagenase to isolate immune cells. Using flow cytometry, the average number of neutrophils (Gr-1⁺ cells) in the lungs of vehicle- or TCDD-treated mice was determined (6 mice per treatment group/day). Mock-infected mice treated with vehicle or TCDD were used as controls (4 mice per treatment group) and average values from these groups are shown at the zero time point. Error bars represent the SEM. An * denotes a significant difference compared to the vehicle treatment group ($p \leq 0.05$). (B) Mice (20 per group) were treated with vehicle or TCDD one day prior to infection with influenza virus. The next day, 300 µg of anti-Gr-1 or rat IgG control was administered (i.p.) two hours prior to infection with 120 HAU influenza virus (A/HKx31). A second 300 µg dose of anti-Gr-1 or rat IgG control was given four days after infection. Neutrophil depletion efficacy was greater than 80 percent. Survival was monitored for all four treatment groups until 14 days after infection. (C) Mice (5 per treatment group) were treated as described in (A), and sacrificed seven days after infection for histological examination of the lungs. Photomicrographs of representative fields were taken with an Olympus MicroFireTM digital camera. Hematoxylin and eosin stain; X100 magnification.

The increase in neutrophil number in TCDD-treated mice is AhR-dependent.

Studies using AhR-deficient mice have shown that the AhR directly mediates suppression of adaptive immune responses caused by exposure to TCDD (Vorderstrasse *et al.*, 2001; Kerkvliet *et al.*, 2002). Based on this, we hypothesized that the excess neutrophilia and the underlying decrease in survival in TCDD-treated mice is likely mediated by the AhR. Since there are no studies to date investigating whether TCDD-mediated neutrophilia is AhR-dependent, we treated AhR-deficient mice with vehicle or TCDD one day prior to infection, and measured the number of neutrophils (Gr-1⁺ cells) in the lungs seven days after infection. In contrast to TCDD-treated wild-type mice, AhR-deficient mice exposed to TCDD did not have excess number of neutrophils in the lungs after challenge with influenza virus (Figure 2.2). Interestingly, no mortality was observed in infected AhR-deficient mice treated with TCDD, whereas

approximately 60 percent of infected wild-type mice treated with TCDD died by day 7 (data not shown). These findings indicate that activation of the AhR directly mediates the excessive number of neutrophils in the lungs of infected mice.

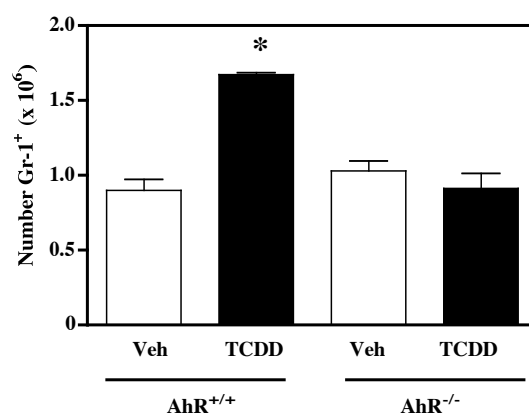


Figure 2.2 Activation of the AhR mediates the enhanced neutrophilia in the lungs of TCDD-exposed mice. Mice were treated as described in Figure 2.1. Using flow cytometry, the average number of neutrophils (Gr-1⁺ cells) in the lungs of infected AhR wild-type (AhR^{+/+}) and AhR-deficient (AhR^{-/-}) mice was determined on day seven post infection, the peak day of enhanced neutrophilia in TCDD-treated wild-type mice (2-4 mice per treatment group). Error bars represent the SEM. An * indicates a significant difference compared to the vehicle treatment group ($p \leq 0.05$). Results are representative of three separate experiments.

Exposure to TCDD does not alter neutrophil function.

During infection with influenza virus, neutrophils produce reactive oxygen species (Hartshorn *et al.*, 1990). While some levels of these reactive oxygen species are host-beneficial, excess levels damage healthy host tissue and are detrimental (McGuire *et al.*, 1996; Suliman *et al.*, 2001). Some reports show that exposure to TCDD leads to an increased production of reactive oxygen species in mice (Slezak *et al.*, 2000; Senft *et al.*, 2002). Therefore, we examined

whether exposure to TCDD enhances the production of reactive oxygen species by pulmonary neutrophils by staining with DCF, an indicator of hydrogen peroxide production (Bass *et al.*, 1983), and HE, an indicator of superoxide anion production (Perticarari *et al.*, 1991). We observed a very small increase in the percentage and mean channel fluorescence (MCF), an indicator of staining intensity, of DCF⁺ neutrophils on days 5 and 9 post infection in mice treated with TCDD (Figure 2.3 A,B). Exposure to TCDD also caused a very slight increase in the percentage of HE⁺ neutrophils on days 5 and 7 post infection (Figure 2.3C). However, the MCF of the HE⁺ neutrophils was equivalent in both treatment groups throughout the course of infection (Figure 2.3D), suggesting that on a per cell basis, superoxide anion levels are not different in neutrophils derived from mice treated with vehicle or TCDD.

Another method to examine neutrophil function is to measure myeloperoxidase (MPO) activity. MPO is an enzyme unique to the cytoplasmic granules of neutrophils and catalyzes the formation of hypochlorous acid from hydrogen peroxide and chloride anion (Rosen *et al.*, 2002). We assessed MPO activity in lung-derived neutrophils seven days after infection (Figure 2.3E), and found that neutrophils from vehicle- or TCDD-treated mice exhibited equivalent MPO activity. Given that hydrogen peroxide and superoxide anion levels were unchanged or only very slightly elevated, and MPO levels were equivalent, we conclude that exposure to TCDD enhances the number of neutrophils in the lungs of infected mice, but they appear functionally equivalent to neutrophils from vehicle-treated, infected mice.

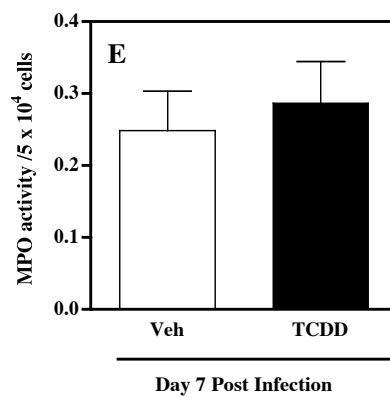
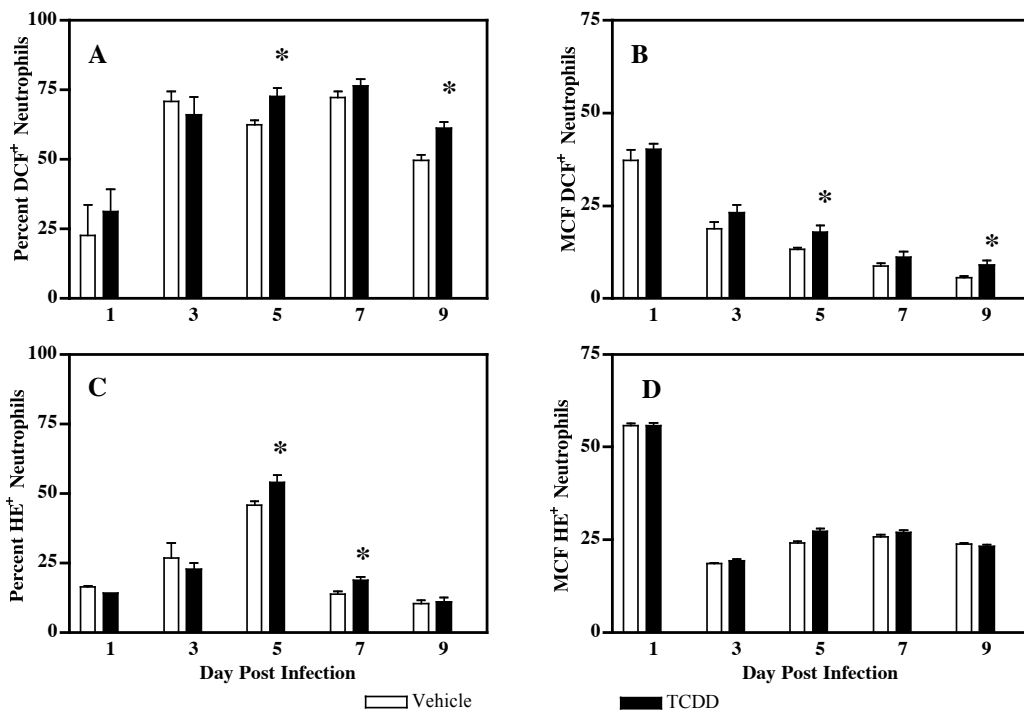


Figure 2.3 Pulmonary neutrophils from vehicle- and TCDD-treated mice are functionally equivalent. Mice were treated as described in Figure 2.1. On the indicated days after infection, mice were sacrificed, and bronchoalveolar lavage (BAL) cells were collected (5-7 mice per treatment group/day). Using flow cytometry, the average percentage of neutrophils (Gr-1⁺ cells) that stained positive for (A) DCF and (C) HE was examined in infected mice treated with vehicle or TCDD. The average mean channel fluorescence (MCF) of (B) DCF⁺ neutrophils and (D) HE⁺ neutrophils was also assessed. (E) Myeloperoxidase (MPO) activity, an indicator of neutrophil function, was measured seven days after infection, using a total of 50,000 neutrophil-enriched BAL cells. Bars represent the average for the vehicle (3 mice) and TCDD treatment group (4 mice). Error bars represent the SEM. An * denotes a significant difference compared to vehicle-treated mice ($p \leq 0.05$).

Profile of neutrophil chemoattractants in the lungs of mice infected with influenza A virus.

To delineate the mechanism underlying the excess neutrophilia, we examined the chemokines keratinocyte chemoattractant (KC), macrophage inflammatory protein-(MIP)-1 α , and MIP-2, which have well-characterized chemoattractant properties for neutrophils (Frevert *et al.*, 1995; Standiford *et al.*, 1995; Tsujimoto *et al.*, 2002). In response to infection, we found that levels of KC, MIP-1 α , and MIP-2 in lung lavage fluid rapidly and coordinately reached peak levels at 12 hours and returned to baseline after 24 hours (Figure 2.4 A-F). In addition to lung lavage fluid, we assessed levels of KC, MIP-1 α , and MIP-2 in lung homogenates at 12 hours, and 1, 3, 5, 7, and 9 days after infection (Figure 2.4 G,H). KC was present in lung homogenates throughout the course of infection; however, there was no effect of exposure to TCDD on the amount of KC (Figure 2.4G). Very low levels of MIP-1 α were detected in homogenates throughout the course of infection (Figure 2.4H), and levels of MIP-2 were below the limit of detection in lung homogenates (data not shown).

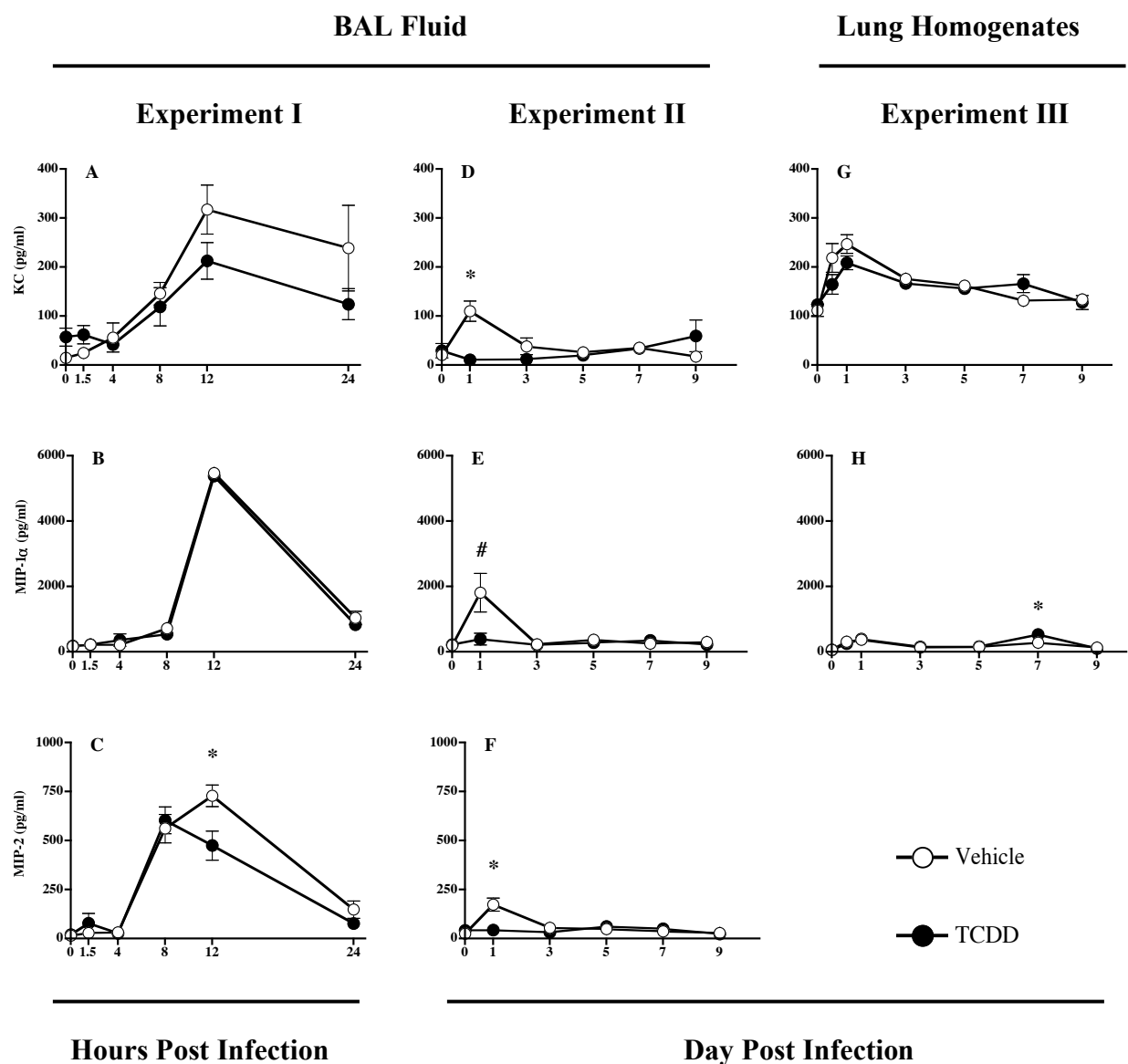


Figure 2.4 Kinetics of chemokine induction in response to infection with influenza virus. Mice were treated as described in Figure 2.1. The levels of KC (A, D, G), MIP-1 α (B, E, H), and MIP-2 (C, F) were measured by ELISA in bronchoalveolar lavage (BAL) fluid or lung homogenates derived from three separate time course studies: (I) 1.5 to 24 hours post infection in BAL fluid (3-4 mice per treatment group/day); (II) days 1 to 9 post infection in BAL fluid (6-7 mice per treatment group/day); and (III) 12 hours to 9 days after infection in lung homogenates (7-8 mice per treatment group/day). Mock-infected mice treated with vehicle or TCDD served as controls (3-4 mice per treatment group for experiments I and II; 6 mice per treatment group for experiment III). Data from mock-infected mice are depicted at the day zero time point. Error bars represent the SEM. An * denotes a significant difference compared to the vehicle treatment group ($p \leq 0.05$), and an # indicates $p \leq 0.09$.

In a separate study we measured the levels of lipopolysaccharide-induced CXC chemokine (LIX), another chemokine with neutrophil recruiting properties (Chandrasekar *et al.*, 2001). Although a less extensive time course was performed, our data show that infection with influenza virus enhances levels of LIX in lung lavage fluid (Figure 2.5). However, exposure to TCDD did not further augment levels of LIX compared to the vehicle treatment group.

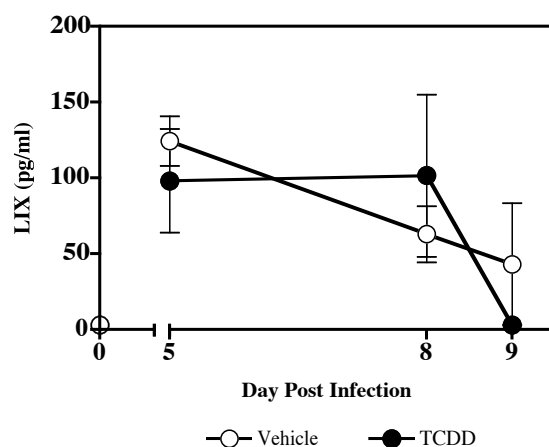


Figure 2.5 Treatment with TCDD does not enhance the levels of LIX in BAL fluid of infected mice. Mice were treated as described in Figure 2.1. On days 5, 8, and 9 after infection, BAL fluid was collected from vehicle or TCDD-treated mice (7-8 mice per treatment group/day). The level of LIX was measured by ELISA. Mock-infected mice treated with vehicle or TCDD served as controls for the infection (2 mice per treatment group). Data from these mice are denoted at the zero time point. Error bars represent the SEM.

Another soluble mediator with neutrophil-recruiting properties is the cytokine IL-6 (Hierholzer *et al.*, 1998). Infection with influenza virus significantly increased levels of IL-6 in lung lavage fluid in both treatment groups (Figure 2.6). This increase occurred very rapidly, however, unlike the other chemokines, did not decline rapidly. We also measured IL-6 in lung homogenates at 12 hours, and 1, 3, 5, 7, and 9 days after infection in mice treated with vehicle or

10 $\mu\text{g}/\text{kg}$ TCDD. In contrast to IL-6 levels in lung lavage fluid, levels of IL-6 in lung homogenates were below the limit of detection (data not shown).

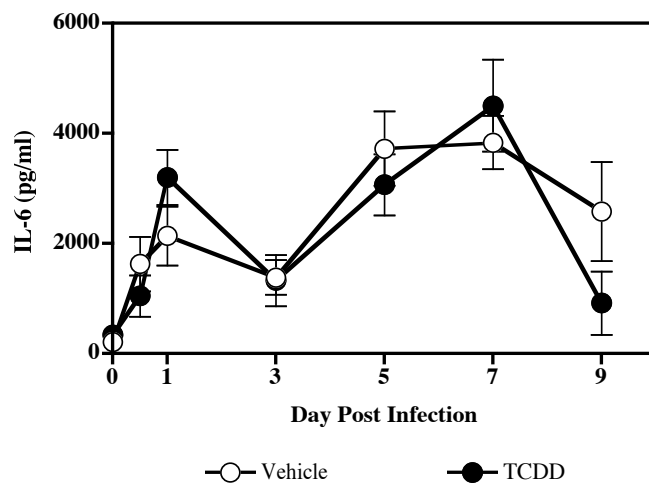


Figure 2.6 Levels of IL-6 are elevated in lung lavage fluid throughout infection with influenza, however, exposure to TCDD does not increase the levels of IL-6. Mice (6-8 mice per treatment group/day) were treated as described in Figure 2.1. On the indicated days relative to infection, mice were sacrificed, and the level of IL-6 was assessed in lung lavage fluid by ELISA. Mock-infected mice treated with vehicle or TCDD served as controls for the infection (5 mice per treatment group). Data from mock-infected mice are depicted at the day zero time point. Error bars represent the SEM. Results are representative of four separate experiments.

In addition to these chemokines and cytokines, we examined complement split product C5a, which is not only chemoattractant for neutrophils (Czermak *et al.*, 1998) but is also elevated in the upper airways of humans during infection with influenza virus (Bjornson *et al.*, 1991). We measured levels of C5a in lung lavage fluid throughout the course of infection in mice treated with vehicle or TCDD (Figure 2.7). Surprisingly, neither infection with influenza virus, nor exposure to TCDD enhanced levels of C5a, suggesting that mice may differ from humans with

regard to enhanced levels of C5a during the immune response to influenza virus. Alternatively, differences in C5a levels between the upper and lower respiratory tract could account for the different expression profiles in our study.

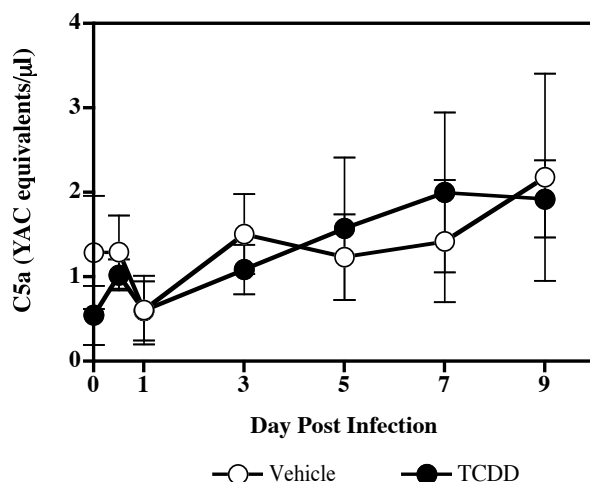


Figure 2.7 C5a is constitutively present in BAL fluid. Mice (6-8 per treatment group/day) were treated as described in Figure 2.1. On the indicated days relative to infection, levels of C5a in BAL fluid from mice treated with vehicle or TCDD were measured by Western blot. Levels of C5a were quantified as yeast-activated complement (YAC) equivalents. Mock-infected mice treated with vehicle (5 mice) or TCDD (6 mice) served as controls. Data from mock-infected mice are shown at the zero time point. Error bars represent the SEM.

In summary, the majority of neutrophil-recruiting mediators examined are produced rapidly after infection with influenza virus. Whereas increased levels of IL-6 were sustained throughout the course of infection, levels of KC, MIP-1 α , and MIP-2 were no longer detected in lung lavage fluid after approximately one day. Together, these results indicate that these molecules are produced transiently at the site of infection, and that their production occurs very

early (within 48 hours) following infection. Given this time frame and the levels detected, it is unlikely that altered expression of these molecules drives the excessive recruitment of neutrophils to the lungs of mice exposed to TCDD. In fact, if anything, levels of KC, MIP-1 α , and MIP-2 were reduced in BAL fluid from TCDD-treated mice.

Equivalent number of neutrophils migrates toward lung lavage fluid from TCDD-treated, infected mice and vehicle-treated, infected mice.

In the absence of an effect of exposure to TCDD on any of the known soluble neutrophil chemoattractants, we determined whether an AhR-mediated deregulation of an unknown soluble neutrophil chemoattractant underlies the recruitment of excess neutrophils to the lungs of TCDD-treated, infected mice. To test this, we measured migration of untreated peritoneal lavage neutrophils toward BAL fluid derived from vehicle- and TCDD-exposed, infected mice. Our data indicate that migration of untreated neutrophils toward lung lavage fluid from vehicle- and TCDD-treated, infected mice was significantly increased compared to migration of untreated neutrophils toward lung lavage fluid from vehicle- and TCDD-treated, mock-infected mice (Fig. 2.8). However, treatment with TCDD did not further enhance this strong infection-associated increase of neutrophil chemotaxis. In other words, we observed an equivalent number of neutrophils migrating toward BAL fluid derived from TCDD-treated, infected mice, as to BAL fluid from vehicle-treated mice (Fig. 2.8). These data suggest that AhR-driven deregulation of an unknown soluble chemoattractant does not underlie the enhanced recruitment of neutrophils to the lungs of TCDD-treated, infected mice. Taken together, the data from this migration study in combination with our other neutrophil chemoattractant data strongly suggest that elevated levels of a soluble, stable neutrophil chemoattractant in the lungs of TCDD-treated, infected mice is not

a likely mechanistic explanation for the excessive number of neutrophils in mice treated with TCDD.

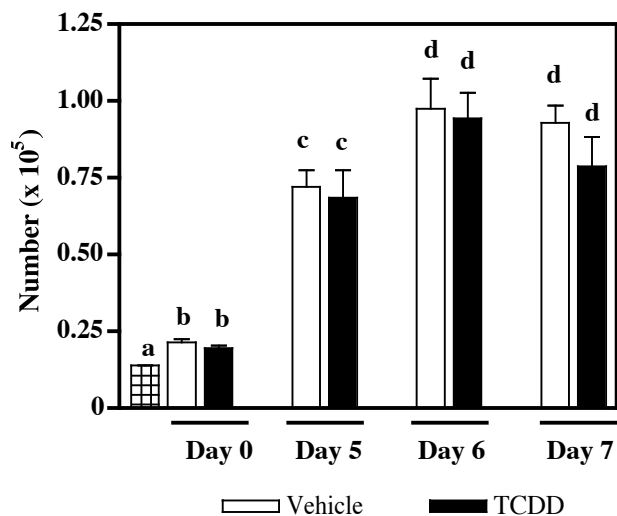


Figure 2.8 An equivalent number of neutrophils migrates toward BAL fluid from vehicle- and TCDD-exposed mice. Mice were treated as described in Figure 2.1. On days 0, 5, 6, and 7 relative to infection, mice were sacrificed and lungs were lavaged with endotoxin-tested PBS to collect BAL fluid (8 mice per treatment group/day). BAL fluid from vehicle- and TCDD-treated, mock-infected mice was collected as controls for the infection (8 mice per treatment group). Data from mock-infected mice are depicted at the day zero time point. Using a 96-well fluorimetric transwell migration assay, the average number of peritoneal neutrophils that migrated from the upper compartments toward BAL fluid in the lower compartments of the transwell assay was measured. Migration of untreated neutrophils toward endotoxin-tested PBS served as a negative control for the assay (checkered bar). Treatment groups with the same letter were not statistically different ($p \leq 0.05$). Error bars represent the SEM. Results are representative of two separate experiments.

Infection with influenza virus induces expression of CD31, CD38, and CD49d on pulmonary neutrophils; however, treatment with TCDD does not markedly alter expression of these adhesion molecules.

Some adhesion molecules important for neutrophil migration are CD11a (LFA-1), CD11b (Mac-1), CD31 (PECAM-1), CD38, and CD49d (VLA-4) (Partida-Sanchez *et al.*, 2001; Stadnyk *et al.*, 2002; Soethout *et al.*, 2003; Tasaka *et al.*, 2003). The effects of AhR agonists on the expression of adhesion molecules on neutrophils have not been previously characterized. However, it is known that treatment with TCDD alters expression of adhesion molecules on dendritic cells (Vorderstrasse and Kerkvliet, 2001), suggesting that activation of the AhR potentially affects expression of adhesion molecules on other immune cells. Based on this logic, we examined whether treatment with TCDD increases expression of adhesion molecules on pulmonary neutrophils following infection with influenza virus. Our data indicate that CD11a and CD11b are constitutively expressed on neutrophils in the lung, whereas CD49d, CD31, and CD38 are not expressed on pulmonary neutrophils in the absence of infection (Figure 2.9). Furthermore, infection increased the expression of all of these molecules on neutrophils in the lung. However, activation of the AhR by TCDD did not markedly up-regulate the expression of any of these adhesion molecules on pulmonary neutrophils. Therefore, altered levels in the expression of these adhesion molecules do not offer a plausible mechanistic explanation for the enhanced pulmonary neutrophilia observed in TCDD-treated mice.

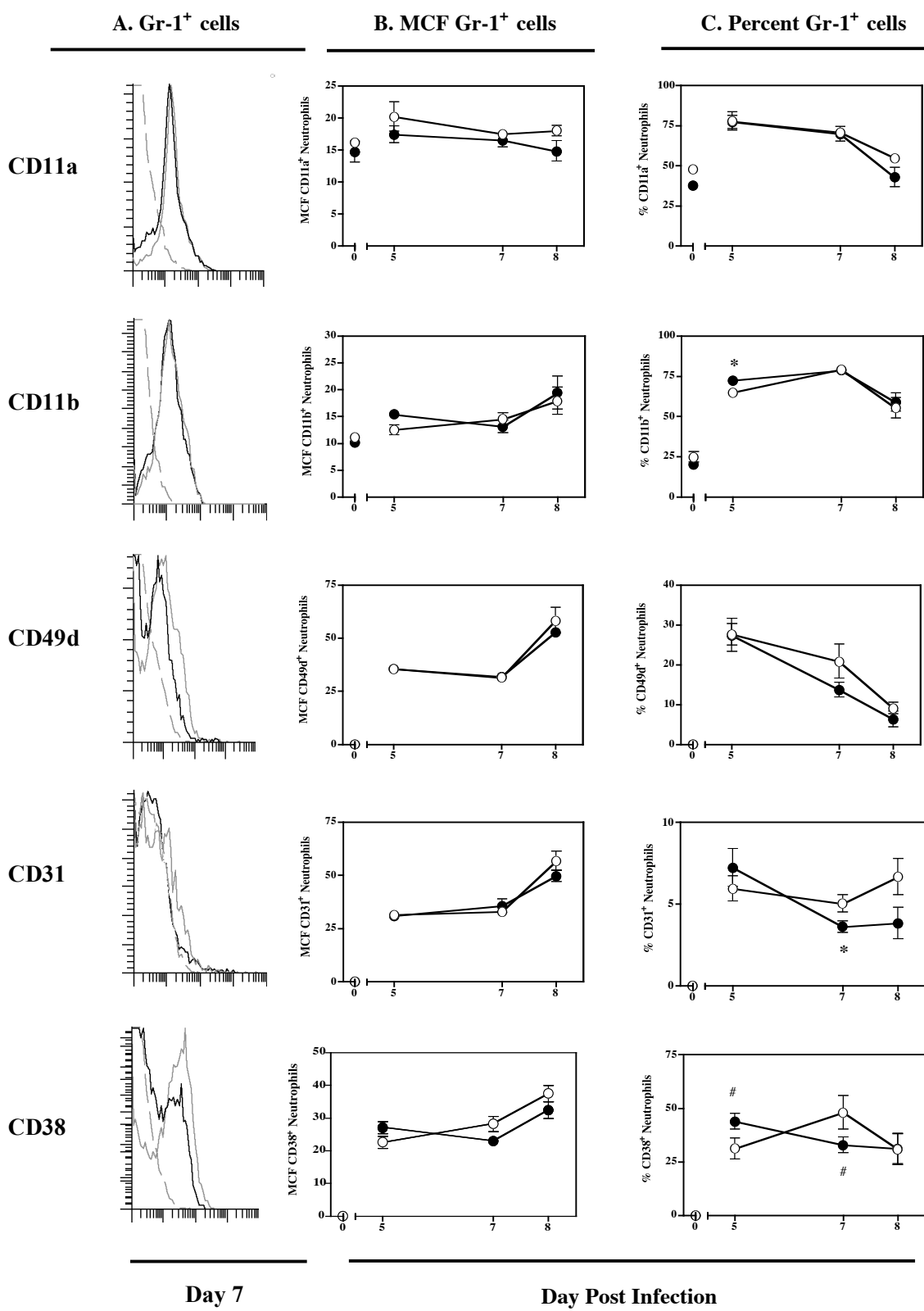


Figure 2.9 Expression profile of adhesion molecules on pulmonary neutrophils in response to infection with influenza virus. Mice (6 per treatment group/day) were treated as described in Figure 2.1. To clear the pulmonary cavity of vascular blood, lungs were flushed with 3 ml endotoxin-tested PBS containing 10 U/ml heparin, prior to digestion with collagenase. **(A)** Representative histograms depict the staining of pulmonary neutrophils (Gr-1⁺ cells) with antibodies against the indicated adhesion molecule on day 7. The dashed grey line depicts background staining with an isotypic control antibody, and the solid grey and solid black lines indicate the staining of Gr-1⁺ cells from vehicle- and TCDD-treated mice, respectively. **(B, C)** The average mean channel fluorescence (MCF) and percentage of each adhesion molecule on Gr-1⁺ cells from lungs of mice treated with vehicle (open circles) or TCDD (closed circles) was examined 5, 7, and 8 days after infection. Mock-infected mice treated with vehicle or TCDD were used as controls (4 mice per treatment group). Data from mock-infected mice are depicted at the zero time point. Error bars represent the SEM. An * indicates a significant difference compared to vehicle-treated mice ($p \leq 0.05$). An # denotes $p \leq 0.09$. We obtained the same results in a separate study in which the pulmonary cavity was not flushed of vascular blood (data not shown).

Neutrophil apoptosis and necrosis are not delayed in the lungs of infected mice exposed to TCDD.

Neutrophils have a very short half-life at the site of infection (six to ten hours), and neutrophil apoptosis is critical for the resolution of inflammation (Pletz *et al.*, 2004). There are numerous reports documenting the effects of TCDD, and other AhR ligands, on apoptosis (Staples *et al.*, 1998a; Prell *et al.*, 2000; Camacho *et al.*, 2001; Dearstyne and Kerkvliet, 2002; Lee *et al.*, 2003; Patterson *et al.*, 2003; Solhaug *et al.*, 2004). However, these studies focus on lymphocyte apoptosis, and to our knowledge there are no reports in which the effects of TCDD on neutrophil apoptosis have been examined. If exposure to TCDD causes a delay or defect in the death of neutrophils, then we would expect to detect fewer apoptotic or necrotic neutrophils in the lungs of TCDD-treated, infected mice. We investigated whether the excess number of neutrophils in the lungs of mice treated with TCDD results from a delay in neutrophil apoptosis (Figure 2.10). Our results indicate that exposure to TCDD did not delay neutrophil apoptosis. We

believe that the slight increase in the percentage of apoptotic neutrophils in cells from mice treated with TCDD (day 8) is not biologically significant because we did not detect this increase in a separate study using the TUNEL staining method (data not shown). Hence, delayed neutrophil death does not underlie the excess neutrophilia in the lungs of TCDD-treated, infected mice.

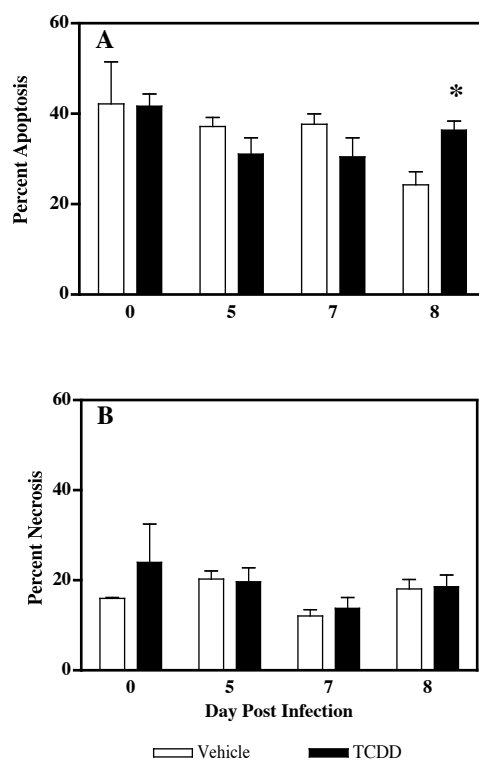


Figure 2.10 Exposure to TCDD does not affect pulmonary neutrophil apoptosis and necrosis. Mice were treated as described in Figure 2.1. On days 5, 7, and 8 post infection, mice treated with vehicle or TCDD were sacrificed, and lungs were digested with collagenase (6 mice per treatment group/day). Mock-infected mice treated with vehicle or TCDD served as controls for the infection (4 mice per treatment group). (A) Apoptosis and (B) necrosis of pulmonary neutrophils was measured by staining with annexin V and 7-amino-actinomycin (7-AAD). Apoptotic neutrophils were defined as Gr-1 positive, annexin V positive, and 7-AAD negative. Necrotic neutrophils were characterized as Gr-1 positive, annexin V positive, and 7-AAD positive. Error bars represent the SEM. An * denotes a significant difference compared to the vehicle treatment group ($p \leq 0.05$). Results are representative of two separate experiments.

DISCUSSION

The lung expresses high levels of the AhR (Yamamoto *et al.*, 2004), and many AhR ligands are airborne pollutants, suggesting that the lung is a likely target for the immunotoxic action of a large group of abundant and persistent toxicants. Previous data from our laboratory (Lawrence *et al.*, 2000; Warren *et al.*, 2000; Vorderstrasse *et al.*, 2003a) and others (Luebke *et al.*, 2002) have shown that exposure to the prototypic AhR agonist TCDD decreases host resistance to infection with influenza virus, and this decrease in survival correlates with enhanced neutrophilia in the lung. However, there are currently no studies that attribute impaired host resistance to a particular cell type. Knowing that excess neutrophils are associated with the pathology that accompanies many inflammatory diseases, it seemed logical to postulate that this increase is detrimental to the host's ability to survive infection. Data presented here strongly support this idea, as survival of TCDD-treated mice was greatly improved following neutrophil-depletion. Furthermore, our findings extend our understanding of how activation of the AhR adversely affects immune function, as we show for the first time that, in addition to the suppressive effects of TCDD on lymphocyte responses, the increased number of neutrophils occurs via an AhR-dependent mechanism.

Findings from these studies also provide novel information regarding the kinetics and magnitude of many aspects of the pulmonary innate immune response to infection with influenza A virus. We measured levels of the neutrophil-recruiting chemoattractants KC, MIP-1 α , MIP-2, LIX, IL-6 and C5a in the lung. All of these mediators were detected and, except for C5a, there was an infection-associated increase in the levels. KC, MIP-1 α , and MIP-2 reached peak levels 12 to 24 hours after infection. IL-6 was also elevated very rapidly, although instead of declining within a few days, levels remained elevated. The rapid production of these soluble mediators in

response to infection with influenza virus is consistent with previous data from our laboratory (Neff-LaFord *et al.*, 2003) and studies by others (Hennet *et al.*, 1992; Van Reeth *et al.*, 1998; Sakai *et al.*, 2000), demonstrating that inflammatory mediators in the lung are elevated very rapidly following viral infection. Moreover, this timing is consistent with the very rapid recruitment of neutrophils to the lung following infection with influenza virus, as neutrophils are detected within 24 hours of infection (Van Reeth *et al.*, 1998; Warren *et al.*, 2000). Yet, since neutrophils continue to immigrate to the lung until the infection is resolved, additional factors are likely responsible for the continued recruitment of neutrophils to the lungs during infection with influenza.

Adhesion molecules, such as CD11a, CD11b, CD49d, CD31, and CD38, are regulatory factors that are clearly important for neutrophil recruitment and extravasation (Partida-Sanchez *et al.*, 2001; Stadnyk *et al.*, 2002; Soethout *et al.*, 2003; Tasaka *et al.*, 2003). However, there are no reports describing the effects of influenza virus infection on the expression of CD11a, CD11b, CD49d, CD31, and CD38 on neutrophils *in vivo*. The roles of CD31 and CD49d in neutrophil migration have been examined in models of *E. coli*- and *Strep. pneumoniae*-mediated bacterial pneumonia (Tasaka *et al.*, 2002; Tasaka *et al.*, 2003). Hartshorn and White (1999) demonstrated that CD11b and CD11c are up-regulated on neutrophils during *in vitro* infection with influenza virus. We show that *in vivo* infection with influenza virus induces expression of CD31, CD38, and CD49d and up-regulates expression of CD11a and CD11b on pulmonary neutrophils. Furthermore, we show that their expression remains elevated at even after the levels of common neutrophil chemoattractants have returned to baseline. This suggests that other factors likely regulate expression of adhesion molecules on neutrophils and their continued immigration to the lung during the response to infection with influenza virus.

Based on reports that exposure to TCDD alters production of other types of cytokines (Prell *et al.*, 1995; Kerkvliet *et al.*, 1996; Warren *et al.*, 2000; Nohara *et al.*, 2002), it was logical to test the hypothesis that treatment with TCDD enhances levels of soluble mediators that are chemoattractant for neutrophils, thereby resulting in more neutrophils at the site of infection. However, exposure to TCDD did not alter levels of known soluble neutrophil chemoattractants in the lungs of infected mice. These findings, especially combined with the findings from the *ex vivo* migration study, suggest that AhR-deregulation of soluble chemoattractants does not underlie the increased recruitment of neutrophils to the lungs of TCDD-treated, infected mice. This conclusion is supported by other studies from our laboratory. For example, two other common pro-inflammatory and neutrophil attracting cytokines not included in the study reported here are IL-1 and tumor necrosis factor (TNF). In separate studies, we characterized the effects of TCDD on IL-1 and TNF levels in lung lavage fluid during the course of infection with influenza A virus. Similar to KC, MIP-1 α , MIP-2, levels peaked very early during infection, and there was no difference in IL-1 or TNF levels in BAL fluid from vehicle- and TCDD-treated mice (Neff-LaFord *et al.*, 2003). Taken together, our findings are also consistent with those of Lang *et al.* (1998), who reported that exposure to TCDD did not alter the production of IL-6 and IL-8 by human lung cells or peripheral blood cells.

The lack of effect of exposure to TCDD on stable, soluble neutrophil chemoattractants or neutrophil adhesion molecules led us to examine whether, instead of enhancing levels of regulatory factors that cause more neutrophils to migrate to the lung, AhR activation decreases the rate of neutrophil apoptosis. Our data do not support this idea either. It is certainly possible that TCDD alters the production of labile, soluble neutrophil chemoattractants, such as arachidonic acid metabolites (please also refer to Chapter 5). For example, LTB₄ has been found in lung

lavage fluid of mice infected with influenza virus (Hennet *et al.*, 1992). AhR-mediated alterations in arachidonic acid metabolism have been examined previously (Lawrence and Kerkvliet, 1998; Lee *et al.*, 1998). However, these studies found no evidence for an increase in LTB₄ levels in TCDD-treated mice. Alternatively, instead of increasing positive signals, exposure to TCDD could prevent or dampen production of essential down-regulators of inflammation, such as IL-10 or transforming growth factor-(TGF)- β . In fact, using lung lavage fluid from vehicle- and TCDD-treated mice, we examined levels of IL-10 and TGF- β at multiple times relative to infection with influenza virus (from day 0 to day 9). We did not detect TGF- β in BAL fluid at any time; however, IL-10 levels increased rapidly after infection in both treatment groups, and returned to baseline levels three days after infection (data not shown). Exposure to TCDD did not alter the infection-associated production of IL-10. These findings indicate that altered levels of LTB₄, IL-10 or TGF- β are not likely the mechanistic explanation for the recruitment of excess neutrophils to the lungs of mice exposed to TCDD.

Thus, elevated number of neutrophils at the site of antigen challenge is likely the result of some other mechanism. One possibility is that inappropriate activation of the AhR exerts its direct action on molecular targets in the lung, rather than via altered cytokine/chemokine production or targets within the hematopoietic system. For example, AhR activation may enhance the expression of adhesion molecules on lung endothelial or epithelial cells, or it may alter the levels of surfactant proteins (SP; please refer to Appendix C). Several reports have attributed pro-inflammatory roles to SP-A and SP-D, including recruitment of neutrophils to the lung (Cai *et al.*, 1999; Schagat *et al.*, 2003). However, other reports suggest anti-inflammatory roles, as SP-A- and SP-D-deficient mice had exacerbated inflammatory processes (LeVine *et al.*, 2001; LeVine *et al.*, 2002). Thus, the precise relationship between SP-A and SP-D and pulmonary inflammation is not

clear. Nevertheless, given the likely role for these lung-derived molecules in neutrophil recruitment and inflammation, it is possible that AhR-mediated alterations in surfactant proteins underlies the enhanced number of neutrophils observed in the lungs of infected mice treated with TCDD.

Information reported here fills a gap in our knowledge of the consequences of exposure to AhR ligands on the inflammatory mediators involved in the response to a common respiratory pathogen. Very little is known about the specific effects of AhR agonists on neutrophils in general, and even fewer studies have examined neutrophils as mediators of the toxicity associated with exposure to AhR ligands. With regard to the lung, other laboratories have reported effects of a variety of AhR ligands on the lung or cultured lung epithelial cells (Topping *et al.*, 1978; Mitchell *et al.*, 1987; Beebe *et al.*, 1990; Kim and Lee, 1996; Gerde *et al.*, 1997; Tritscher *et al.*, 2000; Wei *et al.*, 2002; Lin *et al.*, 2004). However, most of these studies focused on changes in cell growth, the induction of metabolic enzymes, and the relationship between AhR activation and cancer. The relationship between these biochemical effects of AhR ligands and pulmonary inflammation has received less attention. *In vitro* and *in vivo* studies using PAHs or complex mixtures that contain PAHs (e.g., diesel exhaust particles) generally demonstrate pro-inflammatory effects (Ng *et al.*, 1998; Li *et al.*, 2002; Harrod *et al.*, 2003). Whether these reported alterations are mediated directly by the AhR has not been clearly defined. Nevertheless, these studies support the idea that exogenous AhR agonists affect pulmonary host defense mechanisms, which in turn likely alters susceptibility to infectious and inflammatory diseases. Our findings corroborate this idea, demonstrating that TCDD, via an AhR-dependent mechanism, increases the number of neutrophils in the lungs during infection.

Thus, our observation that exposure to TCDD exacerbates the inflammatory response in the lungs suggests a possible mechanistic link between exposure to AhR ligands and chronic inflammatory lung diseases. Excess neutrophils within airways play a clear role in the pathology of these diseases; however, it is not clear why certain individuals are more prone to develop chronic inflammatory diseases of the lung. Exposure to toxicants and decreased resistance to respiratory infection have been suggested to be important factors, yet the mechanistic relationship between these events and the onset of inflammatory disease remains unclear. Interestingly, retrospective study of a population heavily exposed to TCDD demonstrated a relationship between level of exposure to TCDD and increases in COPD (Pesatori *et al.*, 1998). Based on our data, we suggest that inappropriate AhR activation (i.e., by exogenous compounds) increases the number of neutrophils recruited to the lung in response to viral infection, and this excess number of neutrophils is detrimental to the host, causing neutrophil-mediated pathology and diminishing survival.

CHAPTER THREE

Exposure to TCDD does not enhance vascular permeability in the lungs of mice infected with influenza A virus¹

In the context of a sublethal infection with murine-adapted human influenza virus, our laboratory typically observes 90 to 100 percent survival of vehicle-treated mice. In contrast, we (Lawrence *et al.*, 2000; Warren *et al.*, 2000; Vorderstrasse *et al.*, 2003a) and others (House *et al.*, 1990; Burleson *et al.*, 1996; Luebke *et al.*, 2002) observe significantly lower survival of TCDD-exposed mice in response to this sublethal infection. While overall survival rates in TCDD-treated, infected mice vary between experiments, these rates can be as low as 20 percent compared to vehicle-exposed, infected mice (Warren *et al.*, 2000). Perplexingly, we have determined that the reduced survival in TCDD-treated, virus-infected mice is not due to impaired viral clearance (Lawrence *et al.*, 2000; Neff-LaFord *et al.*, 2003). Despite reduced numbers of influenza virus-specific cytotoxic T lymphocytes (CTL_e) in the lungs of TCDD-exposed, infected mice (Warren *et al.*, 2000; Mitchell and Lawrence, 2003), our findings show that these CTL_e adequately clear influenza virus from the lungs of mice treated with TCDD (Lawrence *et al.*, 2000; Neff-LaFord *et al.*, 2003). In fact, our data demonstrate that the kinetics of viral clearance are equivalent in vehicle- and TCDD-treated mice (Lawrence *et al.*, 2000; Neff-LaFord *et al.*, 2003).

¹Data from this chapter were published as part of the following manuscript: Bohn, A. A., Harrod, K. S., Teske, S., and Lawrence, B. P. (2005). Increased mortality with TCDD exposure in mice infected with influenza A virus is not due to severity of lung injury or alterations in Clara cell protein content. *Chemico-Biological Interactions* 155(3):181-99. Data represented in Table 3.1 were generated by Sabine Teske. Data shown in Table 3.2 and 3.3 were generated by Dr. Andrea Bohn.

Knowing that TCDD-treated mice succumb from an otherwise sublethal infection with influenza virus, despite equivalent viral clearance, prompted use to investigate the cause underlying the decreased survival of these mice.

Concomitant with the decreased survival in TCDD-treated, infected mice we observe a profound increase in the number of neutrophils in the lungs of TCDD-treated, infected mice (Warren *et al.*, 2000; Vorderstrasse *et al.*, 2003a; Teske *et al.*, 2005). Furthermore, as described in Chapter 2, we have shown that the elevated number of neutrophils decreases survival to infection with influenza virus (Teske *et al.*, 2005). As presented in detail in Chapter 2, we have investigated several mechanisms that could drive the recruitment of excess neutrophils to the lungs of TCDD-treated mice. Our previous data indicate that elevated levels of known stable, soluble neutrophil-chemoattractants in the lung, up-regulated expression of adhesion molecules on pulmonary neutrophils, or delayed neutrophil apoptosis in the lung are not mechanisms that underlie the excess pulmonary neutrophils in TCDD-exposed, infected mice (Teske *et al.*, 2005).

In the absence of an effect of exposure to TCDD on these mechanisms, we tested whether treatment with TCDD enhances pulmonary vascular damage in infected mice, leading to nonspecific leakage of neutrophils from the bloodstream to the lung. Infection with influenza virus targets epithelial cells throughout the respiratory tract (Nayak *et al.*, 2004). Furthermore, several studies have shown that infection with influenza virus damages the vasculature, leading to pulmonary edema (Burlison *et al.*, 1996; Suliman *et al.*, 2001; Luebke *et al.*, 2002). In our model system, increased vascular permeability would not only provide a mechanistic explanation for the diminished survival of TCDD-treated, infected mice, but also would explain the excessive pulmonary neutrophilia. As described in detail in Chapters 1 and 2, neutrophils are constraint to the bloodstream in the absence of infection. During respiratory viral infection, neutrophils

actively migrate from the bloodstream to the lung. However, during an infection the integrity of both the endothelium and epithelium are potentially compromised, resulting in hyperporous blood vessels and damaged epithelial cells. This infection-mediated endothelial and epithelial cell damage leads to passive leakage of neutrophils to the site of infection (e.g., the lung in our model system). To test whether treatment with TCDD alters endothelial and epithelial integrity, we measured levels of protein, levels of lactate dehydrogenase (LDH), and lung wet weights in vehicle- and TCDD-treated, infected mice. Elevated levels of protein in the lung and increased lung wet weights are markers of pulmonary edema, which are indicative of vascular damage. Analogously, enhanced levels of LDH in the lung is a marker of epithelial cell damage, which is indicative of LDH release from damaged cells.

Our data show a two-fold increase in lung wet weights and wet-dry weight difference seven days after infection with influenza virus compared to mock-infected mice (Table 3.1). However, treatment with TCDD did not exacerbate this infection-associated increase in lung wet weights or the wet-dry weight difference. Hence, wet/dry weight ratios were not significantly altered between the vehicle- and TCDD-treated, infected mice. These findings are consistent with studies conducted by Burleson *et al.* (1996) and Luebke *et al.* (2002), which have also reported that influenza virus infection doubled lung weights, yet exposure to TCDD did not further increase lung weights in those studies.

TREATMENT	wet weight (mg)	dry weight (mg)	wet/dry ratio	wet-dry difference (mg)
Veh, mock-infected	115±6	25±2	4.54±0.07	89±5
TCDD, mock-infected	119±5	25±1	4.77±0.18	94±4
Veh, infected	226±77	41±11	5.35±0.65	185±67
TCDD, infected	263±51	46±6	5.68±0.36	217±45

Table 3.1 Exposure to TCDD does not increase infection-associated pulmonary edema. Female C57BL/6 mice were gavaged with peanut oil vehicle (*Veh*) or TCDD (10 µg/kg body weight) one day prior to intranasal (i.n.) infection with 120 hemagglutinating units (HAU) of influenza virus (A/HKx31; *infected*). Mice received 120 HAU influenza virus in a final volume of 25 µl endotoxin-tested PBS under general anesthesia. Vehicle- and TCDD-treated, mock-infected mice were given 25 µl PBS i.n. under general anesthesia (*mock-infected*). Seven days after infection, mice were sacrificed and lungs were excised, briefly rinsed in PBS, and blotted dry. Immediately thereafter lungs were weighed (*wet weight*), placed in a drying oven at 60°C, and weighed until they reached a constant weight (*dry weight*). The following indicators of pulmonary edema were determined: lung wet weight, lung dry weight, weight/dry weight ratio, and wet-dry weight difference. The average values (4-5 mice per treatment group) for each treatment group are indicated, ± SEM. No statistically significant differences were observed between vehicle- and TCDD-treated mice.

In addition to measuring lung wet weights, we assessed protein levels in lung lavage fluid of vehicle- and TCDD-exposed mice over the course of infection (Table 3.2). Our results indicate that protein levels were significantly increased in both vehicle- and TCDD-treated mice five and seven days after infection compared to mock-infected mice. This finding is consistent with a study by Suliman *et al.* (2001), which documents elevated levels of protein in the lungs of influenza virus-infected mice. However, in contrast to the strong infection-associated increase in pulmonary protein levels, exposure to TCDD did not exacerbate this infection-associated

increase. Taken together, the lung wet weight and protein data strongly suggest that treatment with TCDD does not increase pulmonary edema in infected mice.

Day Post Infection	TREATMENT	
	Vehicle	TCDD
0	24±9(5)	20±3(5)
0.5	36±15(8)	23±5(7)
1	37±10(6)	66±30(7)
3	63±26(7)	69±42(8)
5	153±42(8)*	168±70(6)*
7	226±29(8)*	207±37(8)*

Table 3.2 Protein levels in lung lavage fluid are not altered by treatment with TCDD. Mice were treated as described in Table 3.1. On the indicated days relative to infection vehicle- or TCDD-treated mice were sacrificed. Lungs were lavaged with 1 ml endotoxin-tested PBS. Lung lavage fluid and cells were separated by centrifugation. The level of protein in lavage fluid was determined using a Cobas Mira Plus Chemistry analyzer. Values indicate the average protein concentration in mg/dl, ± SEM. The number in parentheses indicates the total number of animals within that treatment group. Statistically significant differences were not observed when comparing vehicle and TCDD treatment groups at any point in time. The asterisks denote values significantly different ($p < 0.001$) from mock-infected mice (*day 0*), indicating an effect of infection with influenza virus.

Finally, we examined levels of LDH in lung lavage fluid of vehicle- or TCDD-treated mice over the course of infection. Infection with influenza virus has been shown to elevate levels of LDH in lung lavage fluid (Suliman *et al.*, 2001). Likewise to Suliman *et al.* (2001), our data indicate that levels of LDH are clearly increased in lung lavage fluid of vehicle- and TCDD-treated five and seven days after infection compared to mock-infected mice (Table 3.3). Again,

treatment with TCDD did not further elevate this infection-mediated increase in LDH levels, indicating that lung cells are not excessively damaged upon treatment with TCDD.

Day Post Infection	TREATMENT	
	Vehicle	TCDD
0	91±61(5)	59±23(5)
0.5	119±70(8)	69±20(7)
1	154±43(6)	154±42(7)
3	248±78(7)	202±117(8)
5	356±67(8)*	352±90(6)*
7	458±95(8)*	464±78(8)*

Table 3.3 Exposure to TCDD does not enhance the concentration of lactate dehydrogenase (LDH) in lung lavage fluid in mice infected with influenza A virus. Mice were treated as described in Table 3.1. On the indicated days after infection, vehicle- or TCDD-exposed mice were sacrificed and lung lavage fluid was collected as described in Table 3.2. The level of LDH in lung lavage fluid was determined using a Cobas Mira Plus Chemistry analyzer. Values indicate the average LDH concentration in Units/L, ± SEM. The number in parentheses indicates the total number of animals within that treatment group. Statistically significant differences were not observed between the vehicle and TCDD treatment groups at any point in time. The asterisks designate values significantly different ($p < 0.001$) from mock-infected mice (*day 0*), indicating an effect of influenza virus infection.

In conclusion, the findings from these studies illustrate that exposure to TCDD does not increase lung wet weights, levels of protein, and levels of LDH in the lungs of infected mice. Hence, increased pulmonary vascular permeability and increased lung cell damage do not prove to be plausible mechanisms underlying the diminished survival in TCDD-exposed, infected mice. Furthermore, these data strongly suggest that enhanced pulmonary endothelial and

epithelial cell damage, leading to concomitant leakage of neutrophils from the bloodstream to the lung is an unlikely mechanistic explanation for the excess number of neutrophils in the lungs of mice treated with TCDD.

CHAPTER FOUR

Recruitment of excess neutrophils during respiratory viral infection is dependent upon aryl hydrocarbon receptor (AhR)-mediated events within the lung and does not require AhR in the hematopoietic system¹

ABSTRACT

In the context of respiratory viral infection with influenza virus, activation of the aryl hydrocarbon receptor (AhR) significantly increases the number of pulmonary neutrophils. Despite the global prevalence of respiratory viral infections, many facets of neutrophil recruitment to the lungs during viral infection are not very well understood. Likewise, it remains to be elucidated how activation of the AhR increases neutrophil recruitment to the lung during infection with influenza virus. We have previously shown that activation of the AhR by TCDD does not elevate levels of soluble neutrophil chemoattractants in the lung, up-regulate adhesion molecules on pulmonary neutrophils, delay pulmonary neutrophil apoptosis, or increase vascular damage in the lungs of infected mice. In the present study, we examined whether activation of the AhR enhances the number of circulating neutrophils, which in turn could increase the number of neutrophils in the lungs. Our results show that AhR activation does not systemically increase neutrophil numbers, suggesting that the excess number of neutrophils in TCDD-treated

¹With the exception of the data presented in Table 4.2 and Figures 4.6, 4.7, and 4.8, all other data will be submitted for publication in the following manuscript: Teske, S., Bohn, A. A., Cundiff, J.A., and Lawrence, B. P. (2006). Recruitment of excess neutrophils during respiratory viral infection is dependent upon aryl hydrocarbon receptor (AhR)-mediated events within the lung and does not require AhR in the hematopoietic system.

mice is restricted to the site of antigen challenge (i.e., the lung). To determine whether AhR-driven events within or external to the immune system regulate the increased pulmonary neutrophil number, we generated bone marrow chimeric mice in which the immune cells are AhR-deficient, but all other tissues, including the lung, express the AhR. Our data show that TCDD-treated, infected CD45.2AhR^{-/-}→CD45.1AhR^{+/+} chimeric mice have a two-fold increase in the number of neutrophils compared to vehicle-treated, infected CD45.2AhR^{-/-}→CD45.1AhR^{+/+} chimeric mice. These data strongly suggest that potential targets of AhR regulation are within in the lung, and not the immune system. Discerning whether AhR-mediated events are within or external to the immune system aids our understanding of the complex mechanism of AhR-driven deregulation of neutrophil recruitment to the lungs of TCDD-exposed, infected mice.

INTRODUCTION

Although vaccination and improved drug therapy have dramatically reduced mortality from infectious diseases, respiratory viral infections remain among the top ten causes of mortality in the United States (NCHS, 2002) and worldwide (WHO, 2004; WHO, 2005). With the continued emergence of new viral strains, influenza viruses in particular pose a threat to human health and the global economy. The immune response to influenza virus relies on the activation of cells from the innate and adaptive arms of the immune system, leading ultimately to the creation of virus-specific CD8⁺ cytotoxic T lymphocytes (CTL; Doherty *et al.*, 1997) and antibodies (Gerhard, 2001). In a primary infection, the generation of CTL and virus-specific antibodies takes 7 to 10 days, during which time cells of the innate immune system immigrate to the lung and presumably keep the infection at bay. In contrast to the well-characterized role of

lymphocytes in anti-viral immune responses, less is known about the precise role that cells of the innate immune system, and neutrophils in particular, play during respiratory viral infections.

It is becoming increasingly clear that the recruitment of neutrophils to the lung requires exquisite control. Evidence for this includes studies demonstrating that the accumulation of excess neutrophils is associated with host tissue damage and increased mortality (Patel *et al.*, 1999; Shimizu *et al.*, 1999; Fernandez *et al.*, 2001; Teske *et al.*, 2005). On the other hand, depletion of neutrophils can diminish survival following infection (Gonzalez *et al.*, 1987; Sayles and Johnson, 1996; Bliss *et al.*, 2001; Stephens-Romero *et al.*, 2005; Tumpey *et al.*, 2005). Thus, there is growing evidence that the magnitude of neutrophil influx to the lung probably plays a very important role in the host's ability to survive viral infection. Therefore, it is important to understand what factors influence the differential recruitment of neutrophils to the lung upon infection with different subtypes of influenza A virus or among different individuals.

We have recently discovered that activation of the aryl hydrocarbon receptor (AhR) markedly increases the influx of neutrophils to the lungs of mice infected with influenza A virus (Teske *et al.*, 2005). Furthermore, by depleting neutrophils *in vivo*, we were able to improve the survival of infected mice (Teske *et al.*, 2005), suggesting that AhR-mediated recruitment of excess neutrophils to the lung contributes to the decreased survival from influenza virus. The AhR is a member of the Per-Arnt-Sim (PAS) family of transcriptional regulators and plays a role in xenobiotic metabolism and development (Gu *et al.*, 2000). The AhR is activated by a diverse spectrum of ligands, including plant-derived natural compounds, tryptophan metabolites and environmental contaminants (Heath-Pagliuso *et al.*, 1998; Casper *et al.*, 1999; Denison and Nagy, 2003; Jeuken *et al.*, 2003; Palermo *et al.*, 2003). Of the AhR agonists characterized thus far, the pollutant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or "dioxin") binds it with the

highest affinity, and is often used as a prototypical AhR agonist. In addition to TCDD, other pollutants that bind and activate the AhR include coplanar polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAH), such as benzo[a]pyrene and 7,12-dimethylbenzanthracene, which are found in cigarette smoke and diesel exhaust (Behnisch *et al.*, 2003).

In short, humans are exposed to AhR ligands daily through ingestion and inhalation (Schechter *et al.*, 2001; IOM, 2003; Charnley and Doull, 2005). Moreover, diminished host resistance and altered immune function following exposure to PAH-containing pollutants correlates with an increased incidence of influenza and other respiratory infections in humans (Sopori and Kozak, 1998). In rodents, AhR activation impairs survival following infection with influenza A virus (House *et al.*, 1990; Burlison *et al.*, 1996; Lawrence *et al.*, 2000; Warren *et al.*, 2000; Luebke *et al.*, 2002; Vorderstrasse *et al.*, 2003a; Teske *et al.*, 2005), further illustrating the relationship between exposure to AhR ligands and altered host resistance to infection.

The AhR is broadly expressed in mammalian tissues, and cells in the immune system and lung have been reported to express the AhR (Hayashi *et al.*, 1995; Lawrence *et al.*, 1996; Lang *et al.*, 1998; Williams *et al.*, 1996; Yamamoto *et al.*, 2004). Interestingly, some of the highest levels of AhR expression of any organ is found in both the human and rodent lung (Lang *et al.*, 1998; Yamamoto *et al.*, 2004), yet the molecular mechanisms of AhR-regulation within the lung are not well-characterized. Furthermore, in both humans and rodents exposure to AhR agonists has been linked to enhanced pulmonary inflammation, including increased neutrophil influx to the lung (Salvi *et al.*, 1999; Diaz-Sanchez *et al.*, 2000; Warren *et al.*, 2000; Gilmour *et al.*, 2001; Luebke *et al.*, 2002; Dick *et al.*, 2003; Harrod *et al.*, 2003; Vorderstrasse *et al.*, 2003a; Teske *et al.*, 2005). However, the mechanism by which AhR activation enhances the directional migration of neutrophils has proved difficult to determine. The immune system is a well known and very

sensitive target organ for the toxicity of dioxins and related compounds, and studies using AhR-deficient mice demonstrate that their toxicity is AhR-dependent (Vorderstrasse *et al.*, 2001; Kerkvliet *et al.*, 2002; Teske *et al.*, 2005; and our unpublished observations). Therefore, much effort to delineate the mechanism by which AhR activation enhances neutrophil recruitment has focused on an immune-mediated mechanism. However, neither the infection-induced increase in soluble neutrophil chemoattractants nor the up-regulation of adhesion molecules on neutrophils is perturbed by when the AhR is activated by TCDD (Teske *et al.*, 2005). Likewise, the functional activity of neutrophils from infected, TCDD-treated mice was equivalent to neutrophils from vehicle control-treated mice (Teske *et al.*, 2005).

Instead of a specific effect on known soluble neutrophil chemoattractants or neutrophils per se, it is possible that the increase in the number of neutrophils results from non-specific leakage of leukocytes from the blood into the lung. However, the following pieces of evidence do not support this idea: (1) the number of macrophages in lungs of infected mice is the same, regardless of TCDD-treatment (Warren *et al.*, 2000; Vorderstrasse *et al.*, 2003a), (2) AhR activation by TCDD reduces the number of lymphocytes in lungs of infected mice (Warren *et al.*, 2000; Mitchell and Lawrence, 2003), and (3) compared to infected controls, there is no change in the levels of protein or LDH in lavage fluid from infected mice treated with TCDD (Bohn *et al.*, 2005). Collectively, these observations suggest that the directional migration of excess neutrophils to the lung during influenza virus infection is likely the result of AhR-dependent deregulation of a neutrophil-specific recruitment processes. To characterize how activation of the AhR deregulates neutrophil-specific processes, we first determined whether AhR activation enhances the number of neutrophils systemically or only at the site of antigen challenge (i.e., the lung). We then determined whether AhR-mediated events within or extrinsic to the immune

system drive the recruitment of excess neutrophils to the lungs of TCDD-treated, infected mice. The findings from these studies provide novel information regarding how AhR activation alters neutrophil recruitment to the lung, and emphasizes that environmental exposure to AhR agonists may influence disease outcome during infection with a common respiratory virus.

MATERIALS AND METHODS

Animals, TCDD treatment, and infection

Female C57BL/6, C57BL/10, C3H/HeNcr, and C3H/HeJCr mice, and B6-Ly5.2/Cr congenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or NCI-Frederick (Frederick, MD) at four to six weeks of age. A breeding colony of C57BL/6-AhR-deficient ($AhR^{-/-}$) mice was maintained at Washington State University as previously described (Teske *et al.*, 2005). All mice were provided with water and food *ad libitum* and housed under pathogen-free conditions in microisolator cages (2-5 mice per cage). All animal treatment was performed in accordance with protocols approved by the Institutional Animal Care and Use Committee.

TCDD ($\geq 99\%$ pure, Cambridge Isotope Laboratories, Inc., Woburn, MA) was dissolved in anisole and diluted in peanut oil to a concentration of 1 $\mu\text{g}/\text{ml}$. Mice were gavaged with a single dose of TCDD (10 $\mu\text{g}/\text{kg}$ body weight). Vehicle-control mice were gavaged with a single dose of peanut oil-anisole vehicle. Mice were infected intranasally (i.n.) with either 120 hemagglutinating units (HAU) of murine-adapted human influenza virus, A/HKx31 (X-31; H3N2) or 10^7 plaque forming units (PFU) of influenza virus, A/Memphis/102/72 (Mem-72; H3N2; 10^7 PFU of Mem-72 are equivalent to 125 HAU), in 25 μl endotoxin-tested phosphate

buffered saline (PBS). Vehicle-treated mice typically survive an infection with these doses of influenza virus (Lawrence *et al.*, 2000; Warren *et al.*, 2000; Mitchell and Lawrence, 2003). Vehicle- and TCDD-treated, mock-infected mice were used as controls for the infection, and received 25 μ l endotoxin-tested PBS (i.n.). Data from mock-infected mice are defined at the zero time point. Infection was performed under general anesthesia (Avertin; 2,2,2-tribromoethanol, Sigma-Aldrich, St. Louis, MO).

Lung-derived immune cells

To obtain airway-derived immune cells, lungs were lavaged with cold serum-free RPMI 1640 media or PBS (Warren *et al.*, 2000; Teske *et al.*, 2005). Airway-derived immune cells were separated from lavage fluid by centrifugation and enumerated using a Coulter counter (Beckman Coulter Corp., Miami, FL).

To obtain total lung-derived immune cells, lungs were digested with collagenase as previously described (Teske *et al.*, 2005). Briefly, lungs were incubated for 25 min at 37°C, 5% CO₂ with warm RPMI 1640 media, containing 1 mg/ml collagenase type 2 (Worthington Biochemical Corp., Lakewood, NJ) and 30 μ g/ml deoxyribonuclease I (Sigma-Aldrich). After digestion with collagenase, Lympholyte-M media (Cedarlane Laboratories, Hornby, Ontario, Canada) was used to separate immune cells from parenchymal lung cells, dead cells, and erythrocytes. Total lung-derived immune cells were counted using a Coulter counter.

Bone marrow cells

Bone marrow cells were collected from femurs of both hind legs (Fortier, 1994; Vorderstrasse *et al.*, 2004a). Briefly, muscle and adipose tissue were removed from femurs and an incision was made at the upper and lower end of the femur. Using a syringe and 26-gauge needle, each femur was flushed with 2 ml cold RPMI 1640 containing 2.5% FBS and 10 mM HEPES. To disrupt cellular clumps, the cell suspension was drawn several times through a 22-gauge needle and passed through a 70 μ m nylon cell strainer (Fisher Scientific, Santa Clara, CA). Erythrocytes were lysed using an ammonium chloride lysis solution. Bone marrow cells were enumerated using a Coulter counter.

Spleen cells and mediastinal lymph node (MLN) cells

A single-cell suspension of spleen and MLN cells was prepared by pressing each organ between the frosted ends of two microscope slides (Warren *et al.*, 2000). Spleen and MLN cells from individual animals were resuspended in cold RPMI 1640 containing 2.5% FBS and 10 mM HEPES. Cellular debris was removed by sedimentation for 8 min on ice. Erythrocytes were removed from the spleen cell suspension by hypotonic lysis. Using a Coulter counter, spleen and MLN cell counts were determined.

Liver histology

Livers from vehicle- and TCDD-exposed mice were excised seven days after infection and fixed in 10% formalin. After fixation, livers were embedded into paraffin, and 5 μ m tissue slices were prepared. The tissue slices were mounted and stained with hematoxylin and eosin at the Washington Animal Disease Diagnostic Laboratory (Washington State University).

Peripheral white blood cell (WBC) analyses

Mice were sacrificed by overdose with Avertin, and blood was collected by cardiac puncture into EDTA-coated syringes. Complete blood counts (CBCs) were performed by the Veterinary Medicine Clinical Pathology Laboratory (College of Veterinary Medicine, Washington State University). Total WBC counts were determined using a Sereno-Baker, System 9010+CP, hematology analyzer (ABX Diagnostics, Irvine, CA). Blood smears were generated and stained with Wright-Geimsa stain, and differential cell counts were performed manually by counting a total of 100 cells per slide without knowledge of treatment group.

Differential analysis of bronchoalveolar lavage (BAL) cells

Using a cytological centrifuge, BAL cells (1.0×10^5) were spun onto a microscope slide. BAL cells were fixed and stained with hematoxylin and eosin (LeukoStat; Fisher Scientific). The number of macrophages, neutrophils, and lymphocytes were enumerated manually by differential counts of 200 cells per slide. Differential cell counts were performed without knowledge of treatment group.

Immunophenotypic analyses

Cells were stained with different combinations of the following fluorescent conjugated monoclonal antibodies: FITC-labeled anti-Gr-1 or PE-labeled anti-Gr-1; FITC-labeled anti-CD45.2; PE-labeled anti-CD8 or APC-labeled anti-CD8; TC-labeled anti-CD44; APC-labeled anti-CD62L; PE-labeled anti-CD45.1 or TC-labeled anti-CD45.1.

To validate that Gr-1⁺ cells are phenotypically neutrophils, we enriched Gr-1⁺ cells from total lung-derived immune cells using immunomagnetic beads (Miltenyl Biotec, Auburn, CA).

We then generated cytopspins of the enriched pulmonary Gr-1⁺ cells and, using light microscopy, verified that these cells are phenotypically neutrophils. To identify influenza virus-specific CD8⁺ T cells in C57BL/6 mice, MLN cells were incubated with PE-labeled D^bNP₃₆₆₋₃₇₄ tetrameric complexes (Beckman Coulter Inc., San Diego, CA). To assess nonspecific fluorescence, appropriately labeled, isotype-matched antibodies were used as negative controls. Antibodies and isotypic controls were purchased from eBioscience (San Diego, CA) and BD Biosciences (San Jose, CA). Data from 50,000 to 100,000 cells were collected by listmode acquisition using a FACSort flow cytometer (Becton Dickinson, San Jose, CA). WinList software (Verity Software, Topsham, ME) was used for data analyses.

Generation of bone marrow chimeric mice

Four-week old, female C57BL/6 mice (CD45.2⁺ phenotype) and B6-Ly5.2/Cr congenic mice (CD45.1⁺ phenotype) were purchased from NCI-Frederick. The mice received sterile-filtered, acidified water (pH 3.0) supplemented with 1 mg/ml oxytetracycline HCl (tetracycline) and were fed irradiated food (Pico-Vac mouse diet 20, Purina Mills) for the entire duration of the study. Anesthetized (Suprane; deflurane, Baxter Healthcare Corp., Deerfield, IL) CD45.1⁺AhR^{+/+} mice were irradiated with a cumulative dose of 1200 rad given, as two separate doses of 600 rad, 3.5 hours apart (Philips SL-15 linear accelerator, Radiology Dept., College of Veterinary Medicine, Washington State University). Administering the irradiation as two separate doses reduces irradiation-associated toxicity (Spangrude, 1994; Staples *et al.*, 1998b).

One hour after the second dose of irradiation, 1.5 x 10⁶ bone marrow cells from either CD45.2⁺AhR^{+/+} or CD45.2⁺AhR^{-/-} donor mice were injected into the tail vein of the irradiated CD45.1⁺AhR^{+/+} recipient mice. Lethally irradiated CD45.1⁺AhR^{+/+} mice that were not

reconstituted with bone marrow from donor mice served as controls for the irradiation. These mice died within ten days of irradiation. Five weeks after irradiation, CD45.2AhR^{+/+}→CD45.1AhR^{+/+} and CD45.2AhR^{-/-}→CD45.1AhR^{+/+} bone marrow chimeric mice were treated with peanut oil vehicle or TCDD (10 µg/kg body weight) one day prior to i.n. infection with 120 HAU X-31.

Immunohistochemical (IHC) studies

1) Intercellular adhesion molecule-(ICAM)-1: Lungs from vehicle- and TCDD treated mice were fixed in 10% formalin, embedded in paraffin, and 5 µm tissue slices were prepared. Using a modified protocol from Frick *et al.* (2000), slides were deparaffinized with xylene and rehydrated in a series of graded ethanol. Endogenous peroxidase activity was blocked using 5% hydrogen peroxide for 10 min at room temperature. Nonspecific binding was minimized by incubating slides in 3% normal goat serum (Vector Laboratories, Burlingame, CA; diluted in endotoxin-tested PBS) for 30 min at room temperature. Slides were then incubated overnight at 4°C with a purified hamster anti-mouse ICAM-1 primary antibody (1:250; BD Biosciences) in 3% normal goat serum. Negative controls included no primary antibody and isotype-matched control antibody (BD Biosciences). Following the incubation, slides were incubated with a peroxidase-conjugated goat anti-hamster secondary antibody (1:50; Jackson ImmunoResearch, West Grove, PA) in 3% normal goat serum for 50 min at 37°C. ICAM-1 staining was visualized using a 3,3'-diaminobenzidine (DAB) substrate kit (Vector Laboratories). Slides were counterstained with hematoxylin QS[®] (Vector Laboratories) and coverslipped using aqueous mounting medium (Serotec Inc., Raleigh, NC).

2) Vascular cellular adhesion molecule-(VCAM)-1: Frozen lungs from vehicle- and TCDD-treated infected mice were cut into 8 μm sections, placed onto ProbeOn Plus microscope slides (Fisher Scientific), and air-dried for 20 min. Slides were fixed with acetone for 10 min at room temperature and stored at -80°C . VCAM-1 staining was performed using a modified protocol by Essani *et al.* (1997). Briefly, slides were equilibrated to room temperature and incubated for 10 min in endotoxin-tested PBS, containing 0.2% formalin and 0.25% triton-X. After rinsing with wash buffer (endotoxin-tested PBS, containing 0.25% triton-X), non-specific binding was reduced by incubating slides for 10 min in 10% FBS (diluted in wash buffer) at room temperature. Slides were then incubated overnight at 4°C with a purified rat anti-mouse VCAM-1 primary antibody (1:20; BD Biosciences) in blocking solution. Negative controls included no primary antibody and isotype-matched control antibody (BD Biosciences). Following this incubation, slides were rinsed and endogenous peroxidase activity was blocked using 1% hydrogen peroxide for 5 min at room temperature. Slides were then incubated with a peroxidase-conjugated goat anti-rat secondary antibody (1:50; Jackson ImmunoResearch) for 50 min at 37°C in blocking solution. VCAM-1 staining was visualized using a DAB substrate kit (Vector Laboratories). Slides were counterstained with hematoxylin QS[®] (Vector Laboratories) and coverslipped.

3) Platelet endothelial cellular adhesion molecule-(PECAM)-1: PECAM-1 staining was performed according to the VCAM-1 staining protocol, with the following minor changes: After the slides were equilibrated to room temperature, slides were incubated for 10 min in endotoxin-tested PBS. Slides were then blocked for 10 min in 1% normal goat serum (Vector Laboratories; diluted in endotoxin-tested PBS) at room temperature. Following this blocking step, slides were incubated overnight at 4°C with rat anti-mouse PECAM-1 primary antibody

(1:250; BioLegend, San Diego, CA) in blocking solution. The next day, slides were incubated with goat anti-rat secondary antibody (1:100; Jackson ImmunoResearch). Subsequent steps were identical to the VCAM-1 staining protocol.

Statistical analyses

All statistical analyses were conducted using StatView statistical software (SAS, Cary, NC). Using one-way analysis of variance (ANOVA), followed by a Fisher's protected least significant difference post hoc test, mean values from each treatment group were compared at a specific point in time and over time. Where applicable, an unpaired two-sided *t*-test was used to compare mean values from each treatment group at a specific point in time. Values of $p \leq 0.05$ serve as the basis for designation of statistical significance for all studies.

RESULTS

Exposure to TCDD enhances the number of neutrophils in the lungs of different strains of mice infected with influenza A virus X-31 and Mem-72.

Past studies from our laboratory have shown that mice exposed to a single dose of TCDD one day prior to infection with influenza virus have two times more neutrophils in their lungs (Warren *et al.*, 2000; Vorderstrasse *et al.*, 2003a; Teske *et al.*, 2005). All of our previous studies were conducted using C57BL/6 mice and a single strain of influenza virus (X-31). We extended this assessment to determine whether AhR activation by TCDD increases the number of neutrophils in other strains of mice, and following infection with a different subtype of influenza virus. Consistent with our previous studies, seven days after infection, we observed a two-fold increase in the number of neutrophils in the lungs of TCDD-exposed C57BL/10, C3H/HeNCr, and C3H/HeJCr mice (Table 4.1). The data from these studies indicate that the excessive number of neutrophils occurs independent of a particular strain of mice or influenza virus.

Table 4.1 TCDD-mediated excess pulmonary neutrophilia occurs in several strains of mice in response to infection with either influenza virus X-31 or Mem-72.

Mouse strain	Influenza virus strain	Number of Neutrophils (x10 ⁵)	
		Vehicle	TCDD
C57BL/6	A/HKx31	4.32±1.40(6) ^b	10.3±2.28(6) ^{b,c}
C56BL/6	A/Memphis/102/72	4.09±0.72 (8) ^a	8.64±2.38 (8) ^{a,c}
C57BL/10	A/HKx31	5.26±0.91 (7) ^a	7.63±0.97 (8) ^{a,c}
C57BL/10	A/Memphis/102/72	5.81±1.81 (7) ^a	13.4±0.79 (7) ^{a,c}
C3H/HeNCr	A/HKx31	5.90±0.75 (9) ^b	8.35±1.36 (10) ^b
C3H/HeJCr	A/HKx31	6.41±0.96 (10) ^b	9.64±2.02 (10) ^b

^a Enumerated by differential cells counts

^b Identified by flow cytometry (Gr-1⁺ cells)

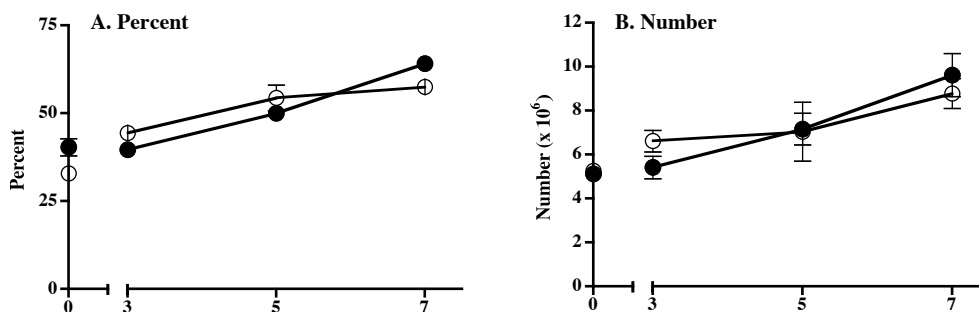
^c Denotes a statistically significant difference from the vehicle treatment group ($p \leq 0.05$)

Female mice (7 to 9 weeks of age) were treated with peanut oil vehicle or 10 µg/kg TCDD one day prior to i.n. infection with X-31 or Mem-72, as described in the Materials in Methods. Seven days after infection, mice were sacrificed and the number of pulmonary neutrophils was determined. The average number of neutrophils found in each treatment group is indicated (\pm SEM). The number in parentheses refers to the total number of animals within that treatment group.

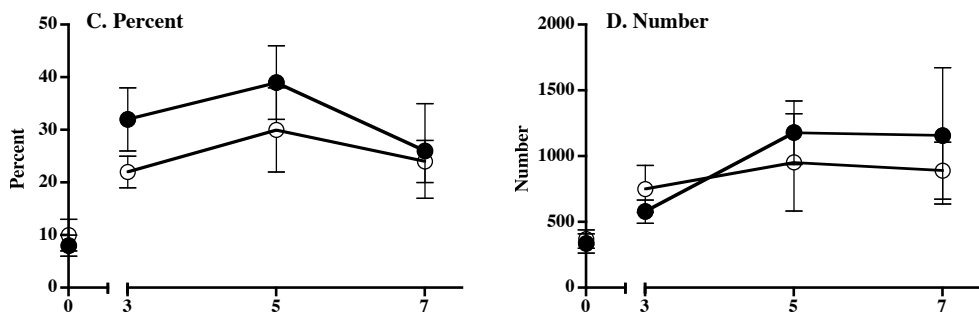
AhR-driven recruitment of excess neutrophils is limited to the lungs of infected mice.

We have previously shown that activation of AhR did not elevate levels of neutrophil chemoattractants in the lung, up-regulate expression of adhesion molecules on pulmonary neutrophils, delay pulmonary neutrophil apoptosis, or enhance vascular damage in the lungs of infected mice (Bohn *et al.*, 2005; Teske *et al.*, 2005). In the absence of an effect of AhR regulation on these mechanisms, an AhR-mediated increase in the number of circulating neutrophils provides a potential alternative mechanistic explanation for the enhanced number of neutrophils in the lungs of TCDD-treated, infected mice. To examine whether activation of the AhR systemically increases neutrophil numbers, we determined the percentage and number of neutrophils in the bone marrow and blood over the course of influenza infection. Compared to mock-infected mice, infection with influenza significantly elevated the percentage and number of neutrophils in the bone marrow (Fig. 4.1 A,B). Unlike the bone marrow, infection with influenza virus did not enhance the percentage and number of neutrophils in the blood (Fig. 4.1 C,D). However, in contrast to the lung (Warren *et al.*, 2000; Vorderstrasse *et al.*, 2003a, Teske *et al.*, 2005), AhR activation did not increase the percentage or number of neutrophils in the bone marrow and blood (Fig. 4.1). We also investigated whether exposure to TCDD enhances the number of band cells in the blood of infected mice. Band cells are immature neutrophils, and dietary exposure to TCDD has been shown to increase the number of band cells in mink (Hochstein *et al.*, 1998). However, unlike the findings by Hochstein *et al.* (1998) our results show that exposure to TCDD did not alter the number of band cells detected in infected mice (data not shown). The absence of an enhanced number of neutrophils in the bone marrow or blood suggests that activation of AhR does not directly increase neutrophil production.

Neutrophils in the bone marrow



Neutrophils in the blood



Day Relative to Infection

Figure 4.1 Exposure to TCDD does not increase the percentage or number of neutrophils in the bone marrow and blood of infected mice. C57BL/6 mice were dosed orally with peanut oil vehicle (open circles) or 10 $\mu\text{g}/\text{kg}$ TCDD (solid circles) one day prior to i.n. infection with 120 HAU influenza A virus (X-31). Mice were sacrificed on the indicated days relative to infection and bone marrow and blood were collected. The average (A) percentage and (B) number of neutrophils (Gr-1⁺ cells) in the bone marrow were determined using flow cytometry. Using microscopy, we validated in separate studies that Gr-1⁺ cells were phenotypically neutrophils. The average (C) percentage and (D) number of neutrophils in the blood were assessed as described in the Materials and Methods. Each treatment group consisted of 5 mice per time point. Mock-infected mice treated with vehicle or TCDD were used as controls for the infection (2-5 mice per treatment group). The average values from mock-infected mice are shown at the zero time point. Error bars represent the SEM. Results are representative of two independent time course experiments.

Additionally, we set out to assess whether treatment with TCDD increases the number of neutrophils at other anatomical sites during respiratory viral infection. To do so, we examined the number of neutrophils in the spleen and liver, because influenza virus does not infect or replicate in these organs. As shown in Fig. 4.2 C and D, exposure to TCDD did not elevate the number of neutrophils in the spleen of infected mice. With regard to the liver, we detected few hematopoietic foci in all four treatment groups (Fig. 4.2 C-F). Likewise, few inflammatory foci containing neutrophils and necrotic hepatocytes were present in all treatment groups (Fig. 4.2 C-F). Based on these observations it does not appear that AhR activation elevates the number of neutrophils in the liver. In summary, the data in Figures 4.1 and 4.2 show that in the context of influenza virus infection AhR activation did not enhance the number of neutrophils in the bone marrow, blood, spleen, or liver, suggesting that the excess number of neutrophils in TCDD-treated, infected mice is restricted to the site of antigen challenge (i.e., the lung).

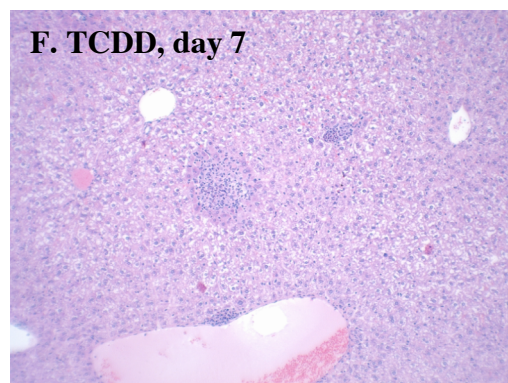
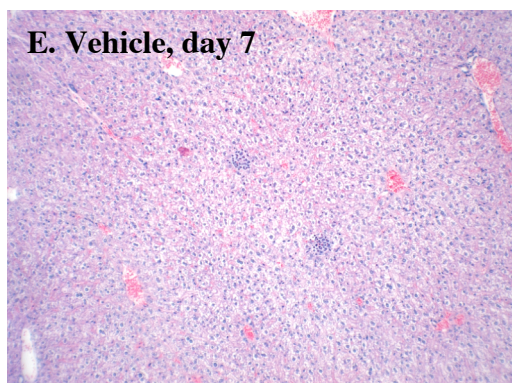
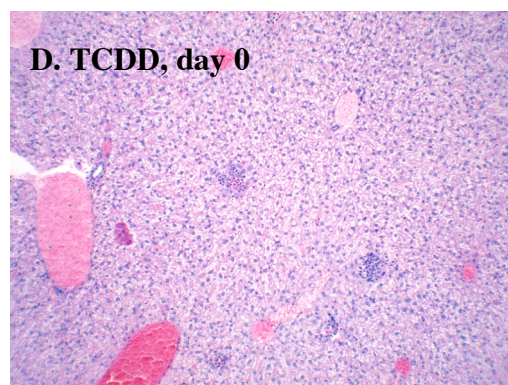
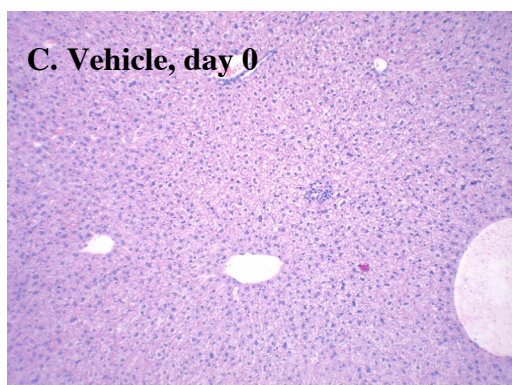
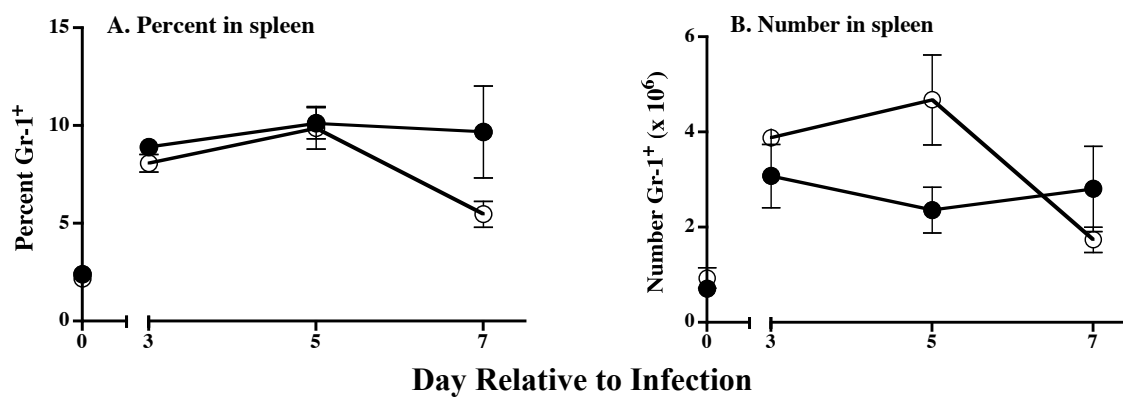


Figure 4.2 Treatment with TCDD does not enhance the number of neutrophils in the spleen or liver. Mice were treated as described in Figure 4.1. Mice were sacrificed on the indicated days relative to infection and spleens and livers were collected. The average (A) percentage and (B) number of neutrophils (Gr-1⁺ cells) in the spleen was determined using flow cytometry (5 mice/treatment group/day). Mock-infected mice treated with vehicle or TCDD served as controls for the infection (5 mice per treatment group). Error bars represent the SEM. For histological analysis of the liver, (E) vehicle- and (F) TCDD-exposed mice (5 per treatment group) were sacrificed seven days after infection. Mock-infected mice treated with (C) vehicle or (D) TCDD served as controls for the infection (5 mice per treatment group). Photomicrographs of representative fields were taken with an Olympus MicroFireTM digital camera. Hematoxylin and eosin stain; X100 magnification. Results are representative of two separate experiments.

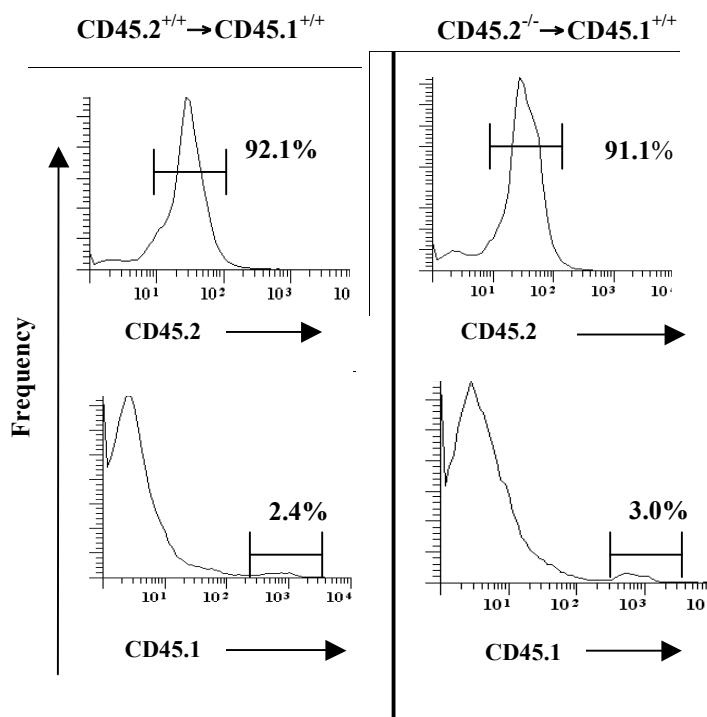
Establishment and validation of bone marrow chimeric mice.

Given the fact that the lung expresses high levels of the AhR compared to immune cells (Lang *et al.*, 1998; Yamamoto *et al.*, 2004), alterations in AhR-regulated events within the lung are a logical mechanism for the recruitment of excess neutrophils to the lungs of TCDD-treated, infected mice. To determine whether AhR-driven events external to the immune system underlie the elevated number of neutrophils in the lungs of TCDD-treated mice, we generated bone marrow chimeric mice in which immune cells lack the AhR, but all other tissues express a functional AhR. To accomplish this, we reconstituted lethally irradiated AhR^{+/+} recipient mice (CD45.1⁺ phenotype) with hematopoietic cells (CD45.2⁺ phenotype) from either wild-type (AhR^{+/+}) or AhR-deficient (AhR^{-/-}) congenic donor mice, generating CD45.2AhR^{+/+}→CD45.1AhR^{+/+} and CD45.2AhR^{-/-}→CD45.1AhR^{+/+} chimeric mice. Five weeks after irradiation, we validated the success of reconstitution of the hematopoietic system in these chimeric mice. As shown in Fig. 4.3A, greater than 90 percent of hematopoietic cells were of donor origin in both CD45.2AhR^{+/+}→CD45.1AhR^{+/+} and CD45.2AhR^{-/-}→CD45.1AhR^{+/+}

chimeric mice, indicating that chimerism was successfully established regardless of AhR status of the donor immune system. Lethally irradiated CD45.1⁺ recipient mice that were not reconstituted with bone marrow from donor mice served as controls for the irradiation. These mice died within ten days of irradiation (data not shown).

In addition to establishing chimerism, we also determined that the bone marrow chimeric mice were immunocompetent. To do so, we monitored survival of CD45.2AhR^{+/+} → CD45.1AhR^{+/+} and CD45.2AhR^{-/-} → CD45.1AhR^{+/+} chimeric mice for 14 days following infection with influenza virus X-31. As illustrated in Figure 4.3B, regardless of the AhR status of immune cells, all chimeric mice survived this infection. Taken together, these data demonstrate that we successfully generated immunocompetent bone marrow chimeric mice.

A.



B.

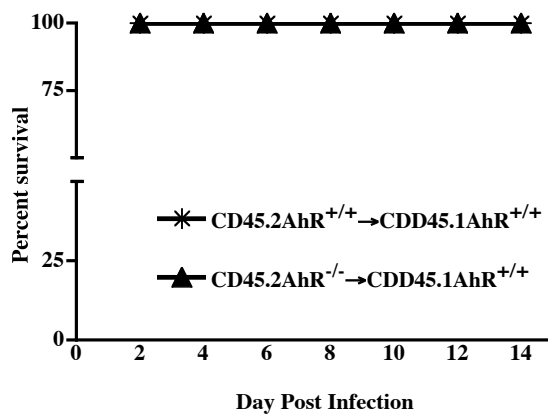


Figure 4.3 $AhR^{-/-}$ bone marrow cells successfully reconstitute the immune system of lethally irradiated $AhR^{+/+}$ mice, generating immunocompetent $CD45.2AhR^{-/-} \rightarrow CD45.1AhR^{+/+}$ bone marrow chimeric mice. Wild-type mice ($AhR^{+/+}$, $CD45.1^{+}$ phenotype) were lethally irradiated and reconstituted with 1.5×10^6 bone marrow cells ($CD45.2^{+}$ phenotype) from either wild-type ($AhR^{+/+}$) or AhR -deficient ($AhR^{-/-}$) congenic donor mice. Five weeks later, $CD45.2AhR^{+/+} \rightarrow CD45.1AhR^{+/+}$ and $CD45.2AhR^{-/-} \rightarrow CD45.1AhR^{+/+}$ chimeric mice were sacrificed and bone marrow cells were collected. **(A)** The representative histograms depict the percentage of $CD45.2^{+}$ (donor-derived) and $CD45.1^{+}$ (host-derived) bone marrow cells in chimeric mice (3 mice per group). Results are representative of three independent experiments. **(B)** The survival of $CD45.2AhR^{+/+} \rightarrow CD45.1AhR^{+/+}$ (asterisks) and $CD45.2AhR^{-/-} \rightarrow CD45.1AhR^{+/+}$ (solid triangles) chimeric mice (3-9 mice per group) was monitored for 14 days following infection with X-31.

Increased number of neutrophils in TCDD-treated, infected mice is dependent on AhR-driven events at the site of antigen challenge, and not the immune system.

Using the bone marrow chimeric mice, we examined whether activation of AhR increases the number of neutrophils in the lungs of infected mice when immune cells lack the AhR. As expected, compared to vehicle-treated, infected chimeric mice, there was a two-fold increase in the number of neutrophils in the lungs of TCDD-exposed, infected mice in which immune cells express a functional AhR (Fig. 4.4A). Interestingly, we also detected two times more $CD45.2AhR^{-/-}$ (donor-derived) neutrophils in the lungs of TCDD-exposed, infected $CD45.2AhR^{-/-} \rightarrow CD45.1AhR^{+/+}$ chimeric mice (Fig. 4.4B). That is, even when the immune system lacks the AhR, exposure to TCDD still results in excess recruitment of neutrophils to the infected lung. This novel finding strongly suggests that the enhanced number of neutrophils in lungs of TCDD-treated, infected mice is mediated by alterations in AhR-regulated events that are external to the immune system.

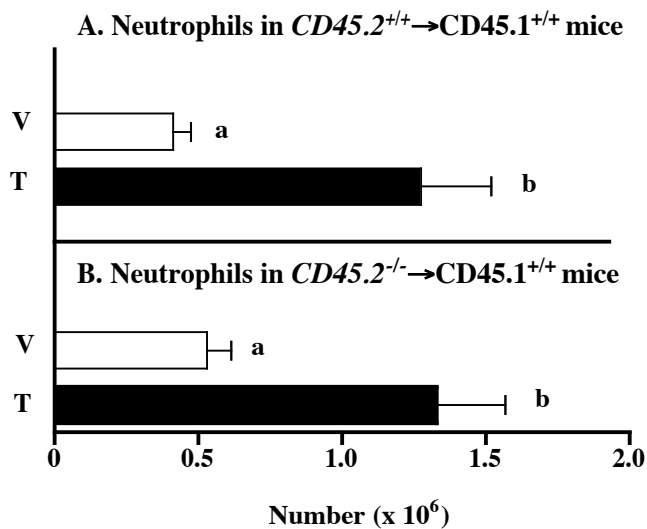
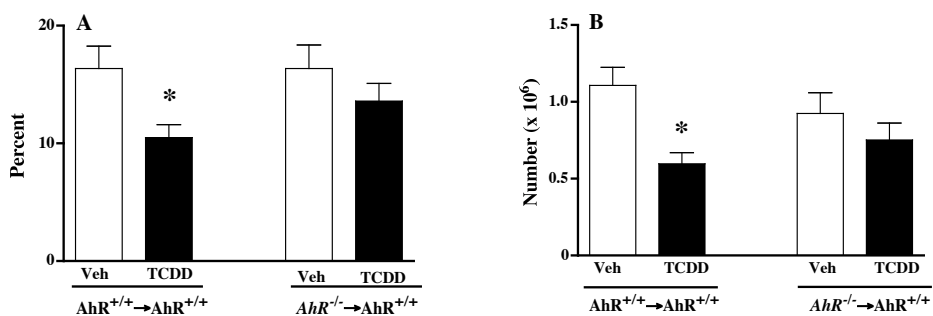


Figure 4.4 Excess number of neutrophils in the lungs of TCDD-exposed, infected mice is mediated by AhR-driven events external to the immune system. Five weeks after irradiation, $CD45.2AhR^{+/+} \rightarrow CD45.1AhR^{+/+}$ and $CD45.2AhR^{-/-} \rightarrow CD45.1AhR^{+/+}$ chimeric mice (7-8 mice per treatment group) were administered peanut oil vehicle (open bars) or 10 $\mu\text{g}/\text{kg}$ TCDD (solid bars) one day prior to infection with 120 HAU X-31. Seven days after infection, mice were sacrificed and total lung-derived immune cells were collected. Using flow cytometry, we assessed the average number of pulmonary $CD45.2^+$ (donor-derived) neutrophils (Gr-1^+ cells) in (A) $CD45.2AhR^{+/+} \rightarrow CD45.1AhR^{+/+}$ and (B) $CD45.2AhR^{-/-} \rightarrow CD45.1AhR^{+/+}$ chimeric mice. Error bars represent the SEM. Statistically significant differences are denoted by different letters ($p \leq 0.05$). Results are representative of three separate experiments.

It has been previously shown that AhR activation suppresses T cell-dependent immune responses, including proliferation and differentiation of CD8⁺ T cells in influenza virus-infected mice (Kerkvliet *et al.*, 1996; Warren *et al.*, 2000; Lawrence *et al.*, 2000; Mitchell and Lawrence, 2003). Moreover, the suppressive effect of TCDD on T cell responses is AhR-dependent (Vorderstrasse *et al.*, 2001; and our unpublished observations), and appears to require the AhR within T cells (Kerkvliet *et al.*, 2002). Therefore, our observation that recruitment of excess neutrophils to the lung does not require the AhR in the hematopoietic system suggests that AhR-mediated deregulation of neutrophil responses may differ from dysregulated T cell responses, which we predict would not be sensitive to TCDD-induced suppression when the hematopoietic system lacks the AhR. To formally test this idea, we determined the number of CD8⁺ T cells in the lung and the number of virus-specific (D^bNP₃₆₆₋₃₇₄⁺) CD8⁺ T cells in the mediastinal lymph node (MLN) of the same group of infected chimeric mice. Consistent with our past findings (Lawrence *et al.*, 2000; Warren *et al.*, 2000), when the immune system is AhR^{+/+}, we observed a two-fold suppression in the number of CD8⁺ T cells in the lungs and a ten-fold decrease in virus-specific CD8⁺ T cells in the MLN of TCDD-treated (Fig. 4.5 B,D left panel). However, when the immune system lacked the AhR (Fig. 4.5 B,D right panel), exposure to TCDD failed to suppress the expansion of CD8⁺ T cell numbers in the MLN and the migration of CD8⁺ T cells to the lungs of infected mice. Therefore, in contrast to the finding that AhR-mediated events extrinsic to the immune system underlie the recruitment of excess neutrophils to the lung, AhR-dependent events within the immune system are required for the suppression of the CD8 T cell response.

CD45.2⁺ CD8⁺ cells in the LUNG



CD45.2⁺ NP⁺CD8⁺ cells in the MLN

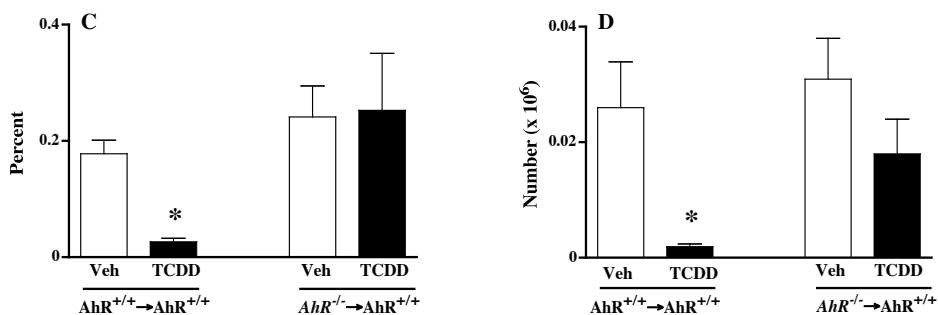


Figure 4.5 The suppressed response of CD8⁺ T cells in TCDD-treated, infected mice requires AhR-mediated events within the immune system. Bone marrow chimeric mice were treated as described in Figure 4.4. Seven days after infection, mice were sacrificed and total lung-derived immune cells and mediastinal lymph nodes (MLN) cells were collected. Using flow cytometry, we assessed the average percentage and number of CD45.2⁺ CD8⁺ (donor-derived) T cells in the lungs (**A**, **B**), and the average percentage and number of CD45.2⁺ D^bNP₃₆₆₋₃₇₄⁺ (donor-derived, virus-specific) CD8⁺ T cells in the MLN (**C**, **D**) of infected chimeric mice. Each treatment group consisted of 5-8 mice. Error bars represent the SEM. Statistically significant differences are indicated by different letters ($p \leq 0.05$). Results are representative of two independent experiments.

Infection with influenza virus strongly induces expression of ICAM-1 in the lungs of vehicle- and TCDD-treated mice.

During respiratory viral infection, directional migration of neutrophils from the bloodstream to the site of infection is governed by elevated levels of neutrophil chemoattractants in the lung, and by the coordinated expression of adhesion molecules on the neutrophil surface, vascular endothelial, and epithelial cells (Wagner and Roth, 2000). In the absence of an effect of AhR regulation on soluble neutrophil chemoattractants in the lung or expression of adhesion molecules on pulmonary neutrophils (Teske *et al.*, 2005), and in conjunction with our findings from the bone marrow chimeric experiment, altered expression of adhesion molecules in the lung is a logical mechanism for AhR-mediated increases in neutrophil migration to the lung. In particular, intercellular adhesion molecule-1 (ICAM-1; CD54), vascular cellular adhesion molecule-1 (VCAM-1; CD106), and platelet endothelial cellular adhesion molecule-1 (PECAM-1; CD31) have been shown to be pertinent for neutrophil migration (Vaporciyan *et al.*, 1993; Piedboeuf *et al.*, 1998; Matsuse *et al.*, 1999; Frick *et al.*, 2000; Bowden *et al.*, 2002; Woo *et al.*, 2005).

These findings prompted to investigate these three adhesion molecules. First, using the software program Genomatix (Munich, Germany), we searched the upstream regulatory regions of the murine ICAM-1, VCAM-1, and PECAM-1 genes for putative dioxin response elements (DREs), which have the 5-mer core 5'-GCGTG-3' sequence. Our results show three putative DREs in upstream regulatory of ICAM-1 (Table 4.2). Likewise, we detected four possible DREs in the upstream regulatory region of PECAM-1 (Table 4.2). In contrast to ICAM-1 and PECAM-1, we did not identify any DREs in the VCAM-1 upstream regulatory region.

DIOXIN RESPONSE ELEMENTS (DREs)

ICAM-1 (CD54)		PECAM-1 (CD31)	
Position	Strand	Position	Strand
289 - 293	-	920 - 924	+
739 - 743	+	1263 - 1267	-
1099 - 1103	-	1271 - 1275	-
		1281 - 1285	-

+ coding strand
 - non-coding strand

Table 4.2 Position of dioxin response elements (DREs) in the upstream regulatory region of ICAM-1 and PECAM-1. Using Genomatix's MatInspector software program, we searched the upstream regulatory region of the murine ICAM-1 and PECAM-1 genes for putative DREs. We defined a DRE as the 5-mer core 5'-GCGTG-3' sequence and the upstream regulatory region as ± 1000 base pairs of the transcription start site. The position of the DRE is given from starting to ending base pair.

Second, using immunohistochemistry (IHC), we investigated expression of ICAM-1, VCAM-1, and PECAM-1 in lung tissue from vehicle- and TCDD-exposed mice five and seven days after infection. Compared to mock-infected mice, VCAM-1 expression was only slightly increased in the lungs of vehicle- and TCDD-treated, infected mice (Fig. 4.6). Furthermore, exposure to TCDD did not alter VCAM-1 expression. In contrast to the low expression of VCAM-1, PECAM-1 was highly expressed in the lungs of vehicle- and TCDD-treated mice regardless of infection (Fig. 4.7). Unlike the constitutive expression of PECAM-1, ICAM-1 expression was greatly increased in the lungs of vehicle- and TCDD-treated mice over the course of infection (Fig. 4.8). However, exposure to TCDD did not grossly alter this infection-associated increase in ICAM-1 expression.

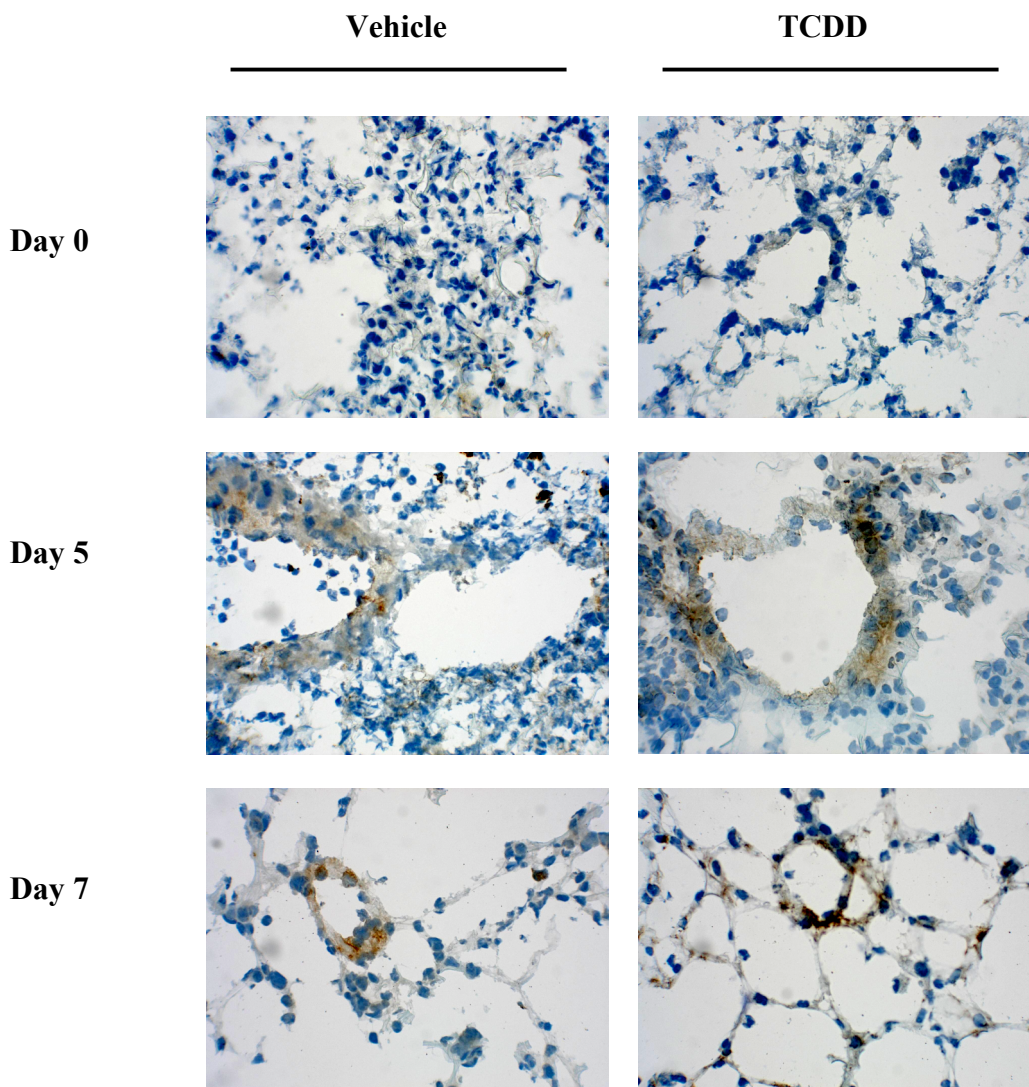


Figure 4.6 Infection with influenza virus slightly enhances expression of VCAM-1 in the lungs of vehicle- and TCDD-treated mice. Mice were treated as described in Figure 4.1. Tissue slices of acetone-fixed, frozen lungs from vehicle- and TCDD-treated, infected mice (5 mice per treatment group/day) were probed with a rat anti-mouse VCAM-1 primary antibody, followed by incubation with a peroxidase-conjugated goat anti-rat secondary antibody. Acetone-fixed, frozen lung tissue slices from vehicle- and TCDD-exposed, mock-infected mice were used as controls (3 mice per treatment group). VCAM-1 staining was visualized using a 3,3'-diaminobenzidine (DAB) substrate kit (brown staining). Slides were counterstained with hematoxylin (blue staining). Representative photomicrographs were taken with an Olympus MicroFire™ digital camera (X50 magnification).

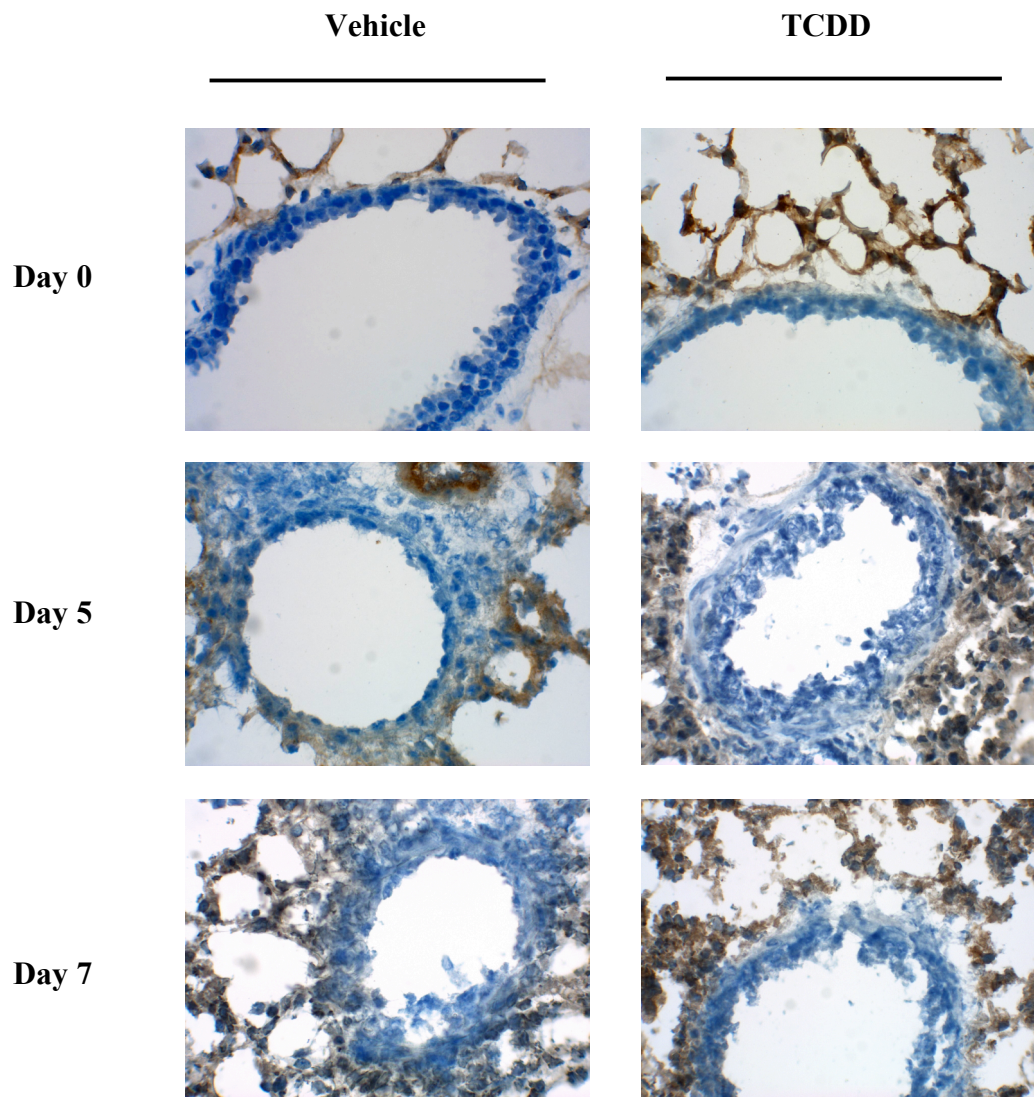


Figure 4.7 PECAM-1 is constitutively expressed in the lungs of vehicle- and TCDD-exposed mice. Mice were treated as described in Figure 4.1. Tissue slices of acetone-fixed, frozen lungs from vehicle- and TCDD-treated, infected mice (5 mice per treatment group/day) were probed with a rat anti-mouse PECAM-1 primary antibody, followed by incubation with a peroxidase-conjugated goat anti-rat secondary antibody. Acetone-fixed, frozen lung tissue slices from vehicle- and TCDD-exposed, mock-infected mice were used as controls (3 mice per treatment group). PECAM-1 staining was visualized using a DAB substrate kit (brown staining). Slides were counterstained with hematoxylin (blue staining). Representative photomicrographs were taken with an Olympus MicroFire™ digital camera (X50 magnification).

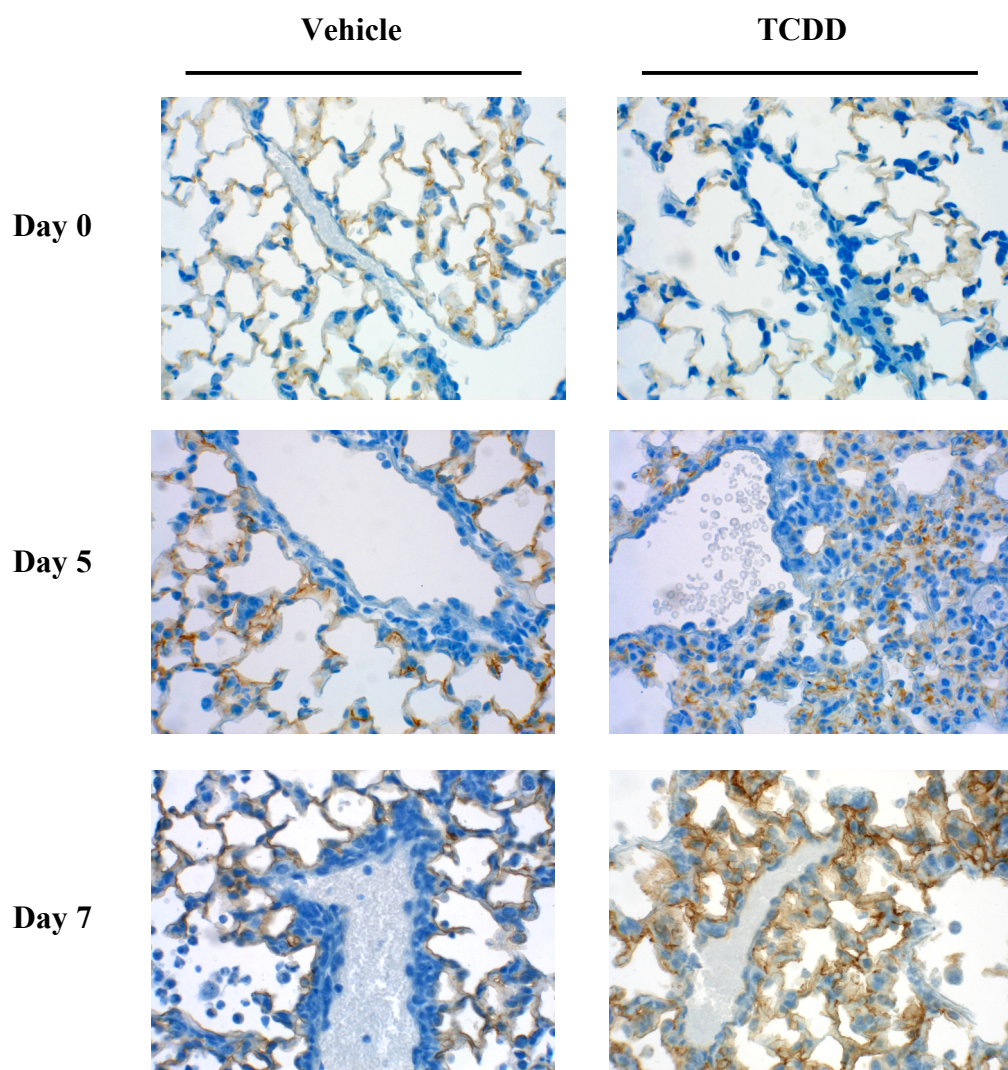


Figure 4.8 Infection with influenza virus significantly induces expression of ICAM-1 in the lungs of vehicle- and TCDD-treated mice. Mice were treated as described in Figure 4.1. Tissue slices of formalin-fixed lungs from vehicle- and TCDD-treated, infected mice (5 mice per treatment group/day) were probed with a hamster anti-mouse ICAM-1 primary antibody, followed by incubation with a peroxidase-conjugated goat anti-hamster secondary antibody. Formalin-fixed lung tissue slices from vehicle- and TCDD-exposed, mock-infected mice were used as controls (3 mice per treatment group). ICAM-1 staining was visualized using a DAB substrate kit (brown staining). Slides were counterstained with hematoxylin (blue staining). Representative photomicrographs were taken with an Olympus MicroFire™ digital camera (X50 magnification).

DISCUSSION

We present here for the first time that the mechanism of AhR-mediated alterations in immune function differs between innate and adaptive immunity. AhR-driven events within the immune system underlie suppression of the CD8⁺ T cell response to infection with influenza virus. In contrast, AhR-driven events external to the immune system mediate the recruitment of excess neutrophils to the lungs of TCDD-treated, infected mice. Furthermore, in the context of respiratory viral infection, we did not detect an increased number of neutrophils in the bone marrow or blood in mice exposed to TCDD. Likewise, activation of AhR did not enhance the number of neutrophils in the spleen or liver, two anatomical sites in which influenza virus does not infect or replicate. Taken together, these findings suggest that AhR-driven events at the site of antigen challenge (i.e., the lung) mediate the excessive number of neutrophils in the lungs of mice infected with influenza virus.

Possible AhR-driven events within the lung that could affect the directional migration of neutrophils include alterations in tumor necrosis factor (TNF), interleukin-(IL)-1, IL-6, keratinocyte chemoattractant (KC), macrophage inflammatory protein-(MIP)-1 α , MIP-2, lipopolysaccharide-induced CXC chemokine (LIX), and complement split product C5a in the lungs of infected mice (Neff-LaFord *et al.*, 2003; Teske *et al.*, 2005). Although levels of most of these chemoattractants increased with influenza virus infection, activation of the AhR did not further enhance this infection-associated increase. This suggests that AhR-driven alterations in known stable, soluble neutrophil chemoattractants are not a logical mechanistic explanation for excess neutrophils in the lungs of TCDD-treated, infected mice.

In fact, in the absence of an effect on known stable, soluble neutrophil chemoattractants, we had previously performed an *ex vivo* neutrophil migration assay to determine if an unknown

stable, soluble chemoattractant is induced by activation of the AhR. Results from this migration assay showed that an equivalent number of peritoneal neutrophils migrated toward BAL fluid from vehicle-treated, infected mice and TCDD-treated, infected mice (please see Chapter 2). Collectively, the past findings from our laboratory in conjunction with the results from the migration assay indicate that the recruitment of excess neutrophils to the lungs of TCDD-exposed mice is mediated by either non-soluble or labile, soluble factors. With regard to possible labile, soluble factors, arachidonic acid metabolites, such as leukotriene-(LTB)₄ and prostaglandin-(PG)E₂, are highly chemoattractant for neutrophils in models of lung inflammation (Cuzzocrea *et al.*, 2003; Alba-Loureiro *et al.*, 2004). Furthermore, in the context of influenza virus infection, LTB₄ has been shown to be elevated in the lungs of influenza virus-infected mice (Hennet *et al.*, 1992). Likewise, LTC₄ was increased in lungs of humans infected with influenza virus (Gentile *et al.*, 2003). However, activation of the AhR did not alter levels of LTB₄, LTC₄, and PGE₂ in several different experimental systems (Lawrence and Kerkvliet, 1998; Lee *et al.*, 1998), suggesting that production of these inflammatory mediators is not affected by AhR activation.

Instead, deregulation of non-soluble pulmonary targets provides a more plausible mechanistic explanation for the enhanced number of neutrophils in the lungs of TCDD-exposed mice. Among the numerous cell-associated targets important for neutrophil recruitment, toll-like receptors (TLRs), cellular adhesion molecules (CAMs), and Fas could be potentially regulated by AhR activation. For example, in mice, activation of TLR-3 has been shown increase production of pro-inflammatory mediators (Alexopoulou *et al.*, 2001). Furthermore, TLR-3 (Guillot *et al.*, 2005) and TLR-7 (Lund *et al.*, 2004) are involved in the immune response to infection with influenza virus. Based on the findings from these reports and the the important

role of TLRs in viral immunity study (Bowie and Haga, 2005), AhR-mediated activation of TLRs, such as TLR-3, is a possible mechanism that could lead to the enhanced recruitment of neutrophils to the lungs of TCDD-treated, infected mice.

Given the role of ICAM-1, VCAM-1, and PECAM-1 in neutrophil recruitment (Matsuse *et al.*, 1999; Frick *et al.*, 2000; Bowden *et al.*, 2002; Woo *et al.*, 2005; Vaporciyan *et al.*, 1993; Piedboeuf *et al.*, 1998), makes these adhesion molecules potential pulmonary targets of AhR-mediated toxicity. Furthermore, we have shown that both ICAM-1 and PECAM-1 have putative DREs in their upstream regulatory, making these mediators potential direct novel targets of AhR-mediated toxicity. However, our data from the IHC studies show that only ICAM-1 was strongly induced in the lungs of influenza virus-infected mice. Although treatment with TCDD did not alter gross expression of ICAM-1 in the lung, localized, site-specific increases in the expression of ICAM-1 could mediate the recruitment of excess neutrophils to the lungs of TCDD-exposed, infected mice. For example, Frick *et al.* (2000) show a site-specific up-regulation of ICAM-1 expression in epithelial cells lining the large airways in mice challenged with *H. influenzae*.

In addition to ICAM-1, AhR-driven altered Fas expression in the lung is a plausible alternative mechanism that underlies the elevated number of neutrophils in TCDD-exposed, infected mice. A recent study has shown that recruitment of excess pulmonary neutrophils can be mediated by Fas expression in the lung (Matute-Bello *et al.*, 2005). Similar to the lack of KC and MIP-2 in the recruitment of excess neutrophils in our model system (Teske *et al.*, 2005), recruitment of enhanced neutrophils occurred independent of these prototypic murine neutrophil chemoattractants in that particular report. Given the parallels between our previous observations and the study by Matute-Bello *et al.* (2005), alterations in pulmonary Fas expression provide yet

another plausible mechanism for an AhR-mediated elevation in the recruitment of neutrophils to the lungs of TCDD-treated, infected mice.

In summary, the findings from this study illustrate the complexity of the mechanism by which AhR activation influences the pulmonary immune response to viral infection. In contrast to the suppression of CD8 T cell responses, which appear to require the AhR within the immune system, AhR-driven enhancement of neutrophil recruitment does not. Furthermore, the influx of neutrophils to the infected lung is not simply due to elevated neutrophil levels systemically, or to enhanced pulmonary vascular permeability (Bohn et al. 2005), nor is it governed by an elevation in soluble neutrophil chemoattractants in the lungs of infected mice (Teske *et al.*, 2005). Instead, we show for the first time that excess neutrophilia is caused by AhR-driven events extrinsic to the immune system, suggesting that AhR-mediated events within the lung influence neutrophil recruitment, and thereby alter the outcome of respiratory viral infection. In addition to understanding how AhR activation affects the immune response to influenza virus, this discovery is significant in a broader sense because it suggests that the lung is a very important and overlooked target of AhR ligands. There is mounting evidence that environmental exposures adversely affect human health. For example, poorer health in disadvantaged communities, increased asthma in urban areas, and distinct clinical outcomes from individuals with the same type of infection point links between environmental exposures and the etiology or differential severity of numerous disease states (Kunzli *et al.*, 2000; Schwela, 2000; Pope *et al.*, 2002; Kaiser *et al.*, 2004). Therefore, the ability of a potent AhR agonist to affect the directional migration of neutrophils to the lung suggests that exposure to ubiquitous AhR ligands may contribute to the etiology of these diseases. It also suggests that as we improve our understanding of the molecular

mechanism, modulation of AhR function and AhR-dependent processes serves as a potential therapeutic target to regulate neutrophil recruitment and susceptibility to disease.

CHAPTER FIVE

Summary and Future Directions

Summary

In contrast to the well-documented immunosuppressive effects of TCDD on adaptive immunity, a limited number of studies have documented the effects of exposure to TCDD on cells of the innate system (Kerkvliet and Oughton, 1993; Moos, 1994; Burleson *et al.*, 1996; Warren *et al.*, 2000; Luebke *et al.*, 2002; Choi *et al.*, 2003; Vorderstrasse *et al.*, 2003a). All of these studies jointly report an increased number of neutrophils at the site of antigen challenge. However, none of the studies investigated the mechanism that underlies this excessive neutrophilia. The goal of this dissertation was to determine the mechanism that drives the recruitment of excess neutrophils to the lungs of TCDD-treated, influenza virus-infected mice. Characterizing the exacerbated neutrophilia not only furthers our understanding of TCDD's immunomodulatory potential, but also helps us understand how AhR activation differentially affects adaptive and innate immunity.

Using AhR-deficient mice, we have shown for the first time that the excess number of neutrophils in the lungs of TCDD-treated, infected mice is directly AhR-dependent. This finding shows that AhR activation not only mediates alterations in adaptive immunity (Vorderstrasse *et al.*, 2001; Kerkvliet *et al.*, 2002), but also leads to alterations in innate immunity. We next determined that the AhR-mediated enhanced pulmonary neutrophilia significantly diminished survival in TCDD-treated mice in response to a sublethal infection with influenza virus. Our findings also show that increased number of neutrophils, and not hyperactive neutrophils, decreased survival in TCDD-treated, infected mice.

With regard to the mechanism that underlies the elevated pulmonary neutrophilia, we have shown that nonspecific leakage of neutrophils from the bloodstream to the lung due to increased vascular permeability did not account for the excess neutrophils in TCDD-treated, infected mice. Likewise, a systemic elevation in the number of neutrophils does not provide a mechanistic explanation for the enhanced number of neutrophils in the lungs of TCDD-exposed mice. Treatment with TCDD also did not elevate levels of soluble neutrophil chemoattractants in the lung, up-regulate expression of adhesion molecules on pulmonary neutrophils, or delay neutrophil apoptosis in the lung.

In the absence of a direct effect of exposure to TCDD on pulmonary neutrophils, we used a technically elaborate approach of CD45.2AhR^{-/-}→CD45.1AhR^{+/+} bone marrow chimeric mice to discern whether AhR-driven events within or external to the immune system underlie the increased number of neutrophils in the lungs of TCDD-treated, infected mice. Our data show that TCDD-treated, infected mice in which the immune cells are devoid of the AhR, but all other tissues express the AhR, had two times more neutrophils in their lungs compared to vehicle-treated, infected CD45.2AhR^{-/-}→CD45.1AhR^{+/+} chimeric mice. This finding indicates that AhR-mediated events external to the immune system underlie the enhanced number of neutrophils in mice exposed to TCDD. Hence, deregulated activation of the AhR at the site of antigen challenge (i.e., the lungs) likely underlies the increased number of neutrophils in mice treated with TCDD. This finding makes adhesion molecules on lung epithelium and endothelium potential targets of AhR-mediated toxicity within the lung. Although our data do not show gross alterations in the expression of the adhesion molecules ICAM-1, VCAM-1, and PECAM-1 in the lungs of TCDD-treated, infected mice, site-specific, localized changes in the expression of these

adhesion molecules could, nevertheless, underlie the deregulated recruitment of neutrophils to the lungs of mice exposed to TCDD.

In summary, our findings indicate that the excess pulmonary neutrophilia in TCDD-treated, infected mice is not only limited to the site of antigen challenge (i.e., the lung), but is likely mediated by events at the site of antigen challenge. This is a novel finding, which suggests that AhR-driven events external to the immune system underlie the excessive recruitment of neutrophils to the lungs of TCDD-exposed, infected mice.

Future Directions

The findings from Chapter 4 of this dissertation indicate that AhR-mediated events within the lung, and not the immune system, underlie the excessive pulmonary neutrophilia in TCDD-treated, infected mice. Furthermore, the results of Chapter 2 demonstrate that potential pulmonary targets of TCDD-mediated toxicity are likely either cell-associated or labile, soluble factors. Perplexingly, our data in Chapter 4 show that exposure to TCDD did not alter the gross expression of adhesion molecules on lung endothelium and epithelium. Given the lack of an effect of exposure to TCDD on the overall expression of these adhesion molecules in the lung, the following paragraphs provide a summary of alternative pulmonary targets, which are potentially subject to TCDD-induced toxicity, and could thereby mediate the elevated neutrophilia in the lungs of TCDD-treated mice.

In addition to the adhesion molecules on lung epithelium/endothelium examined in Chapter 4, alternative pulmonary targets that could potentially underlie the excess number of neutrophils in the lungs of TCDD-exposed mice are Fas and toll-like receptors (TLRs).

Historically, Fas has been primarily implicated in apoptosis of immune cells, especially T cells. However, elevated expression of pulmonary Fas has recently been attributed to increased lung tissue damage and enhanced influx of neutrophils to the lungs of rodents during acute lung injury (Matute-Bello *et al.*, 2005; Neff *et al.*, 2005). Likewise, in humans elevated Fas expression has been shown to mediate the enhanced influx of neutrophils to the lungs of patients afflicted with acute respiratory distress syndrome (ARDS; Albertine *et al.*, 2002). Therefore, altered Fas expression constitutes a potential pulmonary target of AhR-mediated toxicity, mediating the recruitment of excess neutrophils to the lungs of TCDD-exposed, infected mice.

To investigate the role of Fas in our model system, we could measure levels of Fas in lung homogenates of vehicle- and TCDD-treated mice over the course of infection using commercially available enzyme-linked immunosorbent assays (ELISAs; R&D Systems). Using immunohistochemistry (IHC), we could assess localized expression of Fas in lungs of vehicle- and TCDD-treated, infected mice. If Fas expression were enhanced in TCDD-treated, infected mice compared to vehicle-treated, infected mice, then we could perform follow-up studies using *lpr* mice, which express non-functional Fas and are available on a C57BL/6 background from The Jackson Laboratory. Specifically, *lpr* and C57BL/6 wild-type mice would be treated with peanut oil vehicle or TCDD one day prior to infection with influenza virus (A/HKx31). Seven days after infection, the peak day of exacerbated pulmonary neutrophilia in TCDD-treated, infected wild-type mice, *lpr* and C57BL/6 wild-type mice would be sacrificed and number of pulmonary neutrophils would be assessed using flow cytometry. If the excess pulmonary neutrophilia were ablated in TCDD-treated, infected *lpr* mice compared to TCDD-treated, infected wild-type mice, then it would suggest that AhR-mediated altered Fas expression underlies the recruitment of excess neutrophils to the lung. On the other hand, equivalent

neutrophilia in TCDD-treated *lpr* and wild-type mice, would indicate that alterations in Fas are not a mechanistic explanation for the enhanced number of neutrophils.

In addition to Fas expression in the lung, expression of TLRs provide alternative non-soluble pulmonary targets of AhR-mediated toxicity. TLRs play an important role in anti-viral immunity, counteracting infections with many different viruses, such as measles virus, HIV, cytomegalovirus, respiratory syncytial virus, and influenza virus (Bowie and Haga, 2005). In mice, TLR-3 (Guillot *et al.*, 2005) and TLR-7 (Lund *et al.*, 2004) are involved in the immune response to infection with influenza virus.

Using a gene array, we assessed mRNA levels of TLRs in lungs of vehicle- and TCDD-treated mice three days following infection with influenza virus (A/HKx31). Analysis of our gene expression data showed very low expression levels of TLR-5, TLR-6, TLR-7, TLR-8, and TLR-9 in both treatment groups (data not shown). Our gene expression data indicate that TLR-4 gene expression was not altered by exposure to TCDD (data not shown). Furthermore, previous findings from our laboratory show that TCDD-treated, infected C3H/HeJ mice, which encode a non-functional TLR-4, had equivalent pulmonary neutrophilia as mice expressing a functional TLR-4 (please see Appendix B). These data support our TLR-4 gene expression data, suggesting that treatment with TCDD does not affect TLR-4. In contrast to TLR-4, analyses of our gene expression data show decreased levels of TLR-1, TLR-2, and TLR-3 in TCDD-treated, infected mice compared to vehicle-treated, infected mice (data not shown).

In the past antibodies to assess protein levels of murine TLRs have not been commercially available, making it difficult to characterize the role of TLRs in our model system. Currently, a mouse TLR antibody sampler kit is available from Imgenex Corp. (San Diego, CA). Using Western blotting, this kit would allow us to determine a preliminary expression profile of

these particular TLRs in lung homogenates of vehicle- and TCDD-treated, infected mice. Alternatively, given the pertinent role of TLR-3 and TLR-7 in infection with influenza virus, we could focus on these two TLRs. To do so, we would assess expression of TLR-3 and TLR-7 (Imgenex Corp.) in lung homogenates of vehicle- and TCDD-treated over the course of infection using Western blotting. If TLR-3 and/or TLR-7 expression were increased in lung homogenates of TCDD-treated, infected mice compared to vehicle-treated, infected mice, then we could perform follow-up studies using TLR-3- and/or TLR-7-deficient mice.

TLR-3-deficient mice are available from The Jackson Laboratory, however, these mice are on hybrid B6;129 background (B6 mice have high sensitivity to TCDD, whereas 129 mice have low sensitivity to TCDD; Poland *et al.*, 1994). We could obtain breeding pairs of TLR-3-deficient mice and backcross these mice to C57/BL6 mice for several generations to obtain TLR-3-deficient mice with homozygous C57BL/6 background. The latter would exhibit equivalent sensitivity to TCDD as C57BL/6 wild-type mice. We would treat TLR-3-deficient and C57BL/6 wild-type mice with peanut oil vehicle or TCDD one day prior to infection with influenza virus (A/HKx31). Using flow cytometry, we would assess the number of neutrophils in the lungs of these mice seven days following infection. If the increased pulmonary neutrophilia were abrogated in TCDD-treated, infected TLR-3-deficient mice, then this would indicate that AhR-mediated activation of TLR-3 underlies the enhanced recruitment of neutrophils. On the contrary, equivalent number of neutrophils in the lungs of TCDD-treated TLR-3-deficient and C57BL/6 wild-type would strongly suggest that TLR-3 does not mediate the excess number of neutrophils in TCDD-exposed, infected mice. Alternatively, if we detected a difference in TLR-7 expression in the lungs of mice exposed to TCDD using Western blotting, we could obtain breeding pairs of TLR-7-deficient mice from Dr. Iwasaki's laboratory (Yale University, New

Haven, CT). Using TLR-7-deficient mice we would perform experiments (analogous to TLR-3-deficient mice experiments) to determine whether TLR-7 mediates the increased recruitment of neutrophils to the lungs of TCDD-treated, infected mice.

In the absence of an effect of TCDD Fas or TLRs, labile soluble targets, such as bioactive lipids, provide further potential pulmonary targets of AhR-mediated toxicity. Infection with influenza virus has been shown to increase levels of the bioactive lipid leukotriene-(LTB)₄ in mice (Hennet *et al.*, 1992) and humans (Gentile *et al.*, 2003). Furthermore, bioactive lipids, such as LTB₄, prostaglandin PGE₂, and platelet activating factor (PAF) are highly chemoattractant for neutrophils (Cuzzocrea *et al.*, 2003; Woo *et al.*, 2003). Given the number of different bioactive lipids and the price of the assays to measure these mediators, we could first determine which type of bioactive lipid plays a role in the excessive pulmonary neutrophilia in TCDD-exposed, infected mice. To do so, we would purchase 5-lipoxygenase-(5-LO)-deficient mice, which are available on a C57BL/6 background from The Jackson Laboratory. 5-Lipoxygenase-(5-LO) is the key enzyme in the production of leukotrienes, which are a large group of bioactive lipids (Peters-Golden and Brock, 2003). 5-LO converts arachidonic acid to the leukotriene precursor LTA₄, which is then converted to either LTB₄ or LTC₄ (Peters-Golden and Brock, 2003). LTC₄ is further converted to LTD₄ and LTE₄ (Peters-Golden and Brock, 2003). We would treat 5-LO-deficient and C57BL/6 wild-type mice with peanut oil vehicle or TCDD one day prior to infection with influenza virus (A/HKx31) and assess the number of pulmonary neutrophils on day 7 post infection by flow cytometry. This would allow us to determine whether leukotrienes play a role in the elevated pulmonary neutrophilia in TCDD-treated, infected mice. If the findings from this experiment showed that the excess pulmonary neutrophilia were ablated in TCDD-treated, infected 5-LO-deficient mice compared to TCDD-treated, infected C57BL/6

wild-type mice, then this would indicate that the arachidonic acid pathway is involved in the exacerbated neutrophilia in TCDD-treated mice. In this case, we would determine which specific leukotriene underlies the excessive recruitment of neutrophils to the lungs of TCDD-exposed, infected mice. To do so, we would collect bronchoalveolar lavage (BAL) fluid from vehicle and TCDD-treated mice on several time points after infection. To preserve labile leukotrienes in BAL fluid, we would acidify and pass the BAL fluid through a silica column. We would then measure levels of LTB₄, LTD₄, and LTE₄ in BAL fluid using commercially available competitive ELISAs (Amersham Biosciences).

On the contrary, if TCDD-treated, infected 5-LO-deficient mice exhibited excess pulmonary neutrophilia of similar magnitude than TCDD-treated, infected C57BL/6 wild-type mice, then this would suggest that alterations in the arachidonic acid pathway are not underlying the enhanced number of neutrophils in TCDD-treated mice. In this case, we could examine the remaining types of neutrophil-chemoattractant bioactive lipids PGE₂ and PAF, which are produced independently of 5-LO (Peters-Golden and Brock, 2003). To determine if treatment with TCDD elevates levels of these mediators, we could measure PGE₂ and PAF in BAL fluid from vehicle- and TCDD-treated mice over the course of infection using commercially available competitive ELISAs and/or radioimmunoassays (Amersham Biosciences and R&D Systems).

In the absence of an effect of exposure to TCDD on labile soluble bioactive lipids, we could investigate whether TCDD alters levels of matrix metalloproteinases (MMPs) in the lungs of infected mice. MMPs are degradative enzymes, which play a role in lung tissue remodeling (Visse and Nagase, 2003). However, excessive levels of MMPs, especially MMP-2 and MMP-9, have been attributed to some of the pathologies observed in chronic respiratory diseases, such as ARDS (Lanchou *et al.*, 2003), COPD (Cataldo *et al.*, 2003), and asthma (Boulay *et al.*, 2004).

Decreased levels of tissue inhibitors of matrix metalloproteinase (TIMPs), especially TIMP-1, have been shown to prolong MMP activity and subsequently lead to lung tissue damage (Kim *et al.*, 2005; Pons *et al.*, 2005). We have previously examined levels of TIMP-1 in both BAL fluid and lung homogenates on several days relative to infection in the lungs of vehicle- and TCDD-treated mice (please refer to Appendix D). Our data show that treatment with TCDD did not alter levels of TIMP-1 in the lung of infected mice (Appendix D). In addition to TIMP-1, exposure to TCDD could instead alter levels of other TIMP isoforms, such as TIMP-2, TIMP-3, and TIMP-4 (Visse and Nagase, 2003). However, currently there are no reagents or mouse models available to investigate TIMP-3 and TIMP-4. TIMP-2-deficient mice provide the only approach to investigate whether TIMP-2 plays a role in the elevated neutrophilia in TCDD-treated, infected mice. Breeding pairs of TIMP-2-deficient mice are available on a C57BL/6 background from Dr. Soloway's laboratory (Roswell Park Cancer Institute, Buffalo, NY). In contrast to highly limited ways to examine specific TIMP isoforms, activity of MMPs can be readily assessed using zymography. Thereby, using zymography, we could assess MMP activity in BAL fluid from vehicle- and TCDD-treated mice over the course of infection. If MMP activity were increased in BAL fluid of TCDD-exposed, infected mice, then this would indicate that excess lung damage leads to enhanced leakage of neutrophils to the lungs of mice treated with TCDD. On the contrary, equivalent MMP activity in vehicle- and TCDD-treated mice would suggest that altered MMP activity does not underlie the excess pulmonary neutrophilia.

Finally, in the absence of an effect of TCDD on bioactive lipids and MMPs/TIMPs, we could examine levels of surfactant proteins-(SPs). SPs are a mixture of phospholipids and proteins, which are synthesized and secreted by the lung (Wright, 1997). Among SPs, SP-B and SP-C play a role in respiratory physiology, while SP-A and SP-D are involved in pulmonary

immunity (Wright, 1997). On the one hand, SP-A and SP-D have been characterized as soluble, neutrophil-chemoattractant mediators (Cai *et al.*, 1999; Schagat *et al.*, 2003). On the other hand, SP-A and SP-D have been shown to be anti-inflammatory (LeVine *et al.*, 2001; LeVine *et al.*, 2002). We have previously examined levels of SP-A in lung lavage fluid and lung homogenates of vehicle- and TCDD-exposed mice (please refer to Appendix C). Our results show that treatment with TCDD did not alter levels of SP-A in BAL fluid and lung homogenates of infected mice (Appendix C), indicating that AhR-driven alterations in pulmonary SP-A do not underlie the exacerbated neutrophilia in TCDD-treated mice.

Similar to SP-A, we could measure levels of SP-D in BAL fluid and lung homogenates of vehicle- and TCDD-treated mice over the course of infection using ELISAs. This study would allow us to determine whether exposure to TCDD enhances or diminishes levels of SP-D in the lungs of infected mice. Due to the lack of commercially available reagents to assess SP-D, we would need to establish a collaboration with Dr. LeVine (Children's Hospital Medical Center, Cincinnati, OH). Enhanced levels of SP-D in the lungs of TCDD-treated, infected mice, would indicate that SP-D is pro-inflammatory, potentially underlying the enhanced recruitment of neutrophils. In this case, we would obtain breeding pairs of SP-D-deficient mice from Dr. LeVine's laboratory to definitively determine if elevated levels of SP-D correlate with the increased number of neutrophils in TCDD-treated, infected wild-type mice. In contrast, diminished levels of SP-D in the lungs of TCDD-treated, infected wild-type mice would suggest that SP-D is anti-inflammatory. This finding would indicate that decreased levels of SP-D lead to prolonged inflammation, characterized by increased recruitment of neutrophils to the lungs of TCDD-exposed, infected mice.

In summary, the findings from these future studies will not only allow us to further characterize the recruitment of excess neutrophils to the lungs of TCDD-treated, virus-infected mice, but will aid our understanding of the innate immune response to respiratory viral infections. Given the rising incidence of respiratory disease globally, in conjunction with evidence linking exposure to environmental contaminants to exacerbation of respiratory disease in humans (Salvi *et al.*, 1999; Diaz-Sanchez *et al.*, 2000; Baccarelli *et al.*, 2004), makes it pertinent to thoroughly characterize the immune response to respiratory pathogens.

APPENDICES

In the preceding Chapters of this dissertation, we have described findings of our investigation of numerous mechanisms that could potentially underlie the AhR-mediated recruitment of excess neutrophils to the lungs of TCDD-treated, influenza virus-infected mice. In addition to those mechanisms, the forthcoming section contains several other pathways that we examined as possible mechanistic explanations for the enhanced number of pulmonary neutrophils in mice exposed to TCDD. Supporting evidence and findings from our laboratory are provided for each pathway in the following six separate appendices:

Appendix A: Interferon-(IFN) γ

Appendix B: Toll-like receptor-(TLR)-4

Appendix C: Surfactant protein-(SP)-A

Appendix D: Tissue inhibitor of matrix metalloproteinase-(TIMP)-1

Appendix E: Vascular endothelial growth factor (VEGF)

Appendix F: Selectins and cellular adhesion molecules (CAMs)

Appendix A

Interferon-(IFN) γ

Interferon-(IFN) γ is a pro-inflammatory cytokine (Williams *et al.*, 1993), which has been shown to be chemoattractant for neutrophils in a murine model of hyperoxia-mediated lung damage (Yamada *et al.*, 2004) and in a murine model of infection with the parasite *L. sigmodontis* (Saeftel *et al.*, 2001). Our laboratory consistently observes a four- to ten-fold increase in levels of IFN γ in the lungs of TCDD-treated mice (Warren *et al.*, 2000; and our unpublished observations), which occurs concomitant with the excessive pulmonary neutrophilia in TCDD-treated mice. Taken together, these findings prompted us to investigate whether the elevated levels of IFN γ in the lungs of TCDD-exposed, infected mice mediate the enhanced number of pulmonary neutrophils. To do so, we determined the number of neutrophils in the lungs of vehicle- and TCDD-treated, wild-type and IFN γ -deficient mice seven days after infection with influenza virus. As expected, our data show that TCDD-treated, infected wild-type mice had an increased number of neutrophils in the lungs compared to vehicle-treated, infected wild-type mice (Fig. 6.1). Interestingly, TCDD-exposed, infected IFN γ -deficient mice also had an elevated number of neutrophils in their lungs compared to vehicle-exposed, infected IFN γ -deficient mice (Fig. 6.1). The magnitude of the excess pulmonary neutrophilia in TCDD-treated, infected IFN γ -deficient mice was similar to that observed in TCDD-treated, infected wild-type mice. Therefore, these data strongly indicate that the elevated levels of pulmonary IFN γ in TCDD-exposed, infected mice do not mediate the recruitment of excess neutrophils to the lungs of mice treated with TCDD. Hence, although IFN γ and the exacerbated neutrophilia peak on the same day post infection, these two parameters are not causally linked in our model system. The latter finding is supported by our findings from an *ex vivo* neutrophil migration assay (please see

Chapter 2), which suggest that non-soluble mediators likely mediate the increased recruitment of neutrophils to the lungs of TCDD-treated, infected mice.

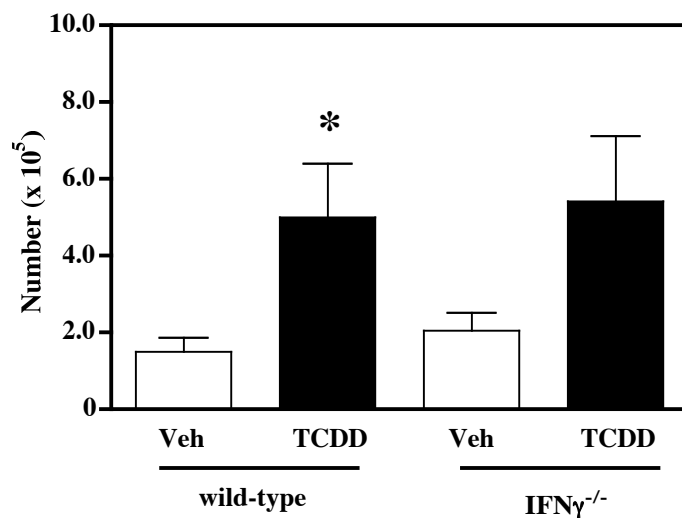


Figure 6.1 TCDD-treated, infected IFN γ -deficient mice have excess number of pulmonary neutrophils. Female C57BL/6 (*wild-type*) and IFN γ -deficient (*IFN γ ^{-/-}*) mice were gavaged with peanut oil vehicle (*Veh*) or TCDD (10 μ g/kg body weight) one day prior to intranasal (i.n.) infection with 10^7 plaque forming units (PFU; equivalent to 125 HAU) influenza virus (A/Memphis/102/72). Seven days relative to infection mice were sacrificed, and lungs were digested with collagenase to obtain total lung-derived immune cells. Using flow cytometry, the average number of pulmonary neutrophils (Gr-1⁺ cells) was determined in vehicle- and TCDD-treated wild-type and IFN γ ^{-/-} mice (5-8 mice per treatment group). Error bars represent the SEM. The asterisk denotes a statistically significant difference compared to the vehicle treatment group ($p \leq 0.05$).

Appendix B

Toll-like receptor-(TLR)-4

Among the currently known twelve toll-like receptors (TLRs) in mice, TLR-4 has been implicated in recruitment of neutrophils to the lungs of mice challenged with lipopolysaccharide (LPS; (Andonegui *et al.*, 2003), *H. influenzae* (Wang *et al.*, 2002), and *E. coli* (Calkins *et al.*, 2002). In addition to these studies using murine models of bacterial challenges, TLR-4 has been shown to underlie the excess number of neutrophils in the lungs of mice exposed to diesel exhaust particles (Inoue *et al.*, 2005). Furthermore, in infants, Gagro *et al.* (2004) have shown that infection with respiratory syncytial virus up-regulates expression of TLR-4. Especially given the role of TLR-4 in response to environmental contaminants (Inoue *et al.*, 2005) and respiratory viral infection (Gagro *et al.*, 2004), made us investigate whether TLR-4 is involved in the enhanced pulmonary neutrophilia in TCDD-treated, influenza virus-infected mice. To accomplish this, we determined the number of neutrophils in the lungs of vehicle- and TCDD-treated C3H/HeN and C3H/HeJ mice seven days upon infection. C3H/HeN mice encode a functional TLR-4 and are, thereby, analogous to C57BL/6 wild-type mice, which have been used for the majority of the studies presented in this dissertation. In contrast to C3H/HeN mice, C3H/HeJ mice have a missense mutation for TLR-4 and encode a nonfunctional TLR-4 (Poltorak *et al.*, 1998).

Using these two strains of mice, we observed a slight increase in the number of neutrophils in the lungs of TCDD-treated, infected C3H/HeN mice compared vehicle-treated, infected C3H/HeN mice (Fig. 6.2). Likewise, TCDD-exposed, infected C3H/HeJ mice had a slight increase in the number of neutrophils in their lungs compared to vehicle-exposed, infected C3H/HeJ mice (Fig. 6.2). Therefore, these data suggest that AhR-mediated deregulation of TLR-

4 does not underlie the increased number of neutrophils observed in the lungs of TCDD-treated, infected mice. Hence, although our data from the *ex vivo* neutrophil migration assay (see Chapter 2) indicate that cell-associated mediators potentially drive the excess recruitment of pulmonary neutrophils, this recruitment likely occurs independently of TLR-4 in our model system.

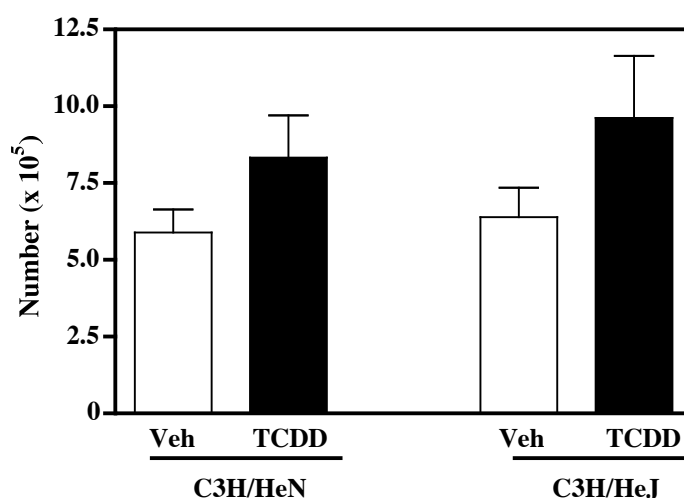


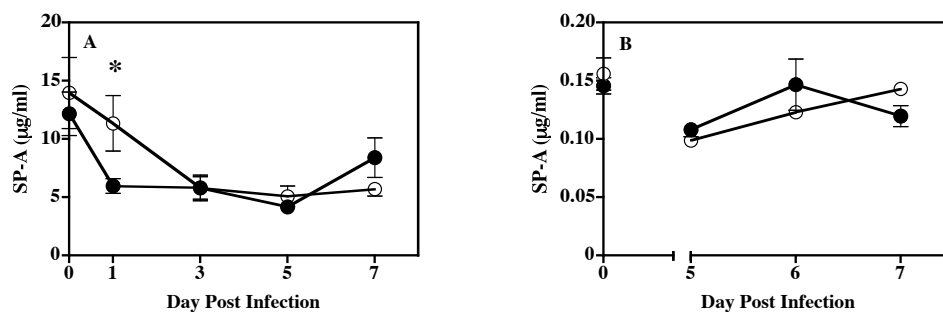
Figure 6.2 Exposure to TCDD slightly increases the number of neutrophils in the lungs of virus-infected C3H/HeN and C3H/HeJ mice. Female C3H/HeN and C3H/HeJ mice were treated with peanut oil vehicle (*Veh*) or TCDD (10 $\mu\text{g}/\text{kg}$ body weight) one day prior to i.n. infection with 10^7 PFU influenza virus (A/Memphis/102/72). Mice were sacrificed seven days relative to infection, and lungs were digested with collagenase to obtain total lung-derived immune cells. Using flow cytometry, the average number of neutrophils (Gr-1⁺ cells) was assessed in the lungs of vehicle- and TCDD-treated C3H/HeN and C3H/HeJ mice (9-10 mice per treatment group). Error bars represent the SEM. No statistically significant differences were observed between vehicle- and TCDD-treated C3H/HeN and C3H/HeJ mice.

Appendix C

Surfactant protein-(SP)-A

Surfactant proteins (SP) are a mixture of phospholipids and proteins that are synthesized and secreted by the lung (Wright, 1997). Among SPs, SP-B and SP-C play a role in respiratory physiology, while SP-A and SP-D are involved in pulmonary immunity (Wright, 1997). SP-A and SP-D have been shown to be chemoattractants for rodent and human neutrophils respectively (Cai *et al.*, 1999; Schagat *et al.*, 2003). Given these findings we assessed whether treatment with TCDD elevates levels of SP-A in the lungs of infected mice, leading to the recruitment of excess neutrophils. Using ELISAs, we measured levels of secreted SP-A in bronchoalveolar (BAL) fluid and levels of total SP-A in lung homogenates of vehicle- and TCDD-treated mice over the course of infection with influenza virus. Our results demonstrate that SP-A was present in BAL fluid of vehicle- and TCDD-treated mice in the absence of infection (Fig. 6.3 A,B). Surprisingly, infection with influenza virus did not increase levels of secreted SP-A; if anything, levels of secreted SP-A decreased with infection (Fig. 6.3 A). With the exception of day 1 post infection (Fig. 6.3 A), levels of secreted SP-A were not significantly different in BAL fluid of vehicle- and TCDD-treated, infected mice. As shown in Fig. 6.3 C, levels of total SP-A were very low in lung homogenates regardless of infection or treatment with TCDD. In summary, our data do not provide any evidence suggesting that exposure to TCDD increases levels of SP-A in the lungs of infected mice. Therefore, enhanced levels of SP-A are not a likely mechanism underlying the excess pulmonary neutrophilia in TCDD-exposed, infected mice.

BAL Fluid



Lung Homogenates

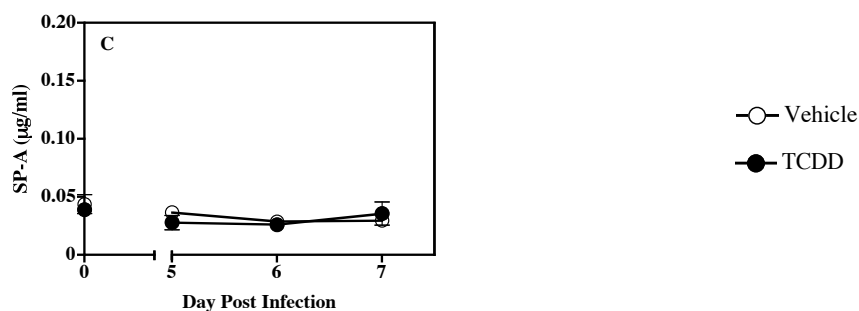


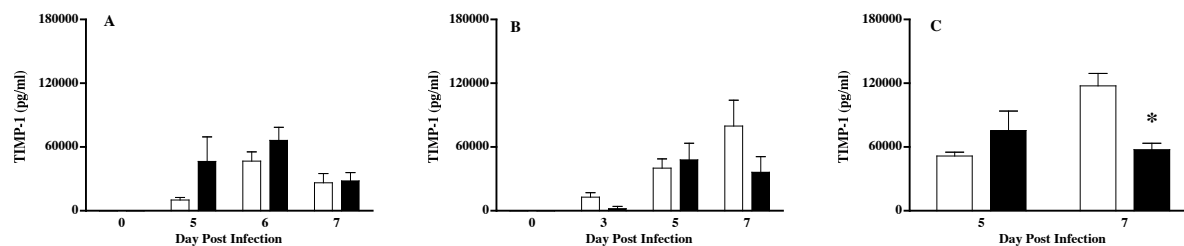
Figure 6.3 Infection with influenza virus does not increase levels of SP-A in the lungs of TCDD-treated mice. Female C57BL/6 mice were treated with peanut oil vehicle or TCDD (10 µg/kg body weight) one day prior to i.n. infection with 120 HAU influenza virus (A/HKx31). On the indicated days relative to infection, using ELISAs, SP-A was measured in (A, B) bronchoalveolar lavage (BAL) fluid and (C) lung homogenates of vehicle- and TCDD-treated mice (5-8 mice per treatment group/day). Mock-infected mice were treated with vehicle or TCDD and served as controls for the infection (4-8 mice per treatment group). Data from mock-infected mice are depicted at the zero time point. Error bars represent the SEM. The asterisk indicates a statistically significant difference compared to the vehicle treatment group ($p \leq 0.05$).

Appendix D

Tissue inhibitor of matrix metalloproteinase-(TIMP)-1

Tissue inhibitors of matrix metalloproteinase-(TIMPs) are endogenous inhibitors of matrix metalloproteinases (MMPs), which are degradative enzymes involved in lung tissue remodeling (Visse and Nagase, 2003). TIMPs bind to MMPs in a 1:1 stoichiometry, therefore inhibiting MMP activity (Visse and Nagase, 2003). Perturbations in the crucial balance of MMPs and TIMPs have been linked to pathologies associated with chronic respiratory diseases (Cataldo *et al.*, 2003; Lanchou *et al.*, 2003; Boulay *et al.*, 2004). Furthermore, using TIMP-1-deficient mice, Kim *et al.* (2005) have shown that TIMP-1 deficiency directly underlies the excess number of neutrophils in lungs of bleomycin-treated mice. Taken together, these findings prompted us to assess whether activation of the AhR alters levels of TIMP-1 in the lungs of infected mice, leading to the elevated pulmonary neutrophilia observed in TCDD-treated, infected mice. Using ELISAs, we measured levels of TIMP-1 in both BAL fluid (Fig. 6.4 A,B,C) and lung homogenates (Fig. 6.4 D,E). Our data indicate that infection with influenza virus strongly increased levels of TIMP-1 in both BAL fluid and lung homogenates of vehicle- and TCDD-exposed mice (Fig. 6.4 A,B,D,E). However, treatment with TCDD did not consistently alter levels of TIMP-1 in lungs of infected mice. Instead, we observed a great degree of variability in levels of TIMP-1, especially on days 5 and 7 post infection (Fig. 6.4). Taken together, these data suggest that AhR-mediated alterations in levels of TIMP-1 are an unlikely mechanism underlying the excess number of neutrophils in the lungs of TCDD-exposed, infected mice. However, alterations in levels of other TIMP isoforms or in MMP activity could still underlie the increased recruitment of neutrophils to the lungs of TCDD-exposed, infected mice (please see Chapter 5).

BAL Fluid



Lung Homogenates

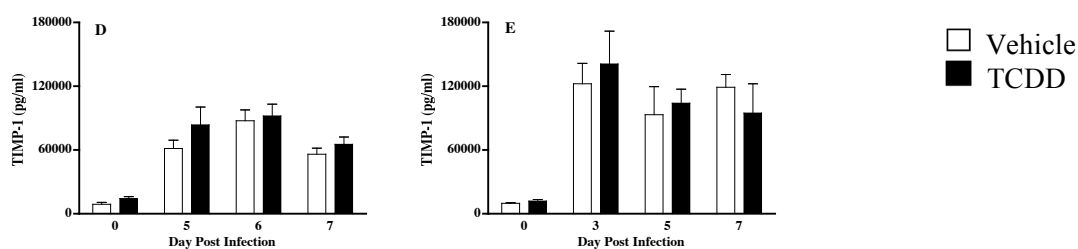


Figure 6.4 Infection with influenza virus strongly increases levels of TIMP-1 in the lungs of vehicle- and TCDD-exposed mice. Mice were treated as described in Figure 6.3. On several days relative to infection with influenza virus, mice were sacrificed. Using ELISAs, levels of TIMP-1 were measured in (A, B, C) BAL fluid and (D, E) lung homogenates of vehicle- and TCDD-treated mice. BAL fluid was collected from three separate time course studies (4-8 mice per treatment group/day) and lung homogenates were from two separate studies (4-8 mice per treatment group/day). Mock-infected mice treated with vehicle or TCDD served as controls for the infection (2-8 per treatment group). Data from these mice are shown at the day zero time point. Error bars represent the SEM. The asterisk indicates a statistically significant difference compared to the vehicle treatment group ($p \leq 0.05$).

Appendix E

Vascular endothelial growth factor (VEGF)

Studies by Ivnitski-Steele *et al.* (2003; 2004) have shown that levels of vascular endothelial growth factor (VEGF) are decreased in TCDD-exposed chick embryos, leading to diminished cardiac development. Furthermore, using a murine model of LPS-induced acute lung injury, Karpaliotis *et al.* (2002) demonstrated that VEGF leads to increased pulmonary damage and concomitant neutrophil influx to the lungs. Based on the findings from these studies, we examined whether treatment with TCDD elevates levels of VEGF, leading to recruitment of excess neutrophils to the lungs of infected mice. Using ELISAs, we measured levels of VEGF in both lung homogenates and BAL fluid of vehicle- and TCDD-treated, infected mice (Fig. 6.5). Our data illustrate that neither exposure to TCDD, nor infection with influenza virus altered total levels of VEGF in lung homogenates (Fig. 6.5A). Contrary to constitutive levels of VEGF in lung homogenates, levels of secreted VEGF in BAL fluid were surprisingly decreased in TCDD-treated mice seven days upon infection (Fig. 6.5B). Given that exposure to TCDD did not increase levels of VEGF in the lungs of infected mice, altered VEGF is an unlikely mechanistic explanation for the exacerbated pulmonary neutrophilia in TCDD-treated mice. This result supports our findings from the *ex vivo* neutrophil migration assay, which indicate that non-soluble mediators drive the enhanced recruitment of neutrophils to the lungs of TCDD-exposed, infected mice (Chapter 2).

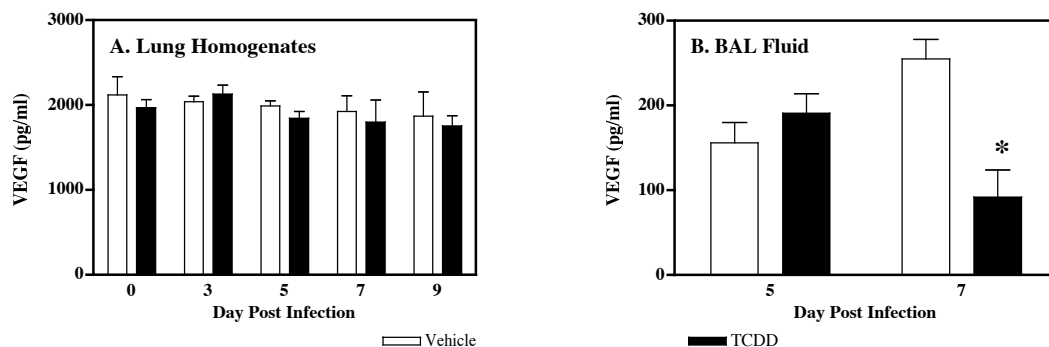


Figure 6.5 Exposure to TCDD does not elevate levels of VEGF in the lungs of infected mice. Mice were treated as described in Figure 6.3 and sacrificed on the indicated days post infection. Using ELISAs, levels of VEGF were measured in (A) lung homogenates and (B) BAL fluid of vehicle- and TCDD-treated mice throughout the course of infection (4-6 mice per treatment group/day). Mock-infected mice treated with vehicle or TCDD served as controls for the infection (3 mice per treatment group). Data from mock-infected mice are depicted at the zero time point. Error bars represent the SEM. The asterisk denotes a statistically significant difference compared to the vehicle treatment group ($p \leq 0.05$).

Appendix F

Selectins and cellular adhesion molecules (CAMs)

During respiratory viral infection neutrophil migration to the lung is governed by soluble neutrophil-chemoattractants at the site of infection, and the expression of adhesion molecules on both the neutrophil surface and the pulmonary endothelium/epithelium (Wagner and Roth, 2000). Adhesion molecules on the lung endothelium and epithelium are either selectins or cellular adhesion molecules (CAMs). Expression of E- and P-selectin (Kamochi *et al.*, 1999; Stone *et al.*, 2002), intercellular adhesion molecule-(ICAM)-1 (Kamochi *et al.*, 1999; Matsuse *et al.*, 1999; Frick *et al.*, 2000), and vascular cellular adhesion molecule-(VCAM)-1 (Bowden *et al.*, 2002; Woo *et al.*, 2005) have been shown to be pertinent for neutrophil migration. In addition to surface expression of these adhesion molecules, a study by Ohno *et al.* (1997) has shown that secreted levels of these mediators alter *in vitro* neutrophil migration.

Given these findings, we determined whether exposure to TCDD elevates levels of E-selectin, P-selectin, ICAM-1, and VCAM-1 in the lungs of infected mice. Specifically, using ELISAs, we assessed whether exposure to TCDD increases total and secreted levels of these mediators. We measured total levels of E-selectin, P-selectin, ICAM-1, and VCAM-1 in lung homogenates and secreted levels in BAL fluid of vehicle- and TCDD-treated mice over the course of infection (Fig. 6.6). Our results show that infection with influenza virus significantly increased total levels of ICAM-1 and VCAM-1 in lung homogenates (Fig. 6.6 E,G). In BAL fluid, secreted levels of E-selectin, P-selectin, and ICAM-1 were significantly elevated over the course of infection (Fig. 6.6 B,D,F). In contrast to this infection-associated increase, exposure to TCDD did not elevate total levels of any of these mediators in lung homogenates (Fig. 6.6 A,C,

E,G). Likewise, exposure TCDD did not elevate levels of E-selectin, P-selectin, ICAM-1, and VCAM-1 in BAL fluid of infected mice (Fig. 6.6 B,D,F, H).

In summary, the lack of an effect of exposure to TCDD on secreted levels of E-selectin, P-selectin, ICAM-1, and VCAM-1 in BAL fluid is supported by our findings from the *ex vivo* neutrophil migration study (Chapter 2). The findings from this migration study indicate that non-soluble mediators likely underlie the recruitment of excess neutrophils to the lungs of TCDD-exposed, infected mice. The absence of an effect of exposure to TCDD on total levels of VCAM-1 in lung homogenates is in support with our findings from the VCAM-1 immunohistochemistry (IHC) study (Chapter 4). The findings from this study show a very slight increase in VCAM-1 expression in the lungs of vehicle- and TCDD-treated, infected mice compared to uninfected mice. In contrast, our data from the ICAM-1 IHC study presented in Chapter 4 show a strong infection-associated increase in pulmonary ICAM-1 expression in vehicle- and TCDD-treated mice. Using two different experimental approaches (IHC and ELISA), we show that infection with influenza virus significantly induced expression of ICAM-1 in this current (Fig. 6.6E) and in our past study (Chapter 4). Based on these findings it is possible that localized increases in the expression of ICAM-1 in the lungs of TCDD-treated, infected mice potentially mediate the excessive recruitment of neutrophils.

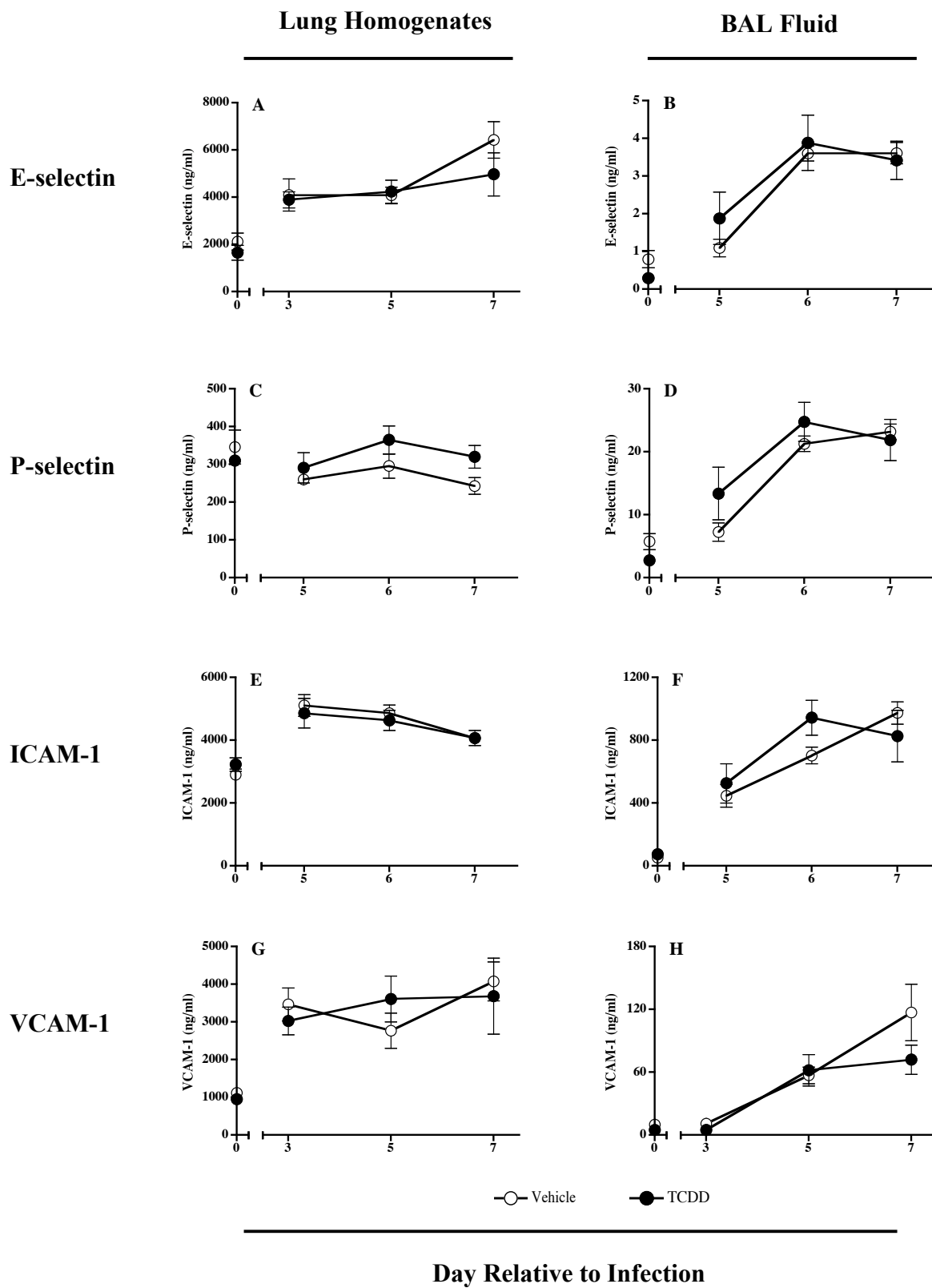


Figure 6.6 Treatment with TCDD does not increase levels E-selectin, P-selectin, ICAM-1, and VCAM-1 in the lungs of infected mice. Mice were treated as described in Figure 6.3 and sacrificed on the indicated days post infection. Using ELISAs, levels of (A, B) E-selectin, (C, D) P-selectin, (E, F) ICAM-1, and (G, H) VCAM-1 were measured in lung homogenates and BAL fluid of vehicle- and TCDD-treated mice throughout the course of infection (4-8 mice per treatment group/day). Mock-infected mice treated with vehicle or TCDD served as controls for the infection (2-8 mice per treatment group). Data from mock-infected mice are shown at the zero time point. Error bars represent the SEM.

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